Camptothecins Inhibit the Utilization of Hydrogen Peroxide in the Ligation Step of Topoisomerase I Catalysis†

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ABSTRACT: The antitumor compounds camptothecin and its derivatives topotecan and irinotecan stabilize topoisomerase I cleavage complexes by inhibiting the religation reaction of the enzyme. Previous studies, using radiolabeled camptothecin or affinity labeling reagents structurally related to camptothecin, suggest that the agent binds at the topoisomerase I−DNA interface of the cleavage complexes, interacting with both the covalently bound enzyme and with the +1 base. In this study, we have investigated the molecular mechanism of camptothecin action further by taking advantage of the ability of topoisomerase I to couple non-DNA nucleophiles to the cleaved strand of the covalent enzyme−DNA complexes. This reaction of topoisomerase I was originally observed at moderate basic pH where active cleavage complexes mediate hydrolysis or alcoholysis by accepting water or polyhydric alcohol compounds as substitutes for a 5′-OH DNA end in the ligation step. Here, we report that a H_2O_2-derived nucleophile, presumably, the peroxide anion, facilitates the release of topoisomerase I from the cleavage complexes at neutral pH, and we present evidence showing that this reaction is mechanistically analogous to DNA ligation. We find that camptothecin, topotecan, and SN-38 (the active metabolite of irinotecan) inhibit H_2O_2 ligation mediated by cleavage complexes not containing DNA downstream of the cleavage site, indicating that drug interaction with DNA 3′ to the covalently bound enzyme is not strictly required for the inhibition, although the presence of double-stranded DNA in this region enhances the drug effect. The results suggest that camptothecins prevent ligation by blocking the active site of the covalently bound enzyme.

Eukaryotic topoisomerase I plays a major role in cellular processes by regulating the DNA topology. The enzyme relaxes negative and positive supercoils arising as a consequence of DNA tracking processes such as transcription and replication (*1*). Mechanistically, topoisomerase I catalyzes DNA relaxation by transiently cleaving, passing, and religating one strand in the DNA double helix. During cleavage, an active site tyrosine residue of the enzyme is used as a nucleophile to break a phosphodiester bond of the DNA backbone, generating a 3′-phosphotyrosyl linkage and a free 5′-hydroxyl group. This 5′-hydroxyl group provides the nucleophile for the religation reaction that restores intact DNA.

Several studies have shown that the resealing reaction of topoisomerase I is inhibited by CPT,[1] a plant alkaloid that holds antitumor activity due to its specific interaction with topoisomerase I catalysis (*2*−*4*). CPT has become the subject of increasing interest during recent years since derivatives of this alkaloid, such as TPT (*5*) and irinotecan (*6*), have been reported to exhibit potent clinical antitumor activity. Presently, these drugs are among the most promising anticancer agents (*7*, *8*). The available evidence suggests that CPT and its derivatives exert their cytotoxicity by inducing high levels of transient topoisomerase I-associated nicks in genomic DNA. Such drug-induced cleavage complexes are converted into permanent double-stranded breaks by collision with replication forks, resulting in DNA fragmentation and cell death (*9*). Since certain CPT-resistant cells have been shown to contain a mutated form of topoisomerase I that is not inhibited by the alkaloids, topoisomerase I appears to be the sole cellular target of camptothecins (*10*).

Investigations of the mechanism by which camptothecins affect topoisomerase I catalysis have shown that the drugs bind to the topoisomerase I−DNA complex after the DNA cleavage step. Radiolabeled CPT interacts reversibly with this complex, whereas there is no detectable binding of the agent to isolated topoisomerase I or to isolated DNA (*11*). Consistently, using synthetic suicide DNA substrates that allow uncoupling of the cleavage and the ligation steps of

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[1] Abbreviations: dATP, 2′-deoxyadenosine 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; dGTP, 2′-deoxyguanosine 5′-triphosphate; dTTP, thymidine 5′-triphosphate; dApdG, 2′-deoxyadenylyl(3′−5′)-2′-deoxyguanosine; DMSO, dimethyl sulfoxide; CPT, camptothecin; SN-38, 7-ethyl-10-hydroxy-CPT; TPT, topotecan, 9-[(dimethylamino)-methyl]-10-hydroxy-20-*S*-camptothecin; DTT, dithiothreitol.

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topoisomerase I catalysis, camptothecins have been shown to selectively block topoisomerase I-mediated DNA ligation whereas they have only little effect on the DNA cleavage reaction (*12*). Other studies have demonstrated that camptothecins preferentially stabilize topoisomerase I-mediated cleavage immediately 5′ to a G, indicating that the nucleotide at this position may be important for drug action (*13*−*15*). These results have led to the suggestion that camptothecins bind to a stereospecific site in the covalent topoisomerase I−DNA cleavage complex and that they probably form a ternary complex with the enzyme and the DNA. Further support for this theory has been provided by affinity labeling studies indicating interactions of CPT with both the enzyme (*16*) and the nucleic acid immediately 3′ to the nick (*17*) within the binary cleavage complexes. However, it is still unclear whether the observed drug−DNA interaction is a prerequisite for inhibition of topoisomerase I catalysis by camptothecins, or just coincidentally favored by these compounds.

To further elucidate the mechanism of drug action, we have investigated the DNA requirements for the inhibitory effect of CPT, TPT, and the active metabolite of irinotecan (SN-38) on the religation reaction of topoisomerase I. For this purpose, we have taken advantage of the recently discovered ability of topoisomerase I to utilize various non-DNA nucleophiles as substitutes for the 5′-OH DNA end in the ligation reaction. This reaction makes it possible to investigate the final transesterification step of topoisomerase I catalysis independently of DNA downstream to the cleavage site, thereby providing a tool for studying the action of camptothecins on the topoisomerase I−DNA complex under conditions that exclude drug−DNA interactions.

We have previously reported enzyme-directed coupling of water or polyhydric alcohols to the cleaved strand of the covalent topoisomerase I−DNA complexes (*18*). However, these reactions were found to be pH-dependent with an optimum at pH 8.5−9.5. To avoid potential effects of a nonphysiological pH on camptothecins, we have searched for stronger non-DNA nucleophiles that can be utilized efficiently in the ligation step of topoisomerase I catalysis at physiologically relevant pH values. In this study, we report efficient topoisomerase I-mediated coupling of H_2O_2 to the cleaved DNA strand at neutral pH and we present evidence showing that this reaction is mechanistically analogous to DNA ligation. We find that coupling of H_2O_2 is inhibited by camptothecins, and we demonstrate that the presence of DNA 3′ to the cleavage site is not a prerequisite for the action of camptothecins, although it enhances their inhibitory effect. On the basis of these results, a model for the molecular mechanism of camptothecins is discussed.

EXPERIMENTAL PROCEDURES

Reagents and Enzymes. DMSO (ACS grade), dApdG, hydrogen peroxide (H_2O_2), *p*-bromophenol, *p*-nitrophenol, tyramine, 4-aminophenol, 2,4-dichlorophenol, 2-methoxyphenol, and CPT were from Sigma. [γ-^{32}P]ATP (7000 Ci/mmol) was from ICN. dATP, dCTP, dGTP, dTTP, and pBR322 were from Boehringer Mannheim. T4 polynucleotide kinase was from New England Biolabs. Sequenase 2.0 was from U.S. Biochemical Corp. SN-38 was kindly provided by S. Sawada (Yakult Central Institute for Microbiological Research, Tokyo, Japan). TPT was a gift from SmithKline Beecham Pharmaceuticals. All camptothecins were dissolved in 50% (v/v) DMSO at 300 μM and stored at −20 °C.

Yeast Strains and Construction of Human TOP1 Plasmids. The *Saccharomyces cerevisiae* top1 null strain RS190 (*19*) was kindly provided by R. Sternglanz (State University of New York at Stony Brook, Stony Brook, NY). Procedures for yeast growth and the construction of pHT100 containing the human topoisomerase I gene under the control of the GAL1-10 promotor have been described previously (*20*). pHT143 was made from pHT100 by cloning a *Sal*I−*Cla*I fragment from this construct, containing the GAL1-10-directed human TOP1 gene, into the 2 μm plasmid pRS426. pHT144 encoding K5 topoisomerase I (*21*) was made from pHT143 by conventional site-directed mutagenesis by introducing an amino acid substitution (Asp to Gly) at position 533 of the human topoisomerase I.

Expression and Purification of Human Topoisomerase I. The topoisomerase I-expressing plasmids pHT143 and pHT144 were transformed into RS190. The cells were grown, and expression was induced as described by Björnsti et al. (*22*). Preparation of crude cell extracts and purification of the wild-type or the K5 human topoisomerase I were performed as previously described (*23*). On the basis of the determination of the protein concentration (*24*) and topoisomerase I activity (*13*), the specific activity was calculated to be approximately 5×10^6 units/mg for both the wild-type and the K5 enzyme.

Synthetic DNA Substrates. Oligonucleotides for construction of topoisomerase I suicide substrates were synthesized on a model 394 DNA synthesizer from Applied Biosystems. Two classes of suicide substrates containing a protruding noncleaved or cleaved strand were used. The sequences of DNA oligonucleotides OL19, OL27, OL22, and OL25 were 5′-GCCTGCAGGTCGACTCTAGAGGATCTAAAAGACTTAGA-3′, 5′-AAAAATTTTTCTAAGTCTTTTAGATCCTCTAGAGTCGACCTGCAGGC-3′, 5′-GCCTGCAGGTCGACTCTAGAGGATCTAAAAGACTTAGAAAAATTTTT-3′, and 5′-AAGTCTTTTAGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGG-3′, respectively, as described previously by Christiansen and co-workers (*25*), and the sequences of the DNA ligator strands OL32 and OL33 were 5′-AGGATGACGATGAGCGCATTGTTAGATT-3′ and 5′-AATCTAACAATGCGCTCATCGTCATCCT-3′, respectively, as published by Christiansen and Westergaard (*26*). The sequence of OL36 was 5′-AGAAAAATTTTT-3′. Purification of full-length oligonucleotides or a variety of successively deleted ones serving as DNA markers was accomplished as described earlier (*25*). The purified scissile strands were 5′-radiolabeled, and the noncleaved strands were 5′-phosphorylated prior to hybridization using the bacteriophage T4 polynucleotide kinase reaction with [γ-^{32}P]ATP or unlabeled ATP serving as the phosphoryl donor, respectively. Excess ATP was removed by spin dialysis on a G-50 column. For hybridization, 10−20 pmol of matching oligonucleotides was heated to 90 °C for 2 min in a buffer containing 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.5) and cooled slowly to room temperature.

Topoisomerase I Reactions Performed on Suicide DNA Substrates. Topoisomerase I catalysis was investigated by using a set of oligonucleotide substrates that allow uncoupling of the cleavage and ligation half-reactions of the

enzyme (*12*, *26*). Active topoisomerase I−DNA cleavage complexes containing the enzyme covalently attached at an internal or a terminal position were generated by preincubating 50 units of human topoisomerase I with 10 fmol of purified DNA substrate [enzyme:DNA = 10:1 (moles/mole)] in a reaction volume of 20 μL at 15 °C for 30 or 60 min, respectively. This reaction was performed in topoisomerase I reaction buffer containing 0.1 mM DTT, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.5). Following termination of the cleavage reaction by the addition of 300 mM NaCl, DNA ligation or coupling of H_2O_2 was performed by continuing incubation for another 10 min at 37 °C in the presence of DNA ligator strands or H_2O_2 at the concentrations indicated in a volume of 300 μL of topoisomerase I reaction buffer. The effect of camptothecins on the ligation step of topoisomerase I catalysis was assayed by adding CPT, SN-38, or TPT to the cleavage mixtures at the concentrations indicated prior to the addition of ligator strands or H_2O_2 . The drugs were stored in 50% DMSO and applied as 5× stock solutions, giving rise to a final concentration of 10% DMSO in the reaction mixtures. The control reactions without drugs (presented in Figures 5−7) were therefore performed in the presence of 10% DMSO alone [10% DMSO had no effect on coupling of H_2O_2 (data not shown)]. All reactions were terminated by the addition of SDS to a final concentration of 0.5% (w/v), and DNA was precipitated with 3 volumes of ethanol. Unless otherwise stated, the samples were subsequently trypsin digested with 1 mg/mL trypsin in 20 μL of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA for 30 min at 37 °C. Finally, the samples were analyzed on denaturing polyacrylamide gels as described below.

Topoisomerase I Reactions Performed on Plasmid DNA. The ability of topoisomerase I to introduce nicks into supercoiled plasmid by coupling of H_2O_2 was assayed by incubating 50 units of human topoisomerase I with 500 ng of negatively supercoiled pBR322 in the absence or presence of 0.5% H_2O_2 (v/v) for 4 h at 37 °C. The reactions were performed in a 50 μL volume containing 0.1 mM DTT, 1.5% glycerol, 5% DMSO, 10 mM bis-Tris propane (pH 7.5), and 0.5 mM EDTA. Following termination of the reaction by the addition of 0.5% SDS, the products were analyzed on 1% agarose gels containing 0.5 μg/mL ethidium bromide or no ethidium bromide. DNA electrophoresed in the absence of intercalator was visualized by subsequent staining of the gel.

Assays for Detecting 3′-Phosphate and 3′-Hydroxyl DNA Ends. 3′-Phosphate termini were detected by taking advantage of the 3′-phosphatase activity of T4 polynucleotide kinase described by Cameron and Uhlenbeck (*27*). Reaction products generated by incubation of the covalent enzyme−DNA cleavage complexes in the presence of 0.5% H_2O_2 (v/v) were analyzed. The reaction mixtures were digested with trypsin as described above, extracted with equal volumes of phenol and chloroform, ethanol precipitated, and dissolved in 20 μL of 0.1 mM Tris (pH 7.0). Following DNA denaturation (90 °C, 2 min), the samples were reacted with 10 units of T4 polynucleotide kinase in 100 mM MES (pH 5.5) and 1 mM $MgCl_2$ in a 30 μL volume for 12 h at 37 °C. To detect recessed 3′-hydroxyl ends, the native reaction products were employed as a template for DNA polymerase extension. To obtain full extension, the reaction products were incubated with 1 unit of Sequenase 2.0 in 24 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 30 mM NaCl, 6 mM DTT, and dATP, dCTP, dGTP, and dTTP (all at 0.5 mM) in a 50 μL volume for 5 min at 37 °C. 3′-Dephosphorylation and polymerase reactions were terminated by the addition of 10 mM EDTA prior to ethanol precipitation.

Denaturing Polyacrylamide Gel Electrophoresis. Following ethanol precipitation and trypsin digestion, the samples were mixed with 1 volume of 80% (v/v) deionized formamide, 50 mM Tris-borate (pH 8.3), 1 mM EDTA, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene xyanol and the mixtures heated to 90 °C for 2 min and applied to a 12% denaturing polyacrylamide gel. Following electrophoresis, the labeled reaction products were visualized by autoradiography as previously described (*18*).

Quantification of Topoisomerase I Catalysis Products on Denaturing Polyacrylamide Gels. The amount of topoisomerase I-mediated DNA cleavage, DNA ligation, and coupling of H_2O_2 was quantified on a model SF Molecular Dynamics PhosphorImager by integrating the area under the curve for each radioactive band using the ImageQuant software from Molecular Dynamics.

RESULTS

Uncoupling of the Cleavage and Ligation Reactions of Topoisomerase I. As previously described (*12*), the individual steps involved in eukaryotic topoisomerase I catalysis can be investigated separately by using synthetic DNA substrates with a highly preferred topoisomerase I interaction site (Figure 1A). These substrates support cleavage while religation is temporarily prevented. Cleavage complexes containing the enzyme covalently attached at an internal or a terminal position can be trapped by using two different classes of DNA substrates containing specific interruptions located next to the cleavage site on the scissile (Figure 1B) or the nonscissile DNA strand (Figure 1C), respectively. Both classes of substrates allow cleavage since they support bipartite DNA interaction of topoisomerase I with two distinct DNA regions, designated region A (encompasses the cleavage site) and region B (located downstream of the cleavage site), which has previously been shown to be required for efficient DNA cleavage (*25*). Religation of the cleaved strand, on the other hand, is prevented due to release of the generated 5′-hydroxyl DNA end which is lost from the reaction site by diffusion. The ligation reaction can be re-initiated, and ligation of DNA to the cleaved strand of the enzyme−DNA complexes can be performed by adding ligator strands containing free 5′-hydroxyl ends (*26*). Active cleavage complexes containing the enzyme covalently attached at an internal position are able to ligate DNA strands with a minimal length of two nucleotides matching the noncleaved strand, referred to as intramolecular ligation (Figure 1B). Cleavage complexes carrying the enzyme at a terminal position can mediate ligation to duplex DNA fragments with blunt ends, referred to as intermolecular ligation in the following (Figure 1C). Moreover, at moderate basic pH, the covalent topoisomerase I−DNA complexes are able to mediate hydrolysis or alcoholysis by using water or short polyhydric alcohols as substitutes for the 5′-OH DNA end in the ligation step (*18*). In this study, we characterize

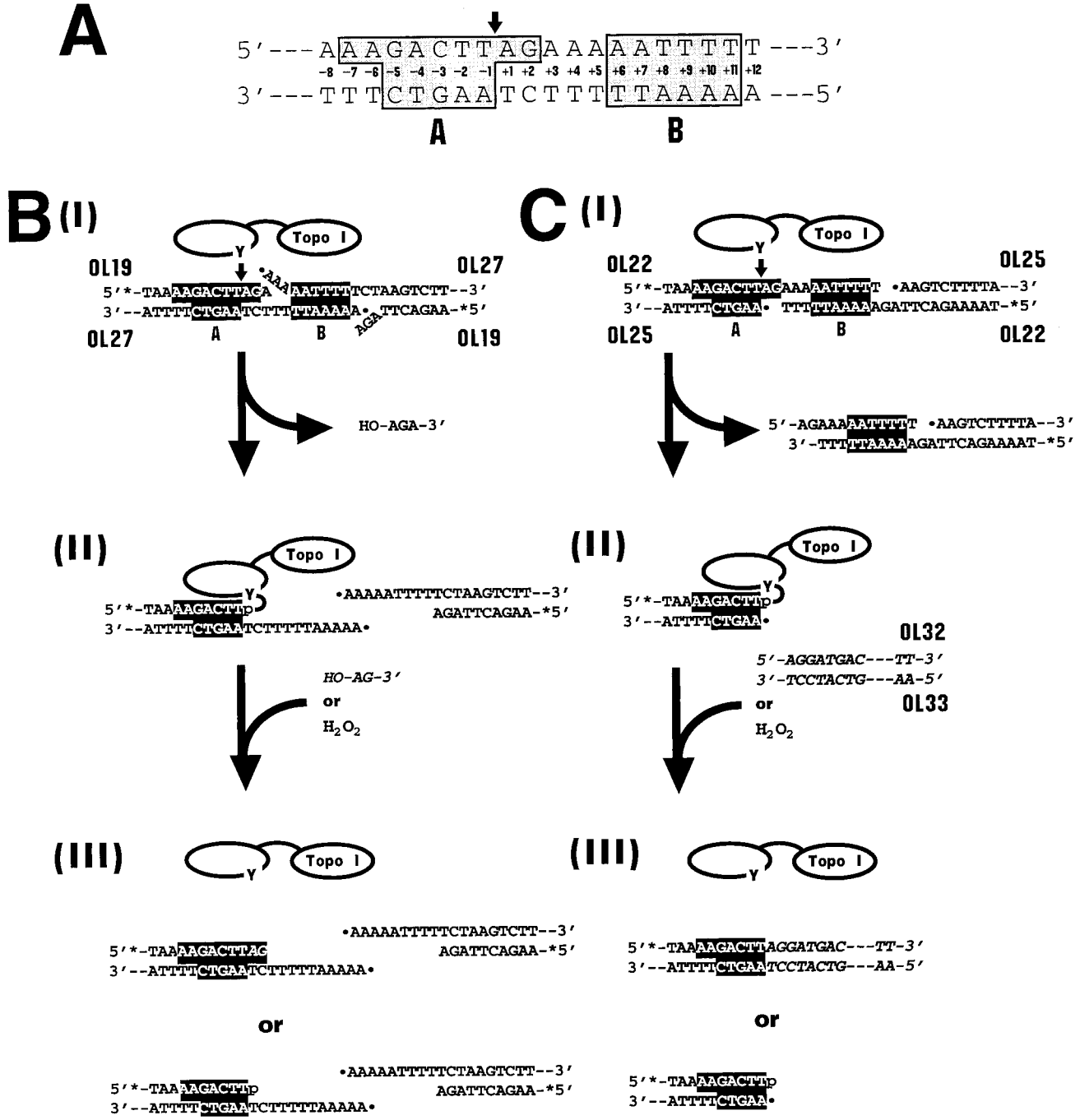

FIGURE 1: Assays for topoisomerase I catalysis. (A) Topoisomerase I recognition sequence. This hexadecameric sequence represents a highly preferred topoisomerase I interaction site found in the rDNA spacers of *Tetrahymena* (*44*, *31*). The topoisomerase I cleavage site is marked by an arrow. The sequence extending upstream from the cleavage site is assigned negative numbers and downstream positive numbers. Regions A and B, framed by shaded boxes, constitute the minimal duplex DNA requirements for topoisomerase I-mediated cleavage. (B and C) Synthetic DNA substrates containing the hexadecameric recognition sequence were utilized to generate cleavage complexes with topoisomerase I bound at an internal or a terminal position. (B) Schematic illustration of an assay employing a suicide DNA substrate supporting covalent binding of topoisomerase I at an internal position. (I) The utilized DNA substrate (OL19★/OL27●) contains a protruding noncleaved strand with a palindromic sequence that supports the formation of double-stranded DNA in region B. (II) Upon DNA cleavage, topoisomerase I is covalently bound to an internal DNA 3′-end via a phosphotyrosyl linkage. Religation is prevented due to the release of the generated 5′-hydroxyl DNA end. (III) DNA ligation or coupling of H_2O_2 to the cleaved strand can subsequently be performed by adding an excess of ligator DNA or H_2O_2 to the reaction mixtures. (C) Illustration of an assay employing a suicide DNA substrate supporting covalent binding of topoisomerase I at a terminal position. (I) The utilized DNA substrate (OL22★/OL25●) has a protruding scissile strand supporting the formation of duplex DNA in region B. (II) Cleavage results in the formation of a covalent topoisomerase I−DNA complex carrying the enzyme at a blunt end. Religation is prevented due to a strand interruption next to the cleavage site on the noncleaved strand resulting in the release of the generated 5′-hydroxyl DNA end. (III) DNA ligation or coupling of H_2O_2 can be performed by adding an excess of a blunt end 28/28-mer (OL32/OL33) or H_2O_2, respectively: (★) 5′-radiolabeling, (●) 5′-phosphorylation, and (Y) active site tyrosine of topoisomerase I.

topoisomerase I-mediated coupling of a strong nucleophile derived from H_2O_2 at neutral pH and we investigate the effect of camptothecins on this reaction using the assays illustrated in panels B and C of Figure 1.

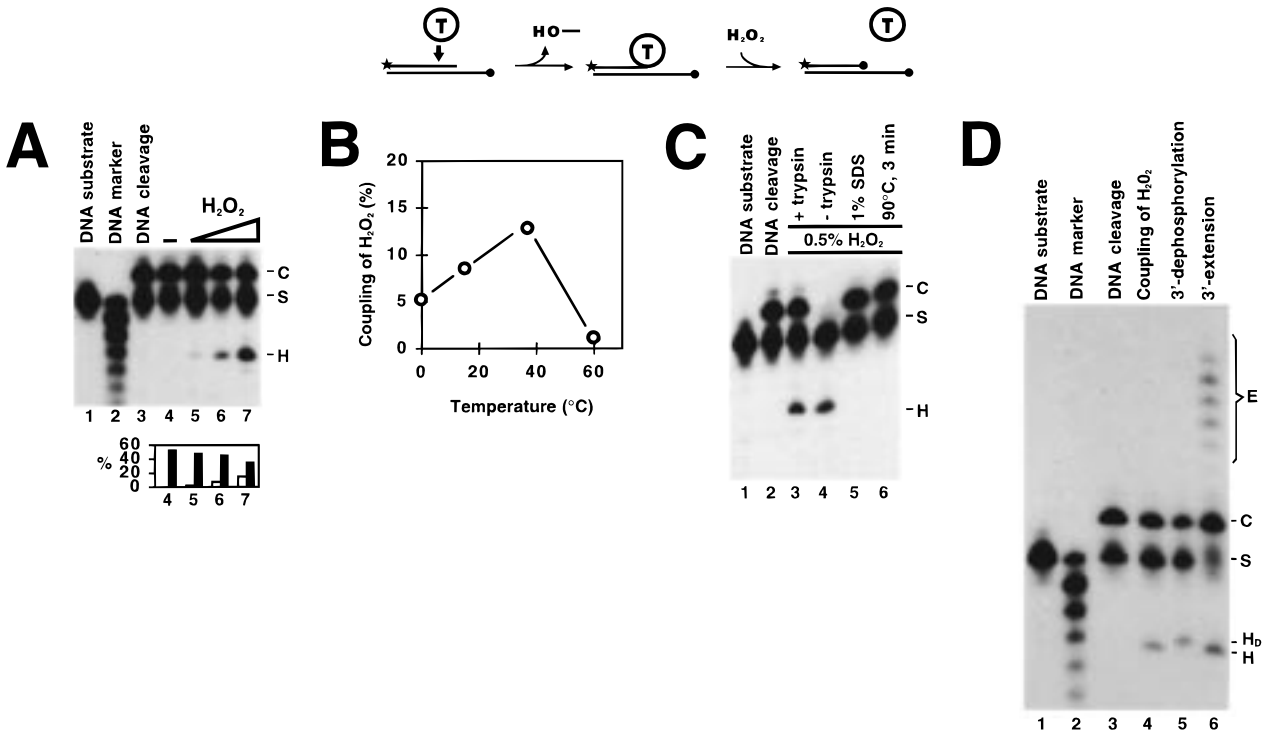

FIGURE 2: Topoisomerase I-mediated coupling of H_2O_2. Topoisomerase I was reacted with a DNA substrate consisting of a 5′-radiolabeled 38-mer scissile strand hybridized to a 5′-phosphorylated 47-mer noncleaved strand (OL19★/OL27●) to generate active cleavage complexes carrying the enzyme at an internal position. Following termination of cleavage by the addition of 300 mM NaCl, coupling of H_2O_2 was studied by incubating the active topoisomerase I−DNA complexes with H_2O_2 under various conditions for 10 min at pH 7.5. Reactions were stopped by the addition of 0.5% SDS (w/v), and the reaction products were ethanol precipitated, digested with trypsin, and analyzed by denaturing gel electrophoresis. The assay is schematically illustrated in the upper panel. (A) Effect of adding increasing concentrations of H_2O_2 to the precleaved DNA substrates: lane 1, DNA substrate incubated without topoisomerase I in the presence of 1.0% H_2O_2 (v/v); lane 2, marker DNA containing 3′-OH ends and decreasing in steps of one nucleotide with the maximal size of 38 nucleotides (this marker was prepared as described in Experimental Procedures); lane 3, precleaved DNA substrate; and lanes 4−7, incubation of active cleavage complexes with 0, 0.01, 0.1, and 1.0% H_2O_2 (v/v) at 37 °C. (A, lower panel) The amount of each reaction product was determined by densitometric scanning, and the percentage of cleavage product (filled columns) and product H (empty columns) out of the total amount of radioactivity was calculated as $C/(H + C + S) \times 100\%$ or $H/(H + C + S) \times 100\%$, respectively. The obtained values are illustrated in a histogram. (B) Temperature dependence of the formation of product H. Cleavage complexes were incubated in the presence of 0.5% H_2O_2 (v/v) at 0, 15, 37, and 60 °C, and the reaction products were subsequently analyzed by denaturing gel electrophoresis as described above. The amount of each reaction product was determined by densitometric scanning, and the percentage of cleavage complexes converted to product H by coupling of H_2O_2 was calculated as $H/(H + C) \times 100\%$ and plotted with respect to temperature. (C) Requirement of the native conformation of topoisomerase I for the formation of product H: lane 1, DNA substrate; lane 2, DNA cleavage control; lane 3, active cleavage complexes incubated in the presence of 0.5% H_2O_2 (v/v) at 37 °C and treated with trypsin before electrophoresis; lane 4, reaction performed as described for lane 3 but with omission of trypsin digestion prior to gel electrophoresis; lane 5, cleavage complexes treated with 1% SDS (w/v) prior to incubation with 0.5% H_2O_2; and lane 6, precleaved substrates incubated at 90 °C for 3 min prior to addition of H_2O_2 (v/v) and further incubation at 37 °C. (D) Analysis of the 3′-ends of the reaction products: lane 1, DNA substrate; lane 2, DNA marker; lane 3, DNA substrate preincubated with topoisomerase I; lane 4, incubation of active cleavage complexes in the presence of 0.5% H_2O_2 (v/v) at 37 °C; lane 5, 3′-dephosphorylation of the reaction products from lane 4 by T4 polynucleotide kinase at pH 5.5 in the absence of ATP; and lane 6, 3′-Sequenase extension of reactions products from lane 4 by incubation with Sequenase 2.0 in the presence of dATP, dCTP, dGTP, and dTTP. E represents polymerase extension products, S DNA substrate, C cleavage product, H the product of topoisomerase I-mediated coupling of H_2O_2, H_D dephosphorylated product H. (★) 5′-Radiolabeled and (●) 5′-phosphorylated.

Topoisomerase I-Mediated Coupling of Non-DNA Nucleophiles at Neutral pH. To identify a non-DNA nucleophile that can efficiently substitute the 5′-OH DNA end in the ligation step of eukaryotic topoisomerase I catalysis under physiologically relevant conditions, we have investigated the ability of topoisomerase I−DNA complexes to mediate coupling of different nucleophilic compounds (not derived from DNA) at neutral pH. The compounds tested were H_2O_2, which can generate the highly nucleophilic peroxide anion (HOO^-) in aqueous solution, and different phenol analogues. Such chemicals have previously been shown to act as nucleophiles supporting strand scission mediated by the Flp recombinase, a specialized member of the type I topoisomerases (*28*, *29*). In this study, we investigate whether these compounds can also act as nucleophiles in the ligation step of topoisomerase I catalysis.

In the assay used, active cleavage complexes were generated by preincubating a 5′-radiolabeled suicide DNA substrate with human topoisomerase I. Following preincubation, the cleavage reaction was stopped by the addition of 300 mM NaCl and incubation was continued in the presence of H_2O_2 or various phenol-derived compounds at pH 7.5 (Figure 2, upper panel). After trypsin digestion, the reaction products were analyzed on a denaturing polyacrylamide gel. The products arising from cleavage with topoisomerase I are shown in Figure 2A (lane 3). Topoisomerase I cleaves off three nucleotides from the substrate used in this experiment. Although smaller by three nucleotides, the cleavage product

(C) migrates more slowly in the gel than the substrate (S) because a topoisomerase I-derived heptapeptide resistant to trypsin remains covalently attached to the cleaved 3′-end (*30*). As shown in lanes 5−7, incubation of the precleaved mixture with H_2O_2 at increasing concentrations resulted in the conversion of cleavage complexes into increasing amounts of a new product (designated H). The product H migrates slighly faster than the 35-mer of the marker DNA containing a 3′-OH end (compare lane 7 with lane 2). This mobility of H is consistent with the new product being a 35-mer containing a 3′-phosphate end which, compared to a 3′-hydroxyl end, increases the negative charge of the DNA strand, thereby causing it to migrate slightly faster than a 3′-hydroxyl 35-mer [this identity of product H was indeed confirmed in later experiments (see below, Figure 2D)]. Thus, since topoisomerase I cleaves three nucleotides from the 38-mer substrate, the size of product H equals the expected size of a cleavage product that is unattached to the topoisomerase I-derived heptapeptide and is consistent with H being the product of a reaction leading to the release of the covalently bound topoisomerase I from the DNA substrate. Incubation of the cleavage complexes with 20 mM tyramine or 20 mM 4-aminophenol also gave rise to new products with approximate sizes of 35-mer, whereas new products could not be observed after incubation with 20 mM *p*-bromophenol, 20 mM *p*-nitrophenol, 20 mM 2,4-dichlorophenol, or 20 mM 2-methoxyphenol (data not shown). Quantification of band intensities revealed that in the presence of tyramine or 4-aminophenol the new products account for less than 5% of the reaction products (data not shown), whereas approximately 30% of the cleavage complexes (Figure 2A, lower panel) are converted to H in the presence of 1% H_2O_2. Thus, within attainable concentrations of the compounds tested, H_2O_2 seems to be the most efficient in generating a new product. We therefore decided to continue the studies of the topoisomerase I ligation step using H_2O_2.

The role of topoisomerase I in the generation of product H was addressed by investigating the effect of temperature and enzyme denaturation on the formation of this product. The temperature dependence of product H formation was studied in the temperature range of 0−60 °C. As shown in Figure 2B, the production of H increased with temperatures up to 37 °C but was largely impaired at 60 °C. This result is consistent with H being the product of a reaction mediated by human topoisomerase I as opposed to a spontaneous chemical reaction that would be expected to proceed at a faster rate with increasing temperatures. Moreover, when the cleavage complexes were denatured either by incubation with 1% (W/V) SDS or by heating to 90 °C for 3 min prior to incubation with H_2O_2, product H was not formed (Figure 2C, compare lanes 5 and 6 with lane 3). Thus, the native conformation of topoisomerase I seems to be a requirement for the formation of product H. On the basis of these results, we conclude that H is the product of a topoisomerase I-mediated reaction and not a product of a chemical oxidation in the presence of H_2O_2. However, it is not possible to distinguish whether product H indeed arises from processing of the cleavage product or from a novel topoisomerase I−DNA cleavage intermediate created in the presence of H_2O_2 despite the fact that the reaction mixtures contained 300 mM NaCl which normally prevents DNA cleavage.

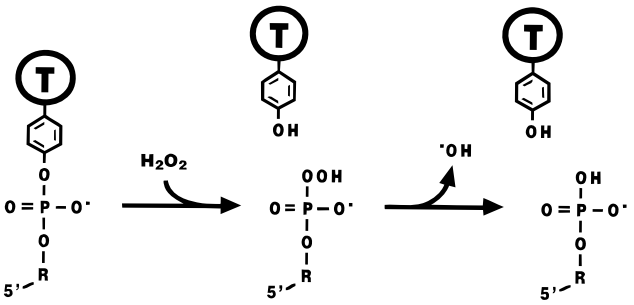

FIGURE 3: Proposed model for topoisomerase I-mediated coupling of H_2O_2. An enzyme-directed nucleophilic attack of the peroxide anion (derived from H_2O_2) on the phosphotyrosyl linkage generated during cleavage leads to the formation of a 3′-peroxyphosphate DNA end. The 3′-peroxyphosphate can be converted to a phosphate group by hydrolysis.

It has previously been shown that DNA covalently attached to topoisomerase I is retarded in the slot of a denaturing polyacrylamide gel due to interaction of the enzyme with the gel matrix (*31*). This characteristic of the enzyme-coupled DNA was employed to examine whether product H is generated from the precleaved complex or represents a de novo cleavage product. As presented in Figure 2C, the omission of trypsin digestion followed by gel electrophoretic analysis of the reaction products caused the enzyme-linked cleavage product (C) to disappear while the mobility of product H was unaffected (compare lanes 3 and 4). This result shows that H is nonproteinaceous and therefore unlikely to be created by topoisomerase I-mediated de novo cleavage in the second incubation step.

On the basis of the experiment described in Figure 2C, it was possible to exclude the possibility that product H contains a topoisomerase I-derived peptide covalently attached to its 3′-end. To further characterize H, the 3′-end of this product was analyzed by assays for phosphate or hydroxyl termini. Bacteriophage T4 polynucleotide kinase was used to identify 3′-phosphate ends, as it specifically exhibits 3′-phosphatase activity on single-stranded DNA at pH 5.5 in the absence of ATP (*27*). As shown in Figure 2D, 3′-dephosphorylation of denatured reaction products changes the mobility of H to that of the 3′-hydroxyl 35-mer of the DNA marker (compare lane 5 with lane 2). In comparison with the untreated product (lane 4), migrating faster than the marker 35-mer, the retardation of the new product (termed H_D) was equivalent to approximately half a nucleotide, which is consistent with the removal of a terminal phosphate group from product H (*32*). Consistent with H having a 3′-phosphate end, the product did not function as a primer for DNA polymerase extension (lane 6), whereas in the same reaction, most of the substrate strand S, containing a free 3′-hydroxyl group, was converted to extension products (collectively referred to as E). The mobility of the cleavage product C, of which the 3′-end is blocked by a topoisomerase I-derived heptapeptide, was unaffected by the dephosphorylation and the extension reaction.

The simplest explanation for all of the obtained results is that H is indeed the product of a topoisomerase I-directed attack of a H_2O_2-derived nucleophile, most probably HOO^-, on the phosphotyrosyl linkage of the cleavage complexes generating a 3′-peroxyphosphate DNA end, which can rapidly be hydrolyzed to a phosphate group (Figure 3). Such activity of topoisomerase I would be analogous to the

previously observed pH-dependent hydrolysis and/or alcoholysis mediated by the enzyme (*18*). The only obvious difference between the observed reactions is that coupling of H_2O_2 occurs more efficiently than both hydrolysis by water or coupling of various alcohol compounds at physiologically relevant pH. This difference can be explained by the peroxide anion being a particularly strong nucleophile compared to water and the polyhydric alcohols used in previous experiments. Activation of this nucleophile by raising the pH is therefore not necessary for the reaction to occur efficiently. Further supporting this notion, coupling of H_2O_2 shows the same pH profile (data not shown) as topoisomerase I-mediated hydrolysis and/or alcoholysis (*18*) but with an overall higher efficiency.

Topoisomerase I-Mediated Coupling of H_2O_2 Is Mechanistically Analogous to DNA Ligation. From the preceding experiments, a clear precursor−product relationship appears to exist between the cleavage product and the product H (see Figure 2A). These results suggest that H_2O_2 acts on the phosphotyrosyl linkage of the cleavage complexes, facilitating the release of the covalently bound enzyme in a reaction most likely to be analogous to DNA ligation. To support of this idea, kinetic experiments have been performed, which showed that intramolecular DNA ligation to 0.5 μM dinucleotide dApdG and coupling of 0.5% H_2O_2 proceed in a time-dependent manner following essentially the same pattern with a maximum of 80 and 35% conversion of cleavage complexes to reaction products, respectively, reached after approximately 2 h of incubation at 37 °C (data not shown).

To further evaluate whether coupling of H_2O_2 can be considered mechanistically analogous to DNA ligation, the ability of H_2O_2 and DNA 5′-OH ends to compete in the ligation step of topoisomerase I catalysis was investigated. In one experiment, active cleavage complexes were incubated with a fixed concentration of H_2O_2 and increasing concentrations of ligator strand (dApdG) and in a second with a fixed concentration of ligator strand and increasing concentrations of H_2O_2 (Figure 4A). In the first case, we observed a decrease in the formation of product H (lanes 4−7) which paralleled the increasing concentrations of dApdG in the reaction. Quantification of the reaction products (Figure 4A, lower panel) showed that the decrease in the amount of product H correlated with an increase in the ligation of dApdG (compare lane 4 with lanes 5−7). In the second case, a decrease in the amount of DNA ligation was found to correlate with an increase in H_2O_2 coupling (compare lane 8 with lanes 9−11 and see the lower panel of Figure 4A). Thus, intramolecular DNA ligation and coupling of H_2O_2 appear to be competitive reactions.

In the experiments described above, the substrate was a topoisomerase I-cleaved suicide DNA substrate prevented in normal religation. To investigate whether H_2O_2 can compete with the 5′-OH DNA end generated during cleavage, the ability of topoisomerase I to introduce nicks into negatively supercoiled pBR322 by coupling of H_2O_2 was investigated. Following incubation of the plasmid with topoisomerase I in the absence or presence of 0.5% H_2O_2, the reaction products were analyzed on 1% agarose gels with or without ethidium bromide to detect the presence of closed relaxed or open (nicked) relaxed reaction products (*23*). The result shown in Figure 4B demonstrates that topoisomerase I action in the presence of H_2O_2 converted all of the supercoiled plasmid into an open relaxed form (compare lanes 3 and 7). In comparison, relaxation by topoisomerase I in the absence of H_2O_2 led to the formation of closed relaxed products (compare lanes 4 and 8). The observed nicking of the plasmid was not due to a nonenzymatic oxidative damage of the DNA backbone since incubation of supercoiled pBR322 with H_2O_2 in the absence of topoisomerase I had no effect on the mobility of the plasmid (compare lanes 1 and 5 with lanes 2 and 6). The most straightforward interpretation of these results is that the nicking of the plasmid observed in the presence of H_2O_2 and topoisomerase I is caused by the ability of the peroxide anion to inhibit strand resealing by competing with the 5′-OH DNA end created during cleavage.

The competitive nature of DNA ligation and coupling of H_2O_2 strongly suggests that the two reactions proceed by essentially the same molecular mechanism and lends further credence to the notion that H is indeed the product of an enzyme-directed nucleophilic attack on the phosphotyrosyl linkage.

Camptothecins Act by Directly Targeting the DNA-Linked Topoisomerase I. Previous studies have indicated that camptothecins inhibit topoisomerase I-mediated religation by forming a ternary complex with the DNA−topoisomerase I intermediate of the cleavage reaction (*11*). By using affinity labeling reagents structurally related to CPT, it has been shown that the drug interacts with the covalently bound enzyme (*16*) and in addition preferentially with the nucleotide located immediately 3′ to the point of cleavage (*17*), although interactions with nucleotides at other positions within the cleavage complexes have not been excluded. On the basis of these observations, two principally different mechanisms of drug action can be hypothesized. The drugs may inhibit religation through their interaction with the covalently bound topoisomerase I by direct blockage of the active site of the enzyme, or they may prevent correct positioning of the 5′-OH DNA end in the catalytic pocket of topoisomerase I by directly interacting with the DNA end to be ligated. To distinguish between these two possibilities, we have investigated the ability of camptothecins to inhibit topoisomerase I-mediated coupling of H_2O_2. This reaction is expected to be inhibited by camptothecins, only if the alkaloids act by blocking the active site of topoisomerase I. If, on the contrary, the drugs act by interacting with the incoming 5′-OH DNA end, thereby preventing its positioning for ligation, utilization of a non-DNA nucleophile in the ligation step is expected to be unaffected by camptothecins.

The effect of camptothecins on topoisomerase I-mediated coupling of H_2O_2 was investigated by the assays schematically illustrated in Figure 5 (far left). Synthetic DNA substrates containing strand interruptions located next to the cleavage site on the scissile or the nonscissile strand were preincubated with topoisomerase I to generate active cleavage complexes carrying topoisomerase I at an internal or a terminal position, respectively (see Figure 1). The precleaved substrates were subsequently incubated with 0.5% H_2O_2 in the absence or presence of 60 μM CPT, SN-38, or TPT. For comparison, the effects of camptothecins on intra- and intermolecular DNA ligation were investigated in the same experiments. Consistent with previous reports (*33*, *34*), intramolecular DNA ligation to the dinucleotide dApdG was inhibited efficiently by all three drugs (Figure 5A, compare

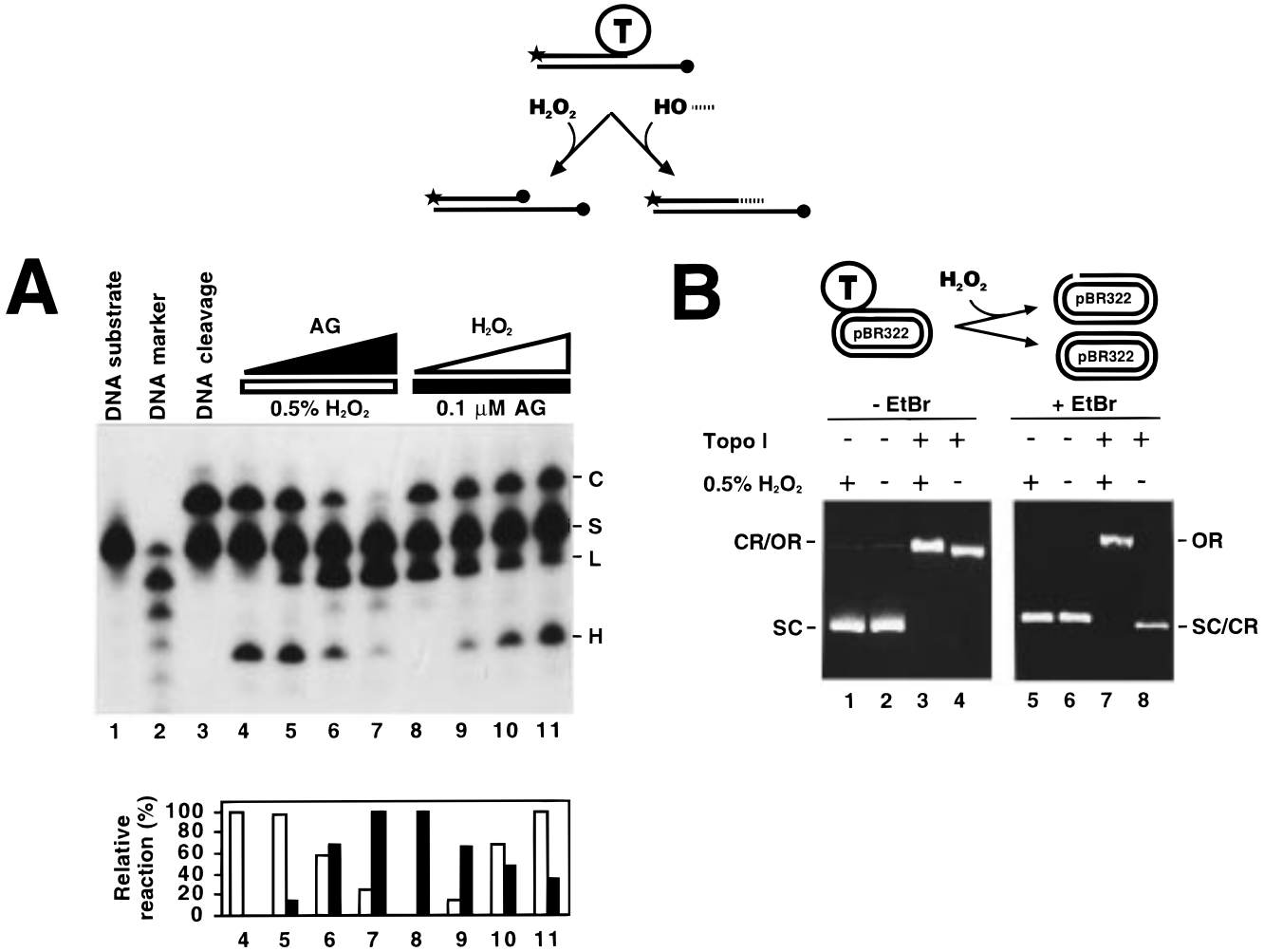

FIGURE 4: Mechanism of topoisomerase I-mediated coupling of H_2O_2. (A) Competition between H_2O_2 and DNA in the ligation step of topoisomerase I catalysis. Preincubation of the DNA substrates (OL19★/OL27●) with topoisomerase I, assays for DNA ligation and coupling of H_2O_2, processing of the samples, and analysis of the reaction products on a denaturing polyacrylamide gel were performed as described in Experimental Procedures. The reaction scheme is depicted in the upper panel: lane 1, DNA substrate; lane 2, DNA marker; lane 3, DNA substrate preincubated with topoisomerase I; lanes 4−7, incubation of active cleavage complexes with 0.5% H_2O_2 (v/v) and 0, 0.05, 0.25, and 1.0 μM dApdG, respectively; and lanes 8−11, incubation of active cleavage complexes with 0.10 μM dApdG and 0, 0.05, 0.25, and 1.0% H_2O_2 (v/v), respectively. (A, lower panel) The amount of each reaction product was determined by densitometric scanning, and the percentage of cleavage products converted by coupling of H_2O_2 (empty columns) or DNA ligation (filled columns) was calculated as described in the legend of Figure 2B. For comparison, the relative reaction percentages were calculated by setting the maximal amount of reaction with H_2O_2 or DNA ligation to 100% within each of the two series (lanes 4−7 and lanes 8−11). The obtained values are illustrated in a histogram. (B) The ability of topoisomerase I to mediate coupling of H_2O_2 to double-stranded DNA. Supercoiled plasmid pBR322 was incubated for 4 h at 37 °C in the presence or absence of topoisomerase I or H_2O_2: lane 1, incubation of pBR322 with 0.5% H_2O_2 (v/v); lane 2, pBR322 incubated in reaction buffer; lane 3, incubation of pBR322 with topoisomerase I in the presence of 0.5% H_2O_2 (v/v); lane 4, incubation of pBR322 with topoisomerase I in the absence of H_2O_2 (upon termination of the reactions, the products were analyzed by electrophoresis in a 1% agarose gel); and lanes 5−8, reactions identical to those in lanes 1−4, the only difference being that these samples were subjected to agarose gel electrophoresis in the presence of 0.5 μg/mL ethidium bromide. S represents DNA substrate, C cleavage product, H the product of topoisomerase I-mediated coupling of H_2O_2, L the DNA ligation product, SC supercoiled plasmid pBR322, CR the closed relaxed plasmid, and OR the open relaxed (nicked) plasmid.

lanes 5, 9, and 13 with lanes 4, 8, and 12). Likewise, coupling of H_2O_2 was strongly impaired by CPT and its analogues when topoisomerase I was bound at an internal position (compare lanes 7, 11, and 15 with lanes 6, 10, and 14). Similar results were obtained using cleavage complexes containing topoisomerase I covalently attached at a terminal position. As shown in Figure 5B, intermolecular ligation to a short double-stranded DNA fragment and coupling of H_2O_2 to a blunt end were both inhibited in the presence of 60 μM CPT, SN-38, or TPT (compare lanes 5, 9, and 13 with lanes 4, 8, and 12 and lanes 7, 11, and 15 with lanes 6, 10, and 14). The observed inhibition of topoisomerase I-mediated coupling of H_2O_2 was unaffected by the presence of 900 mM NaCl which excludes intermolecular DNA interaction of the covalently bound enzyme (data not shown). The presence of DNA downstream of the cleavage site seems therefore not to be an absolute requirement for drug action. Due to the pronounced structural differences between DNA and H_2O_2, it is unlikely that the drugs interact specifically with the non-DNA nucleophile like they do with DNA. Moreover, the possibility that the observed inhibition of H_2O_2 coupling reflects specific properties of H_2O_2 can be excluded, since topoisomerase I-mediated coupling of glycerol at pH 8.5 is also inhibited by camptothecins (data not shown). Thus, the concordance between the results obtained on DNA ligation and coupling of non-DNA nucleophiles strongly

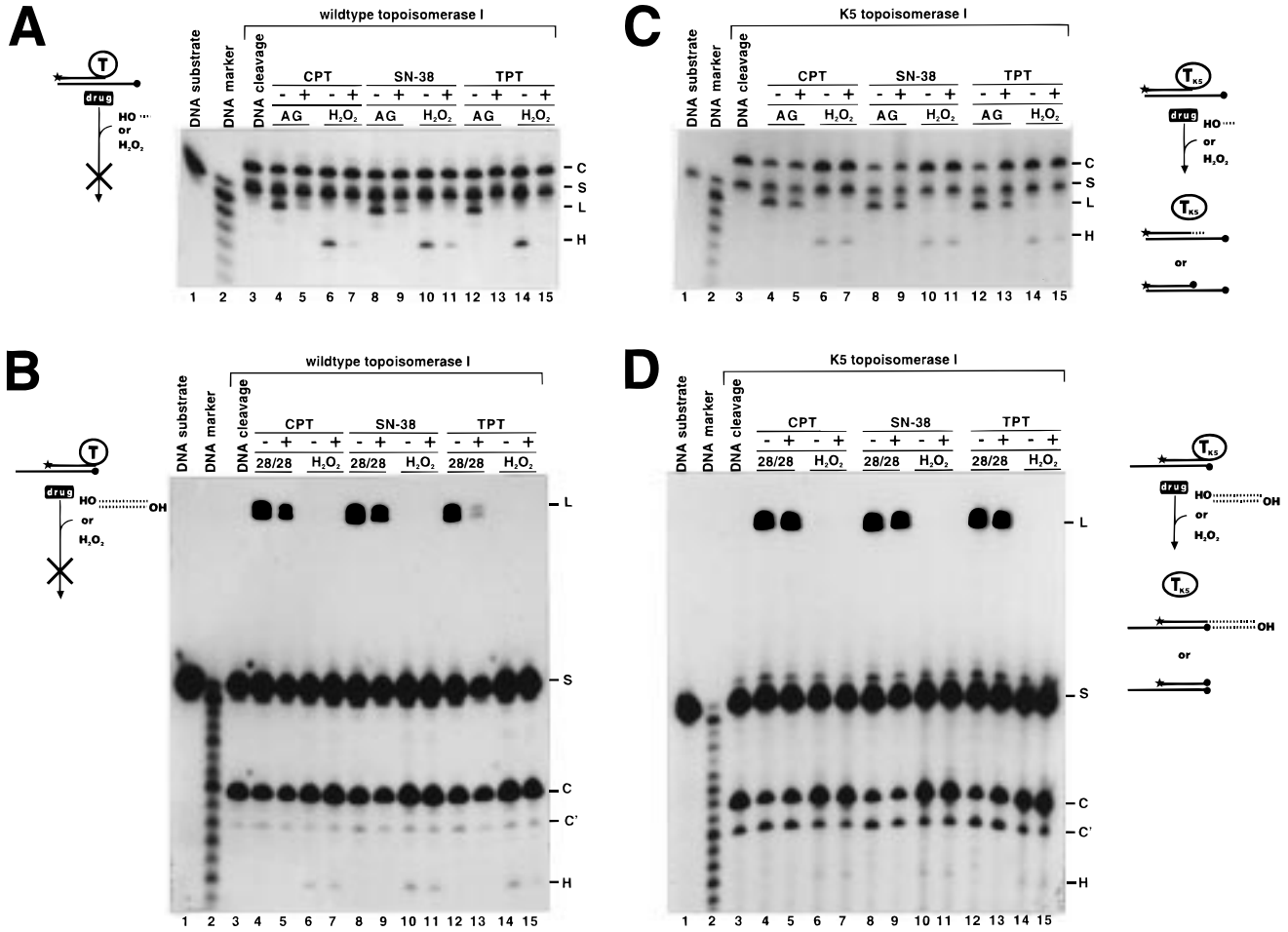

FIGURE 5: Effect of camptothecins on topoisomerase I-mediated coupling of H_2O_2 and DNA ligation. Topoisomerase I was reacted with suicide DNA substrates consisting of OL19★ hybridized to OL27● or OL22★ hybridized to OL25● to generate active cleavage complexes with the enzyme covalently bound at an internal or a terminal position, respectively. The cleavage reaction was terminated by adding NaCl to a final concentration of 300 mM to the reaction mixtures. Coupling of H_2O_2 or DNA ligation was subsequently performed in the presence or absence of 60 μM camptothecins (see flowcharts). (A) Effect of camptothecins on the reactions mediated by wild-type human topoisomerase I covalently attached to DNA at an internal position: lane 1, DNA substrate; lane 2, DNA marker; lane 3, DNA substrate preincubated with wild-type topoisomerase I; lane 4, incubation of active cleavage complexes with 0.10 μM dApdG; lane 5, like lane 4 except that ligation was performed in the presence of 60 μM CPT; lane 6, active cleavage complexes incubated with 0.5% H_2O_2 (v/v); lane 7, precleaved complexes incubated with 0.5% H_2O_2 and 60 μM CPT; lanes 8−11, like lanes 4−7, the only difference being that CPT was replaced by SN-38; and lanes 12−15, like lanes 4−7 except that TPT was used instead of CPT. (B) Effect of camptothecins on intermolecular DNA ligation or coupling of H_2O_2 to a blunt end. In this experiment, the cleavage complexes were made by preincubating topoisomerase I with the substrate OL22★/OL25● leading to covalent attachment of the enzyme at a blunt end. To obtain DNA ligation, 0.033 μM of a 28/28-mer duplex DNA (OL32/OL33) was added to the reaction mixture. Otherwise, the reactions were performed and the mixtures loaded on the gel as described for panel A. (C) Like panel A with the difference being that the K5 mutant of human topoisomerase I was used. (D) Like panel B except that K5 topoisomerase I replaces the wild-type enzyme. S represents DNA substrate, C the cleavage product, H the product of topoisomerase I-mediated coupling of H_2O_2, and L the DNA ligation product.

suggests a mechanism of drug action by which camptothecins exert their inhibitory effect primarily by interacting with the covalently bound enzyme and not by dislocating the incoming nucleophile through a direct interaction with it.

An amino acid substitution (Asp533Gly) of one of the residues proposed to interact with CPT (*35*) in topoisomerase I has been reported to cause CPT resistance of the human leukemia cell line (CPT-K5) (*10*, *21*). It has previously been shown that intramolecular DNA ligation mediated by this enzyme is significantly less affected by CPT than ligation mediated by the wild-type enzyme (*36*, *37*). To determine the effect of the point mutation (Asp533Gly) on the sensitivity of H_2O_2 coupling to camptothecins, the experiments described above were repeated using the K5 enzyme instead of wild-type topoisomerase I. As shown in Figure 5C, incubation of cleavage complexes carrying the K5 enzyme at an internal end with H_2O_2 in the presence of CPT (lane 7), SN-38 (lane 11), or TPT (lane 15) causes only a slight decrease in the amount of product H formed, compared to reactions performed in the absence of drugs (lanes 6, 10, and 14). Consistent with previous results, K5 topoisomerase I-mediated intramolecular ligation to dApdG also appeared to be essentially unaffected by the drugs (compare lanes 5, 9, and 13 with lanes 4, 8, and 12). Moreover, intermolecular DNA ligation and coupling of H_2O_2 to a blunt end are largely unaffected by the three drugs when activated cleavage complexes containing the K5 enzyme covalently attached at a terminal position were used. The fact that the single point mutation of K5 topoisomerase I protects DNA ligation as well as coupling of H_2O_2 from camptothecins indicates that the mechanism of drug action most probably is similar in both reactions.

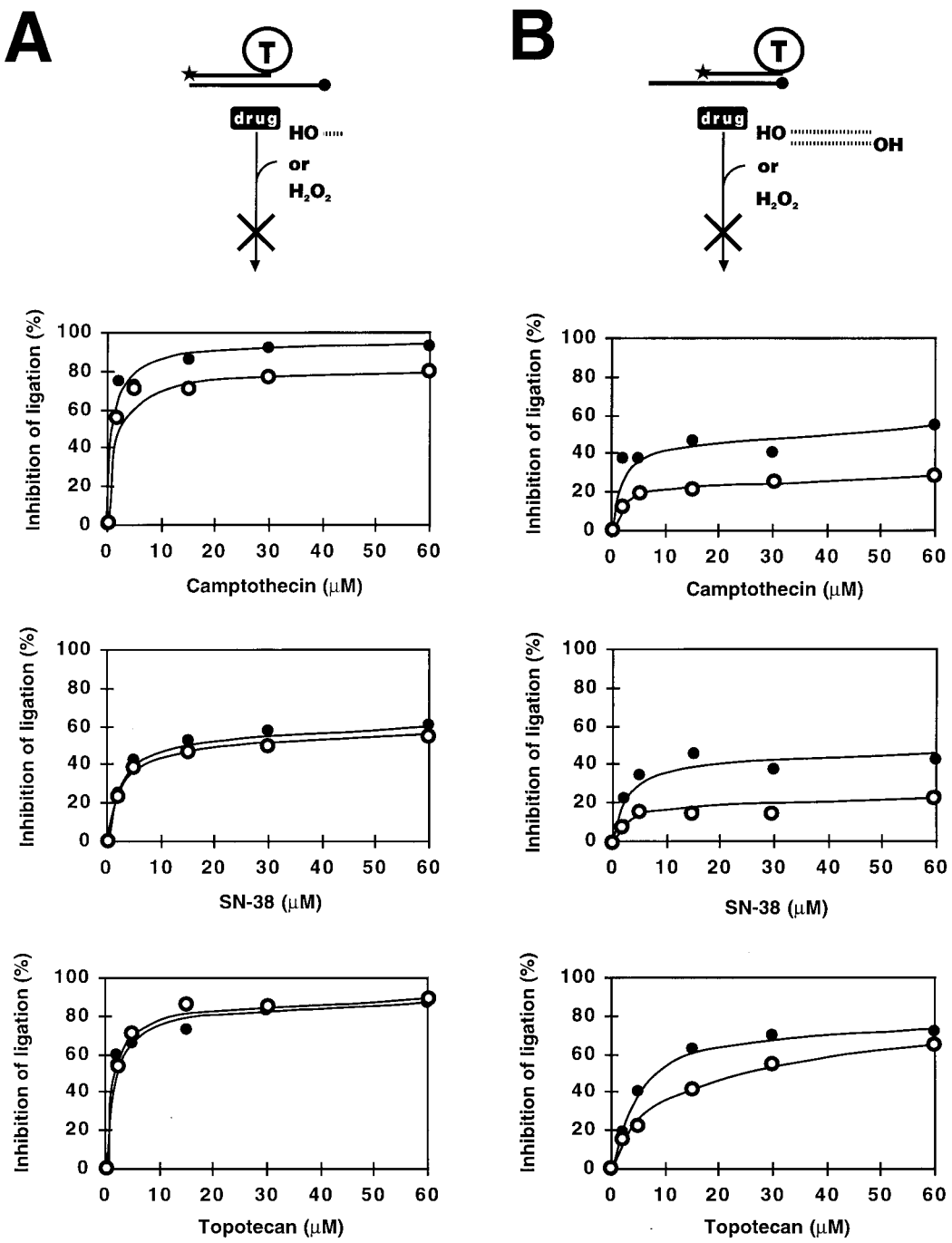

FIGURE 6: Effects of local DNA environments on CPT action. The drug dose−response relationships of coupling of H_2O_2 or DNA ligation mediated by cleavage complexes containing topoisomerase I at an internal or a terminal end were investigated as described in Experimental Procedures (see also the flowcharts, upper panel). Following gel electrophoresis, the reaction products were quantified by densitometric scanning and the percentage of inhibition of DNA ligation or coupling of H_2O_2 was calculated as $(L_{-drug} - L_{+drug})/L_{-drug} \times 100\%$ or $(H_{-drug} - H_{+drug})/H_{-drug} \times 100\%$, respectively. An average of three independent experiments were plotted with respect to the drug concentration. (A) Inhibition of reactions mediated by topoisomerase I covalently attached to DNA at an internal position. Active cleavage complexes were incubated with 0.10 μM dApdG (●) or 0.5% H_2O_2 (v/v) (○) in the presence of 0, 2, 5, 15, 30, or 60 μM CPT, SN-38, or TPT as indicated. (B) Inhibition of reactions mediated by topoisomerase I covalently attached to DNA at a terminal position. Active cleavage complexes were incubated with 0.033 μM 28/28-mer (●) or 0.5% H_2O_2 (v/v) (○) in the presence of 0, 2, 5, 15, 30, or 60 μM CPT, SN-38, or TPT as indicated.

Interaction of Camptothecins with DNA at Position +1 within the Ternary Complex Is Not a Requirement for but Enhances Inhibition. To determine the influence of the local DNA environment on the action of camptothecins, we compared the dose−response curves of drug action on DNA ligation or H_2O_2 coupling mediated by cleavage complexes carrying topoisomerase I at an internal or a blunt end, respectively (Figure 6).

Panel A shows the effects of increasing concentrations of CPT, SN-38, or TPT on DNA ligation (filled circles) or H_2O_2 coupling (open circles) mediated by cleavage complexes containing topoisomerase I covalently attached at an internal position. The dose−response curves for these two reactions are very similar, showing that both reactions are inhibited with approximately equal efficiency by the individual drugs. The addition of concentrations of drugs as low as 5 μM leads

to a significant inhibition of the reactions. In both cases, constant levels of approximately 90, 60, or 90% of inhibition are reached by the addition of ≥20 μM CPT, SN-38, or TPT to the reaction mixtures. Thus, consistent with previous reports, our observations support the common effect of camptothecins being able to inhibit topoisomerase I-mediated ligation (*38*). In panel B, the effects of camptothecin on intermolecular DNA ligation or coupling of H_2O_2 mediated by cleavage complexes carrying topoisomerase I at a blunt end are presented. Consistent with observations recently published by Pourquier and co-workers (*39*), intermolecular DNA ligation is inhibited by the camptothecins, although to a lesser extent than intramolecular DNA ligation. Thus, the addition of ≥20 μM CPT, SN-38, or TPT inhibits DNA ligation to a blunt end by 50, 40, or 70%, respectively. In comparison, the inhibitory effect of the drugs on coupling of H_2O_2 to a blunt end is less pronounced. This reaction is inhibited by 20, 20, or 60% following the addition of ≥20 μM CPT, SN-38, or TPT, respectively.

Consistent with the results presented in Figure 5, the dose−response curves demonstrate that the presence of DNA 3′ to the point of cleavage is not a prerequisite for the action of CPT, SN-38, or TPT, since coupling of the non-DNA nucleophile to a blunt end is clearly inhibited by the drugs. However, the efficiency with which the drugs inhibit the topoisomerase I-mediated reactions depends on the type of cleavage complexes utilized in the experiments, with the inhibitory effect being more pronounced when topoisomerase I is bound at an internal position.

In our assay, SN-38 appears to be a less efficient inhibitor of topoisomerase I-mediated ligation than both CPT and TPT (compare the middle panels with the upper and lower panels of Figure 6A,B). This observation contradicts previous data suggesting that SN-38 is the most potent topoisomerase I poison of the three drugs (*40*, *41*). The apparent discrepancy between our results and those obtained by other researchers can most easily be explained by the different composition of the DNA substrates used in the respective studies. In previous experiments, the effect of SN-38 was investigated in terms of its ability to stabilize cleavage complexes formed on intact double-stranded DNA (*41*). The experiments described here, on the other hand, were performed with cleavage complexes created on short synthetic suicide DNA substrates containing, at the most, only short regions of partly double-stranded DNA downstream of the cleavage site. Intramolecular DNA ligation to a dinucleotide supporting the formation of double-stranded DNA at positions +1 and +2 was inhibited relatively poorly by SN-38 (Figure 6A). This result indicates that stretches of double-stranded DNA longer than two base pairs downstream of the cleavage site may be necessary for SN-38 to act optimally. To test this hypothesis, we compared the inhibitory effect of SN-38 and CPT on intramolecular DNA ligation to a 12-mer ligator strand that hybridizes to the noncleaved strand of the topoisomerase I−DNA complexes, thereby creating double-stranded DNA from position +1 to +12 (Figure 7, upper panel). The utilization of this 12-mer ligator strand instead of the dApdG ligator used in Figure 6A does indeed increase the inhibition efficiency of SN-38 relative to that of CPT (compare Figure 7 with Figure 6A; note that the absolute levels of inhibition presented in Figures 6 and 7 cannot be directly compared since these change with the used of different ligator strands). This observation suggests that compared to that of CPT the action of SN-38 is enhanced by the presence of an extended region of double-stranded DNA downstream of the cut site of the cleavage complexes. Therefore, the relatively low efficiency of SN-38 observed in this study (Figure 6) does not necessarily contradict previous observations (*40*, *41*) but may rather reflect differences in the utilized substrates.

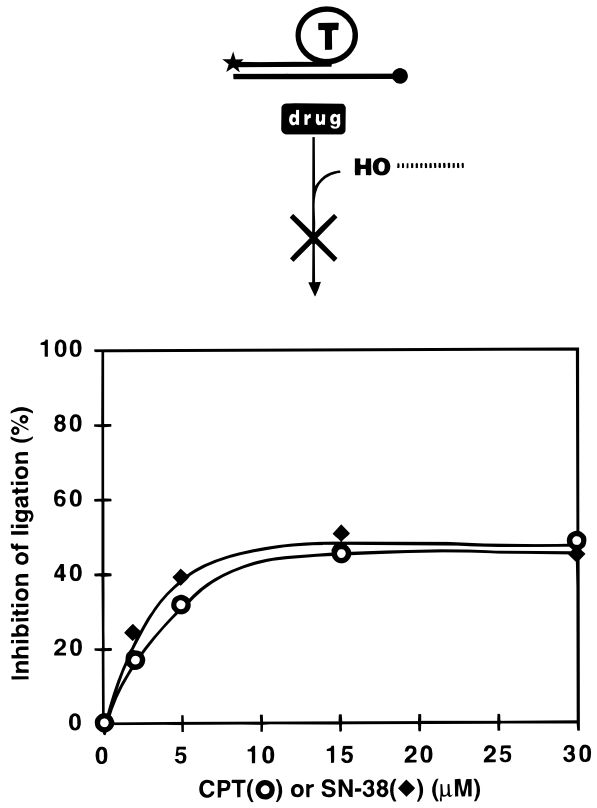

FIGURE 7: Inhibition of intramolecular DNA ligation by SN-38 and CPT. The drug dose−response relationship eof DNA ligation to a 12-mer ligator strand (OL36) mediated by precleaved complexes was investigated as described in Experimental Procedures (upper panel). The utilized 12-mer hybridizes to the noncleaved strand of OL19★/OL27●, creating double-stranded DNA at positions 1−12. Active cleavage complexes were incubated with 30 fmol of OL36 in the presence of 0, 2, 5, 15, and 30 μM CPT (○) or SN-38 (◆) as indicated. Following gel electrophoresis, the reaction products were quantified by densitometric scanning and the percentage of inhibition of DNA ligation was calculated as $(L_{-drug} - L_{+drug})/L_{-drug} \times 100\%$. An average of three independent experiments were plotted with respect to the drug concentration.

DISCUSSION

This study was motivated by previous investigations demonstrating that eukaryotic topoisomerase I can catalyze alcoholysis or hydrolysis of the 3′-phosphotyrosyl linkage formed during DNA cleavage. Topoisomerase I-mediated alcoholysis or hydrolysis has an optimum at pH 8.5−9.5 and is barely detectable at neutral pH (*18*). The biological relevance of these reactions might therefore be limited. Here, we report efficient topoisomerase I-mediated coupling of H_2O_2 to the cleaved strand of the covalent enzyme−DNA complexes at physiologically relevant pH, and we propose a model for this reaction by which the H_2O_2-derived peroxide anion acts as a nucleophile to facilitate the release of

topoisomerase I from the cleavage complexes. Peroxide has previously been shown to act as a nucleophile in reactions assisted by the specialized type I topoisomerase, FLP recombinase (*28*, *29*). The body of available evidence strongly suggests that coupling of H_2O_2 is mechanistically analogous to DNA ligation. There is a clear precursor−product relationship between the cleavage product and the product of H_2O_2 coupling, indicating that the target of the reaction is the 3′-phosphotyrosyl linkage formed upon DNA cleavage by topoisomerase I. More specifically, topoisomerase I seems to direct a nucleophilic attack of peroxide 3′ to the phosphate group of the phosphotyrosyl linkage since coupling of H_2O_2 yields a product with a free phosphate at its 3′-end. The same chemical bond serves as the target for the 5′-OH DNA end during normal DNA ligation. Moreover, we find that coupling of H_2O_2 mediated by precleaved suicide DNA substrates is inhibited in a competitive manner by the presence of a DNA ligator strand and vice versa. These results suggest that the two nucleophiles occupy the same or closely related sites within the active site pocket of the covalently bound enzyme. Further supporting this notion, camptothecins, which have been shown to inhibit specifically the religation reaction of topoisomerase I (this study and refs *12* and *38*), were found also to inhibit coupling of H_2O_2.

Incubation of topoisomerase I in the presence of H_2O_2 leads to the formation of nicks in supercoiled plasmid. This reaction is strictly dependent on topoisomerase I and is most easily explained by topoisomerase I accepting the peroxide anion as a substitute for the 5′-OH DNA end generated during cleavage. H_2O_2 is a product of oxygen metabolism and might under certain conditions be present at relatively high concentrations in living cells (*42*). Thus, it is possible that the reaction described here can have important biological implications, suggesting a role of topoisomerase I in the generation of DNA damage during for instance oxidative stress.

In this study, we have used the ability of active cleavage complexes to accept H_2O_2 (peroxide) as a nucleophile in a reaction analogous to DNA ligation to investigate the molecular mechanism of the topoisomerase I specific antitumor agents CPT, TPT, and SN-38. These agents are known to enhance topoisomerase I-mediated cleavage by inhibiting the normal religation reaction of the enzyme. Studies using radiolabeled camptothecin (*11*) or affinity labeling reagents structurally related to camptothecin (*16*, *17*) suggest that the drug binds at the topoisomerase I−DNA interface of the cleavage complex, interacting with both the covalently bound enzyme and preferentially with the +1 base of the scissile DNA strand. This mode of drug interaction has recently been supported by modeling CPT into the crystal structure of human topoisomerase I cleavage complexes, showing a possible interaction of the drug with Arg364, Asp533, and Asn722 on the protein and with the +1 base of both the scissile and nonscissile DNA strands (*35*). Given the binary binding mode of camptothecin, two explanations can account for the inhibitory effect of this compound and its derivatives. First, the drugs may inhibit religation directly through their interaction with topoisomerase I most probably by occupying the catalytic pocket of the covalently bound enzyme. Second, the direct interaction between the drugs and the 5′-OH DNA end created during cleavage may cause a dislocation of this end, preventing its correct positioning for ligation. To determine which of these models explains the molecular action of camptothecins most appropriately, we investigated the effect of camptothecins on the utilization of H_2O_2 (peroxide) in the ligation step of topoisomerase I catalysis. We find that coupling of H_2O_2 mediated by cleavage complexes carrying topoisomerase I at an internal position is inhibited by CPT, SN-38, and TPT to the same degree as DNA ligation. With a molecular structure fundamentally different from DNA, it is unlikely that peroxide is bound specifically by the camptothecins. Thus, the drugs most probably inhibit coupling of this nucleophile by interacting directly with the covalently bound enzyme and blocking catalytic residues of the active site pocket. Due to the mechanistic similarities between coupling of H_2O_2 and DNA ligation, the same mode of drug action is likely to account for the observed inhibition of both reactions. This is further supported by the finding that a single point mutation (Asp533Gly) of the K5 topoisomerase I causes both reactions to be largely unaffected by the camptothecins. Taken together, our results suggest that camptothecins inhibit topoisomerase I-mediated coupling of H_2O_2 as well as DNA ligation through its direct interaction with the enzyme and not through a direct interaction with the nucleophile to be ligated. Further support for this mode of camptothecin action has recently been provided by Straub and co-workers, who found that CPT inhibits DNA ligation in a competitive manner (*34*) and by theoretical insertion of CPT into the resolved topoisomerase I structure (*35*), indicating that CPT and 5′-OH DNA ends tend to occupy the same or closely related sites within the active site pocket of topoisomerase I.

In concordance with camptothecins inhibiting the religation reaction of topoisomerase I by interacting directly with the active site of the enzyme, we find that CPT, SN-38, and TPT also inhibit coupling of H_2O_2 to a blunt end. Thus, interaction of camptothecins with DNA downstream of the cleavage site is not a requirement for drug action. However, compared to the reaction performed with cleavage complexes containing an activated blunt end, coupling of H_2O_2 mediated by cleavage complexes carrying the topoisomerase I at an internal end is inhibited considerably more efficiently by the drugs. The enhanced effect of camptothecins on reactions mediated by these cleavage intermediates most probably reflects an improved drug−enzyme binding induced by an additional interaction of the drug with the protruding noncleaved DNA strand of the cleavage complexes. This notion correlates with previous observations indicating that the stability of topoisomerase I-linked nicks induced by camptothecins depends on the DNA sequence 3′ to the cleavage site (*41*). However, in contrast to previous investigations in which camptothecins have been shown to interact specifically with the +1 base of the scissile strand (*17*), we find that the presence of double-stranded DNA at this position is not necessary to support efficient inhibition by the drugs. Accordingly, coupling of H_2O_2 mediated by the cleavage complexes that has the potential to form double-stranded DNA downstream of position +3 is inhibited to the same degree as intramolecular ligation to a dinucleotide that hybridizes to position +1 and +2 upon addition of the individual camptothecins. One explanation for this result might be that a putative drug interaction with the +1 base

of the nonscissile strand is sufficient to enhance drug binding. Such an interaction has indeed been hypothesized on the basis of the recently resolved structure of topoisomerase I. Alternatively, our results may reflect the ability of the covalently bound enzyme to bring a DNA end located several bases downstream of the cleavage site into the correct position for the drug to interact with it. It has previously been shown that topoisomerase I is able to seal gaps on partially double-stranded DNA by interacting with 5′-OH DNA ends located even further away from the point of cleavage (*43*).

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