IMPACT OF THE C-TERMINAL DOMAIN OF TOPOISOMERASE IIα ON THE DNA CLEAVAGE ACTIVITY OF THE HUMAN ENZYME[†]

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Abstract

The enzymatic function of the C-terminal domain of eukaryotic topoisomerase II is not well defined. This region of the enzyme is highly variable, hydrophilic, and contains nuclear localization signals and phosphorylation sites. In contrast to eukaryotic topoisomerase II, type II enzymes from chlorella virus completely lack the C-terminal domain. These viral enzymes are characterized by a robust DNA cleavage activity, high coordination between their two active site tyrosyl residues, and reduced sensitivity to anticancer drugs. As a first step towards characterizing the contribution of the C-terminal domain of human topoisomerase IIα to enzyme function, the protein was truncated at amino acid 1175, which corresponds to the C-terminal residue of *Paramecium bursaria* chlorella virus-1 topoisomerase II as determined by BLAST sequence alignment. Although the overall catalytic activity of the resulting enzyme, hTop2αΔ1175, was lower than that of full-length topoisomerase IIα, the mutant protein displayed a double-stranded DNA cleavage activity that was ~2−to 3−fold higher. While the DNA breaks created by hTop2αΔ1175 were primarily double-stranded, cuts generated by topoisomerase IIα were primarily single-stranded. Thus, the enhanced cleavage observed for hTop2αΔ1175 appears to be due, at least in part, to an increase in active site coordination. Finally, hTop2αΔ1175 displayed a distinctly lower susceptibility to anticancer agents than did topoisomerase IIα, despite the fact that it showed a similar binding affinity for etoposide. Therefore,

[1]The abbreviations used are

PBCV-1

Paramecium bursaria chlorella virus-1

CVM-1

Chlorella virus Marburg-1

PCR

polymerase chain reaction.

[2]Although partially relaxed plasmid molecules can be seen in the reactions catalyzed by topoisomerase IIα and hTop2αΔ1175, the DNA relaxation patterns generated by the two enzymes differ. In the reaction catalyzed by the full-length enzyme, the negatively supercoiled band is rapidly shifted to an intermediate superhelical density, which gradually shifts to the position of fully relaxed products. This pattern is indicative of a distributive reaction, in which topoisomerase IIα dissociates from partially relaxed plasmids during the course of the assay. In contrast, in the reaction catalyzed by hTop2αΔ1175, the negatively supercoiled band persists and appears to be shifted primarily to the position of fully relaxed products with minimal accumulation of partially relaxed intermediates. This pattern is indicative of a processive reaction, in which hTop2αΔ1175 remains largely bound to plasmids throughout the relaxation process.

the C-terminal domain of human topoisomerase IIα appears to play significant roles in modulating the DNA cleavage/ligation reaction of the enzyme and its response to anticancer agents.

Topoisomerase II is an essential enzyme that is responsible for resolving knots and tangles in chromosomal DNA that are generated during normal nuclear processes (1-7). Vertebrate species encode two distinct isoforms of the enzyme, topoisomerase IIα and β (8,9). Topoisomerase IIα expression is linked to the proliferative status of cells. Enzyme levels rise dramatically during periods of active growth and this isoform is required for replication and mitosis (10,11). In contrast, the expression of topoisomerase IIβ appears to be independent of cell growth and the specific physiological functions of the enzyme have yet to be defined (12,13). Although this isoform is dispensable at the cellular level, deletion of topoisomerase IIβ leads to neurological defects in developing mouse embryos (14).

Topoisomerase II functions by passing an intact double helix through a transient double-stranded break that it generates in a separate segment of DNA (2,3,5,7,15). Although the DNA strand passage activity of the enzyme is essential for cell survival, the double-stranded DNA breaks that it creates have the potential to destabilize the genome (3,5,7,16,17). As a result, topoisomerase II normally cleaves and ligates the genetic material very rapidly. However, physiological conditions that increase levels of topoisomerase II-associated DNA strand breaks convert the enzyme to a potent cellular toxin that fragments the genetic material (3,5,7,16, 18).

Because of its DNA cleavage activity, topoisomerase II plays a central role in cancer. The enzyme is the cytotoxic target for some of the most successful anticancer drugs currently in clinical use (3,5,7,19-21). All of these agents act by increasing cellular levels of topoisomerase II-mediated DNA strand breaks (3,5,7,19). In contrast, emerging evidence suggests that strand breaks generated by the enzyme may initiate chromosomal translocations that trigger specific types of leukemia (17,22-27).

It is difficult to study the DNA cleavage/ligation reaction of eukaryotic topoisomerase II because the enzyme normally maintains low equilibrium levels of strand breaks (2,3,5,7). Recently, however, novel viral type II topoisomerases were discovered that possess a dramatically (30– to 50–fold) higher DNA cleavage activity than their eukaryotic counterparts. These enzymes are encoded by the chlorella viruses, *Paramecium bursaria* chlorella virus-1 (PBCV-1)[1] and *Chlorella* virus Marburg-1 (CVM-1) (28-31). The basis for the high DNA cleavage activity of chlorella virus topoisomerase II is not known. However, both viral enzymes are considerably (~45-60 kDa) shorter than eukaryotic topoisomerase II and lack the entire C-terminal domain typical of the eukaryotic enzyme. The remaining portion of chlorella virus topoisomerase II displays nearly 50% amino acid sequence identity to human topoisomerase IIα. Furthermore, virtually every amino acid residue that is invariant or highly conserved within the eukaryotic type II family also is retained in chlorella virus topoisomerase II.

The enzymatic function of the C-terminal domain of eukaryotic topoisomerase II is not well defined. This region of the enzyme is highly variable, hydrophilic, and contains nuclear localization signals as well as phosphorylation sites (11,32,33). Based on structural studies, it has been proposed that the C-terminal domain of prokaryotic type II topoisomerases interacts with DNA, but comparable studies have not been carried out with eukaryotic topoisomerase II (34-37).

Human topoisomerase IIα is 1581 amino acids in length. Truncation of the protein at residue 1296 yields an enzyme that is active, but unable to complement a yeast strain encoding a temperature-sensitive topoisomerase II (33). This failure to complement presumably results from deletion of nuclear localization signals. Truncation of the human enzyme at amino acid

1121 yields an enzyme that is neither able to complement a deficient yeast strain nor able to catalyze DNA strand passage (33). Similar findings have been reported for yeast and *Drosophila* topoisomerase II (33,38-41).

The impact of the C-terminal domain on the DNA cleavage/ligation reaction of human topoisomerase IIα has not been analyzed. However, the enhanced DNA cleavage activity of chlorella virus topoisomerase II suggests that the C-terminal region of the enzyme may affect its ability to generate DNA strand breaks. Therefore, the human enzyme was truncated at amino acid 1175, which corresponds to the terminal amino acid in PBCV-1 topoisomerase II as determined by BLAST sequence alignment. The double-stranded DNA cleavage activity of the truncated human enzyme, hTop2αΔ1175, was 2–3–fold higher than that of full-length topoisomerase IIα. Increased cleavage appears to be due, at least in part, to a greater coordination between the two active site tyrosyl residues. Finally, similar to the viral enzyme, hTop2αΔ1175, displayed a decreased sensitivity toward anticancer drugs. On the basis of these results, it is concluded that the loss of the C-terminal domain contributes to the robust DNA scission activity and low drug sensitivity of chlorella virus topoisomerase II.

EXPERIMENTAL PROCEDURES

Enzymes and Materials

Negatively supercoiled pBR322 DNA was prepared using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Kinetoplast DNA (kDNA) was isolated from *Crithidia fasciculata* as described by Englund *et al*. (42). [γ-^{32}P]ATP (~5000 Ci/mmol) was obtained from ICN. Etoposide was from Sigma, amsacrine was from Bristol-Myers Squibb, and CP-115,953 was from Pfizer Global Research. Drugs were stored at 4°C as 10 or 20 mM stock solutions in 100% DMSO. Etoposide labeled with [^{3}H] (654 mCi/mmol) was obtained from Moravek Biochemicals and stored at —80 °C in 100% methanol. All other chemicals were analytical reagent grade.

Cloning of hTop2αΔ1175

A fragment of the human topoisomerase IIα gene in the YepWob6 plasmid was digested with KpnI and SmaI (43). PCR was utilized to introduce a stop codon at 1175 and a SmaI cut size downstream of the stop codon. The following sequences were used for the forward and reverse primers respectively: CATTGGTCAGTTTGGTACCAG-GCTACATGGTGGCAAGGATTCTGCTAG and CAGTGTATATTGCCCGGGATGATGTTA-TTAATAAATGTAGCCAAGTCTTCTTTCCACAAATCTGATGG. DNA was denatured at 95 °C for 6 min and subjected to 30 cycles of PCR using the following program: denaturation at 95 °C for 1 min, annealing at 53 °C for 1 min and primer extension at 72 °C for 4 min. Following the last round of PCR, primers were allowed to extend for an additional 8 min at 72 °C and the final product was purified by gel electrophoresis. The new insert was digested with SmaI and KpnI and ligated into the previously digested YepWob6 plasmid, and transformed into *Escherichia coli* DH5α. The truncated human topoisomerase IIα construct was sequenced to confirm the primary structure of the gene. The resultant plasmid carrying *hTop2αΔ1175* was transformed into *Saccharomyces cerevisiae* JEL-1Δ*top1* by heat shock.

Expression and Purification of Recombinant Type II Topoisomerases

PBCV-1 topoisomerase II was expressed in *S. cerevisiae* JEL-1Δ*top1* and purified by a modification (31) of the procedure of Lavrukhin *et al*. (28). Human topoisomerase IIα and hTop2αΔ1175 were expressed in *S. cerevisiae* JEL-1Δ*top1* and purified as described by Kingma *et al*. (44).

Decatenation of Kinetoplast DNA (kDNA)

DNA decatenation reactions were carried out as described by Miller *et al*. (45). Assay mixtures contained 1 mM ATP, 5 nM kDNA, and 0–20 nM topoisomerase IIα or hTop2αΔ1175 in a total of 20 μL of topoisomerase II activity buffer (10mM Tris-HCl, pH 7.9, 175 mM KCl, 5 mM MgCl$_2$, 0.1 mM NaEDTA, 2.5% glycerol). Decatenation was initiated by the addition of enzyme, followed by incubation for 30 min at 37 °C. Reactions were terminated by the addition of 3 μL of stop solution (0.5% SDS, 77 mM EDTA). Proteinase K was added (2 μL of a 0.8 mg/mL solution) and reactions were incubated for 30 min at 45 °C to digest the enzyme. Samples were mixed with agarose gel loading buffer (60% sucrose in 10 mM Tris-HCl, pH 7.9) and subjected to electrophoresis in 1% agarose gels in 100 mM Tris-borate, pH 8.3, and 2 mM EDTA. Gels were stained for 30 min with 0.5 μg/mL ethidium bromide and DNA bands were visualized by UV light and quantified using an Alpha Innotech digital imaging system.

DNA Relaxation

DNA relaxation assays were carried out as described by Fortune and Osheroff (46). For catalytic reactions, assay mixtures contained 1 nM topoisomerase IIα or hTop2αΔ1175, 1 mM ATP, and 10 nM negatively supercoiled pBR322 DNA in a total of 20 μL of topoisomerase II activity buffer. Samples were incubated at 37 °C for 0–30 min. For non-turnover reactions, assay mixtures contained 110 nM human enzymes, 5 nM negatively supercoiled pBR322 DNA, and 50 μM ATP in a total of 20 μL of topoisomerase II activity buffer. Samples were incubated for 0–60 s at 37 °C. In all cases, reactions were stopped by the addition of 3 μL of stop solution. Samples were processed and products were quantified as in the preceding section.

DNA Cleavage

Plasmid DNA cleavage reactions were based on the procedure of Fortune and Osheroff (46). Unless stated otherwise, reaction mixtures contained 10 nM negatively supercoiled pBR322 DNA in a total of 20 μL of DNA cleavage buffer (human type II topoisomerases, 10 mM Tris-HCl, pH 7.9, 100 mM KCl, 5 mM MgCl$_2$, 0.1 mM NaEDTA, and 2.5% glycerol; PBCV-1 topoisomerase II, 10 mM Tris-HCl, pH 8.5, 62.5 mM NaCl, 62.5 mM KCl, 0.1 mM NaEDTA, 2.5 mM MgCl$_2$, and 2.5% glycerol). Where noted, 0-200 μM etoposide, amsacrine, or CP-115,953 was added to cleavage reactions. DNA cleavage was initiated by the addition of topoisomerase II (final enzyme concentrations were hTop2αΔ1175 or topoisomerase IIα, 0.2-1 μM; PBCV-1 topoisomerase II, 0.02 μM). Reaction mixtures were incubated for 6 min at 37 °C (human) or 25 °C (PBCV-1) to establish cleavage/religation equilibria. Cleavage intermediates were trapped by the addition of 2 μL of 5% SDS and 2 μL of 250 mM NaEDTA, pH 8.0, (human) or 2 μL of 1.15% SDS and 2 μL of 115 mM NaEDTA, pH 8.0 (PBCV-1). Proteinase K was added (2 μL of 0.8 mg/mL) and mixtures were incubated for 30 min at 45 °C to digest the type II enzyme. Samples were mixed with 2 μL of agarose gel loading buffer, heated for 2 min at 45 °C, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate, pH 8.3, and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. Double- and single-stranded DNA cleavage was monitored by the conversion of negatively supercoiled plasmid to linear and nicked circular molecules, respectively. DNA bands were quantified by digital imaging as described above.

DNA sites cleaved by hTop2αΔ1175 or human topoisomerase IIα were mapped as described by a modification (47) of the procedure of O'Reilly and Kreuzer (48). A linear 4330 bp fragment (HindIII/EcoRI) of pBR322 plasmid DNA singly labeled with [^{32}P]phosphate on the 5'-terminus of the HindIII site was used as the cleavage substrate. Reaction mixtures contained 0.35 nM DNA molecules and 60 nM enzyme in a total of 50 μL of DNA cleavage buffer. Assays were carried out in the absence or presence of 50 μM etoposide. Reactions were started by the addition of enzyme and mixtures were incubated for 10 min at 37 °C. Cleavage intermediates were trapped and processed as described above. Products were precipitated twice

in 100% ethanol, dried, resuspended in 40% formamide, 8.4 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanole FF, and subjected to electrophoresis in 6% polyacrylamide gels. Gels were fixed in 10% methanol/10% acetic acid for 5 min and dried. DNA cleavage products were visualized using a Bio-Rad Molecular Imager FX.

It should be noted that DNA cleavage reactions were performed in the absence of ATP. The nucleotide cofactor does not influence the mechanism of topoisomerase II-mediated DNA scission (5). Furthermore, the omission of ATP from cleavage assays is advantageous in that the topological state of the DNA is not altered during the reaction. Thus, negatively supercoiled substrates are neither relaxed nor catenated over the course of the assay (5). Alterations in DNA topology have the potential to become problematic, especially in light of the fact that the efficiency of DNA strand passage differs between full-length human topoisomerase IIα and hTop2αΔ1175 (see Figures 2 and 3).

Topoisomerase II-DNA Binding

The ability of topoisomerase IIα or hTop2αΔ1175 to bind DNA was assessed using a nitrocellulose filter-binding assay (31). Linear pBR322 DNA radiolabeled with [^{32}P]phosphate was prepared as described in the preceding section. Binding mixtures contained 5 nM DNA and 10–400 nM enzyme in a total of 20 μL of DNA binding buffer (10 mM Tris-HCl, pH 7.9, 5 mM KCl, 0.1 mM NaEDTA, and 2.5% glycerol) and were incubated for 6 min at 37 °C. Nitrocellulose membranes (0.45 μm HA, Millipore) were prepared by incubation in DNA binding buffer for 10 min. Samples were applied to the membranes and filtered in vacuo. Membranes were washed 3 times with 1 mL of DNA binding buffer, dried, and submerged in 8 mL of scintillation fluid (Econo-Safe, Research Products International). Radioactivity remaining on the membranes was quantified using a Beckman LS 5000 TD Scintillation Counter. The percent DNA bound to topoisomerase II was determined based on the ratio of the radioactivity on the membranes vs. that of the input DNA.

DNA Religation

DNA religation mediated by topoisomerase IIα or hTop2αΔ1175 was monitored according to the procedure of Robinson and Osheroff (49). Enzyme—DNA cleavage/religation equilibria were established as above for plasmid DNA cleavage. Religation was initiated by shifting reactions from 37 °C to 0 °C. Reactions were stopped 0–30 s after the temperature shift by the addition of 2 μL of 5% SDS followed by 2 μL of 250 mM NaEDTA, pH 8.0. Samples were processed and analyzed as described for topoisomerase II plasmid DNA cleavage reactions. Religation was monitored by quantifying the loss of linear DNA.

Topoisomerase II-Etoposide Binding

The ability of human topoisomerase IIα, hTop2αΔ1175, or PBCV-1 topoisomerase II to bind etoposide was monitored as described by Kingma and Osheroff (50). Nitrocellulose membranes (0.45 μm HA) were presoaked in DNA cleavage buffer for 10 min. Binding mixtures contained 3 μM enzyme and 25-100 μM [^{3}H]etoposide in a total of 60 μL of cleavage buffer. Samples were incubated at 25 °C for 6 min and applied to the nitrocellulose membranes in vacuo. Filters were immediately washed 3 times with 1 mL of ice-cold wash DNA cleavage buffer, dried, and submerged in 8 mL of scintillation fluid. Radioactivity remaining on the membranes was quantified as described above. The amount of radioactive etoposide remaining on the filter in the absence of enzyme was subtracted for binding calculations.

RESULTS

Because of the deleterious effects of DNA strand breaks in proliferating cells, eukaryotic type II topoisomerases normally maintain very low levels of DNA scission intermediates. Typically,

less than 0.2% of a population of human topoisomerase IIα is in a DNA cleavage complex at any given time (2,3,5,7). In marked contrast, topoisomerase II from chlorella virus PBCV-1 or CVM-1 maintains a dramatically higher level of cleavage intermediates (typically >25%) (29-31). The major structural difference between the chlorella and human type II topoisomerases is the lack of a C-terminal domain in the viral enzyme. Therefore, to determine the impact of the C-terminal domain on the DNA cleavage activity of topoisomerase IIα, we truncated the human enzyme at amino acid 1175. This position corresponds to the terminal residue in PBCV-1 topoisomerase II, as determined by BLAST sequence alignment. The resulting enzyme, hTop2αΔ1175, was expressed in yeast and purified along with full-length topoisomerase IIα and PBCV-1 topoisomerase II.

Catalytic Activity of hTop2αΔ1175

Before addressing the effects of the C-terminal domain on the DNA cleavage activity of human topoisomerase IIα, we determined whether the truncated enzyme retained overall catalytic activity. Two independent assays were used to assess the ability of hTop2αΔ1175 to catalyze the DNA strand passage reaction. First, the ability of the truncated enzyme to decatenate kDNA was characterized (Figure 1). hTop2αΔ1175 was able to unlink catenated DNA rings, albeit with an activity that was ~2-fold lower than that of full-length topoisomerase IIα.

To further characterize the influence of the C-terminal region on catalytic activity, the ability of hTop2αΔ1175 to relax negatively supercoiled plasmid DNA was determined. Under optimal reaction conditions for human topoisomerase IIα, hTop2αΔ1175 was able to remove negative supercoils from plasmid molecules (Figure 2). However, reaction rates were lower than observed for the full-length enzyme. In addition, as determined by the loss of the negatively supercoiled band relative to the production of partially relaxed plasmid species, the reaction catalyzed by the truncated enzyme appeared to be more processive.[2] This latter result was unexpected. If the C-terminal domain of eukaryotic topoisomerase II interacts with DNA, its presence might enhance enzyme-nucleic acid binding, and hence increase processivity. However, as discussed below, the assumption that the C-terminal domain of human topoisomerase IIα increases the affinity of the enzyme for DNA is not correct.

The decreased catalytic activity of hTop2αΔ1175 could be due to deficiencies in DNA strand passage or enzyme turnover. To distinguish between these two possibilities, a non-turnover DNA relaxation assay was utilized. Reaction mixtures contained a 22-fold stoichiometric excess of either full-length topoisomerase IIα or hTop2αΔ1175 over plasmid molecules. This excess eliminates the need for enzyme turnover and dissociation during the reaction, since complete relaxation of the plasmid can be achieved if each topoisomerase II molecule catalyzes only a single DNA strand passage event. Human topoisomerase IIα relaxed the DNA substrate within 60 s (Figure 3). In contrast, hTop2αΔ1175 displayed little DNA relaxation over the course of the assay. This finding suggests that hTop2αΔ1175 catalyzes the DNA strand passage event more slowly than the full-length enzyme. Taken together, the above results confirm that the C-terminal domain of the human enzyme is dispensable for catalytic activity. However, this portion of the protein contributes to the catalytic efficiency of topoisomerase IIα.

DNA Cleavage Activity of hTop2αΔ1175

The most dramatic enzymatic difference between human topoisomerase IIα and chlorella virus topoisomerase II is the enhanced double-stranded DNA scission activity of the viral enzyme (29-31). Therefore, the ability of hTop2αΔ1175 to cleave DNA was compared to that of the full-length human enzyme. As seen in Figure 4, loss of the C-terminal domain of topoisomerase IIα increased levels of double-stranded DNA cleavage. Over a range of enzyme concentrations, hTop2αΔ1175 generated 2 to 3 times more double-stranded DNA breaks than did full-length topoisomerase IIα. While this difference does not approach the enhancement observed with

the viral enzyme (Figure 4, *insert*), the loss of the C-terminal domain clearly enhances the ability of topoisomerase IIα to cleave DNA.

DNA Binding

Several factors may contribute to the increased DNA cleavage activity of hTop2αΔ1175. One possibility is that the truncated enzyme binds more tightly to its DNA substrate. To test this hypothesis, the ability of hTop2αΔ1175 to bind DNA was compared to that of full-length human topoisomerase IIα. As seen in Figure 5, the affinity of the two enzymes for DNA was similar. Thus, the increase in DNA scission that accompanies the loss of the C-terminal domain is not due to an enhanced affinity for nucleic acids.

DNA Religation

A number of topoisomerase II-targeted anticancer drugs raise levels of enzyme-generated DNA strand breaks by inhibiting the religation of cleaved DNA molecules (3,5,7,51). Thus, the rate of DNA religation mediated by hTop2αΔ1175 was determined (Figure 6). The truncated enzyme resealed DNA breaks at a rate that was slightly faster than that of human topoisomerase IIα. This result indicates that the loss of the C-terminal domain does not increase levels of double-stranded DNA breaks by impairing the religation reaction of the enzyme.

Coordination Between Enzyme Subunits

PBCV-1 topoisomerase II displays significantly greater coordination between its two active site tyrosyl residues than does human topoisomerase IIα. In this regard, the overwhelming majority of DNA breaks created by the chlorella virus enzyme are double-stranded in nature. Under optimal reaction conditions, PBCV-1 topoisomerase II generates nearly five double-stranded DNA breaks for every nick (i.e., single-stranded break) that it creates (Figure 7). In contrast, topoisomerase IIα displays markedly lower coordination between its active site tyrosyl residues (52). For every double-stranded break that the human enzyme creates, it generates 2–5 nicks (i.e., cleavage intermediates in which one or the other active site tyrosyl residue, but not both, has cleaved the DNA) (Figure 7). It is not obvious how this coordination contributes to the catalytic activity of topoisomerase IIα. However, the ability of the two active site tyrosyl residues to work in concert clearly should enhance the generation of double-stranded DNA breaks at the expense of single-stranded cuts.

To determine whether the C-terminal domain of topoisomerase IIα affects active site coordination, the ratio of single-to double-stranded DNA breaks generated by hTop2αΔ1175 was calculated. Results indicate that active site coordination of the truncated human enzyme was intermediate between those of full-length topoisomerase IIα and PBCV-1 topoisomerase II. Based on the averages of three independent DNA cleavage titrations, hTop2αΔ1175 produced approximately one nick for every double-stranded DNA break that it generated (Figure 7). This marked rise in active site coordination can account, at least in part, for the increase in double- stranded DNA breaks that accompany the loss of the C-terminal domain of topoisomerase IIα.

Sensitivity of hTop2αΔ1175 to Anticancer Drugs

Compared to human topoisomerase IIα, PBCV-1 topoisomerase II displays low sensitivity to DNA cleavage-enhancing anticancer drugs. When global levels of DNA cleavage are monitored using plasmid substrates, the viral enzyme is nearly refractory to a number of topoisomerase II-targeted agents (29).

It is not apparent why PBCV-1 topoisomerase II is resistant to anticancer agents. One possibility is that the C-terminal domain of the enzyme contributes to drug binding.

Consequently, the ability of topoisomerase IIα, hTop2αΔ1175, and PBCV-1 topoisomerase II to bind [³H]etoposide was assessed using a nitrocellulose filter technique (Figure 8). Results indicate that the three enzymes bind similar amounts of the drug. Therefore, the insensitivity of the viral enzyme toward anticancer agents, at least etoposide, is not due to a lack of drug binding.

Etoposide, amsacrine, and CP-115,953 increase double-stranded DNA cleavage mediated by full-length topoisomerase IIα 5– to 10–fold (Figure 9). Since the C-terminal domain of the human enzyme does not appear to contribute to drug binding, the effects of these anticancer agents on the DNA cleavage activity of hTop2αΔ1175 were determined. As seen in Figure 9, the truncated enzyme was less sensitive to the drugs. In most cases, the relative DNA cleavage enhancement observed was approximately 50% that seen with full-length topoisomerase IIα.

Despite the diminished susceptibility of hTop2αΔ1175 for anticancer drugs, the loss of the C-terminal domain does not appear to alter the DNA cleavage specificity of the truncated enzyme, either in the absence or presence of etoposide (Figure 10). It should be noted that the DNA samples shown in Figure 10 were subjected to electrophoresis under denaturing conditions. Therefore, the overall levels of DNA cleavage seen in the autoradiogram reflect both single- and double-stranded DNA breaks. Although hTop2αΔ1175 generates higher levels of double-stranded DNA breaks than full-length human topoisomerase IIα, the truncated enzyme produces considerably lower levels of single-stranded breaks. Consequently, the intensity of the DNA cleavage pattern observed with the full-length enzyme in the absence of etoposide is somewhat greater than that seen for hTop2αΔ1175.

Finally, in most cases, the intensity of etoposide-induced DNA cleavage bands generated by the truncated enzyme was lower than that of the corresponding bands produced by topoisomerase IIα. However, in a few instances, the intensity of bands generated by hTop2αΔ1175 in the presence of etoposide was comparable to or slightly greater than those produced by the full-length enzyme. Whether this result reflects differences in levels of single-stranded DNA cleavage or subtle effects of the C-terminal domain on DNA cleavage site utilization in the presence of anticancer drugs is not known at the present time.

Taken together, the above findings indicate that the C-terminal domain of topoisomerase II substantially influences the degree to which the enzyme responds to anticancer agents, but not the sites at which it forms DNA cleavage complexes.

DISCUSSION

The C-terminal domain of eukaryotic type II topoisomerases varies considerably from species to species (38,41,53,54). It is believed that this portion of the protein contributes to the physiology of topoisomerase II, as it contains phosphorylation sites and nuclear localization signals (11,32,33,55). However, the contribution of the C-terminal domain to the enzymatic activity of topoisomerase II has yet to be defined. Structural studies indicate that the C-terminal domain of prokaryotic type II topoisomerases interacts with nucleic acids (34-37). Unfortunately, comparable studies have not been carried out with eukaryotic enzymes.

In contrast to eukaryotic topoisomerase II, type II enzymes from chlorella virus completely lack the C-terminal domain. These viral enzymes are characterized by a robust DNA cleavage activity and a high coordination between their two active site tyrosyl residues (28-31). Therefore, as a first step toward characterizing the contribution of the C-terminal domain of human topoisomerase IIα to enzyme function, the human protein was truncated at amino acid 1175, which corresponds to the C-terminal residue of PBCV-1 topoisomerase II.

The overall catalytic activity of the resulting enzyme, hTop2α∆1175, was lower than that of the full-length protein, however, it displayed a double-stranded DNA cleavage activity that was ~2— to 3—fold higher than that of topoisomerase IIα. While the truncated enzyme did not necessarily produce more total DNA breaks than its full-length counterpart, the cuts that it generated were primarily double-stranded in nature. In contrast, the DNA breaks created by topoisomerase IIα were primarily single-stranded. Coupled with the findings that the full-length and truncated enzymes cleaved DNA at a similar spectrum of sites and displayed comparable rates of DNA ligation, we conclude that the enhanced double-stranded DNA cleavage observed for hTop2α∆1175 is due largely to an increase in active site coordination.

It is not obvious why the two active site tyrosyl residues of human topoisomerase IIα normally do not act in a concerted fashion or why the enzyme would evolve a C-terminal domain that contributed to this lack of coordination. One possibility is that this absence of coordination contributes to the preservation of genomic stability by decreasing the likelihood that topoisomerase IIα would generate double-stranded DNA breaks in the genetic material in the absence of strand passage.

Despite the high DNA scission activity of chlorella virus type II topoisomerases, these enzymes are relatively insensitive to the DNA cleavage-enhancing effects of anticancer drugs (29,31). Remarkably, hTop2α∆1175 also displayed a distinctly lower susceptibility to anticancer agents. This loss of sensitivity does not result from a decreased affinity for drugs (at least for etoposide). Therefore, the C-terminal domain of topoisomerase II appears to play a role in the response of the enzyme to anticancer agents.

The C-terminal domain of human topoisomerase IIα has long been known to play an important role in the physiological regulation of the enzyme. Results of the present study indicate that this region of the protein also plays a part in modulating topoisomerase II-mediated catalysis. The mechanistic basis for the contributions of the C-terminal domain to enzyme function is not known. It is clear from the properties of hTop2α∆1175 that the C-terminal domain does not contain any essential "active site" amino acid residues. Thus, it is possible that this portion of the enzyme may act by altering the conformation of topoisomerase IIα. Alternatively, based on the finding that the C-terminal domain of bacterial DNA gyrase plays a role in mediating enzyme-DNA binding, this region of the human enzyme may subtly alter interactions between the active site of topoisomerase IIα and its nucleic acid substrate. Ultimately, structural studies will be required to address this important issue.

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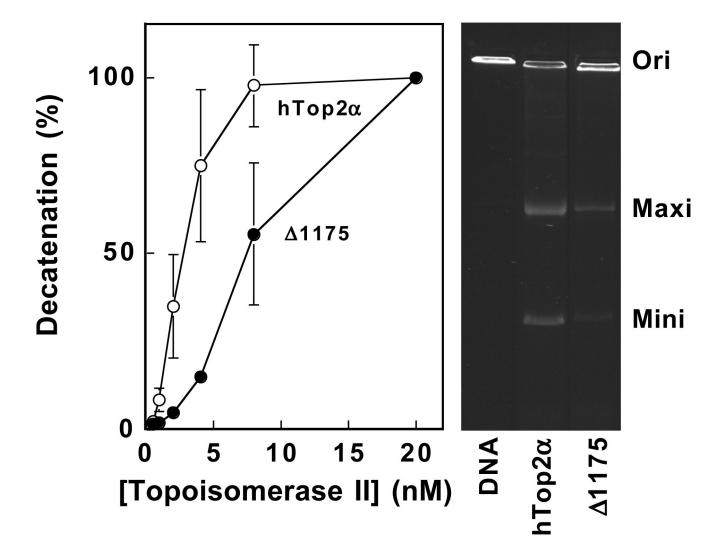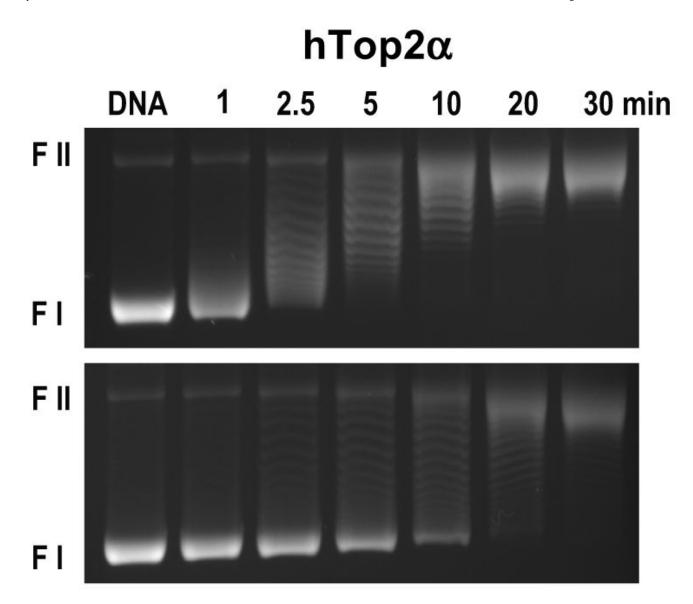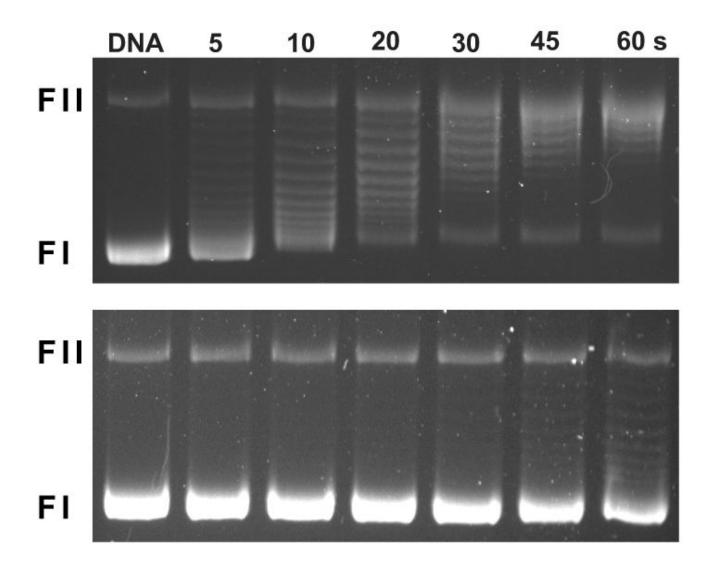
Figure 1.
Decatenation of kDNA by human topoisomerase IIα and hTop2αΔ1175. The percent
decatenation was determined by quantifying minicircles that were released from kDNA
networks by human topoisomerase IIα (hTop2α, open circles) or hTop2αΔ1175 (Δ1175, closed
circles) and is shown on the left. Error bars represent the standard deviation of three
independent experiments. The image at the right is an ethidium bromide-stained gel showing
kDNA decatenation in the absence of enzyme (DNA), or in the presence of human
topoisomerase IIα or hTop2αΔ1175. Reaction mixtures contained 4 nM enzyme. Positions of
the kDNA networks at the origin, as well as those of the released DNA maxicircles and
minicircles, are indicated.

Figure 2.
Relaxation of negatively supercoiled pBR322 plasmid DNA by topoisomerase IIα and
hTop2αΔ1175. The relaxation of negatively supercoiled pBR322 plasmid DNA by human
topoisomerase IIα (hTop2α, top) and hTop2αΔ1175 (bottom) is shown. Relaxation reactions
were carried out from 0-30 min using 1 nM enzyme. Ethidium bromide-stained gels that are
representative of 3 independent experiments are shown. Negatively supercoiled DNA (form I,
FI) and nicked circular DNA (form II, FII) are indicated. A DNA control in the absence of
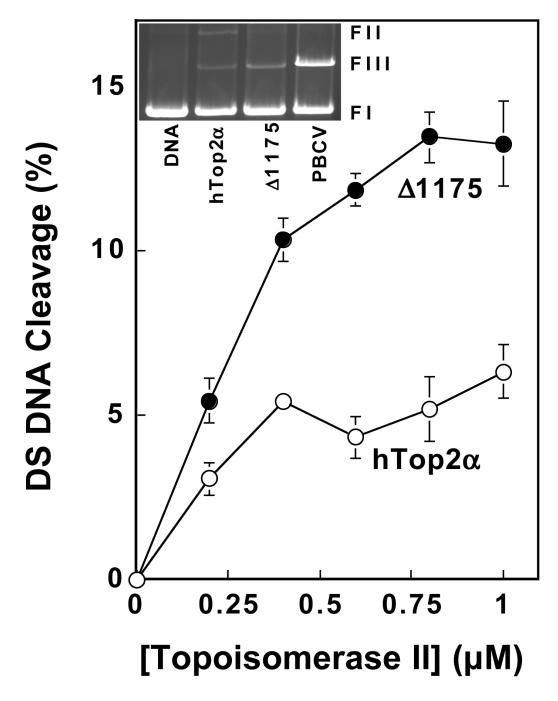enzyme is shown (DNA).

Figure 3.
Non-turnover relaxation of negatively supercoiled pBR322 plasmid DNA by topoisomerase IIα and hTop2αΔ1175. The non-turnover relaxation of negatively supercoiled pBR322 plasmid DNA by human topoisomerase IIα (hTop2α, top) and hTop2αΔ1175 (bottom) is shown. Relaxation reactions were carried out from 0-60 s using 110 nM enzyme and 5 nM DNA. Ethidium bromide-stained gels that are representative of 3 independent experiments are shown. Negatively supercoiled DNA (form I, FI) and nicked circular DNA (form II, FII) are indicated. A DNA control in the absence of enzyme is shown (DNA).

Figure 4.
Double-stranded DNA cleavage by type II topoisomerases. The ability of human topoisomerase IIα (hTop2α, open circles) and hTop2αΔ1175 (Δ1175, closed circles) to cleave negatively supercoiled pBR322 plasmid DNA is shown. A range of enzyme (0.2-1 μM) was used at a constant concentration of 10 nM plasmid. Double-stranded DNA cleavage is expressed as the percentage of circular plasmid substrate that is converted to linear molecules. Error bars represent the standard deviation of three independent experiments. The inset shows an ethidium bromide-stained agarose gel of a DNA cleavage reaction in the absence of enzyme (DNA), in the presence of 0.6 μM human topoisomerase IIα (hTop2α) or hTop2αΔ1175 (Δ1175), or 0.02 μM PBCV-1 topoisomerase II (PBCV). Double-stranded DNA cleavage

converts negatively supercoiled plasmid (form I, FI) to linear molecules (form III, FIII). The position of nicked circular DNA (form II, FII) also is indicated.
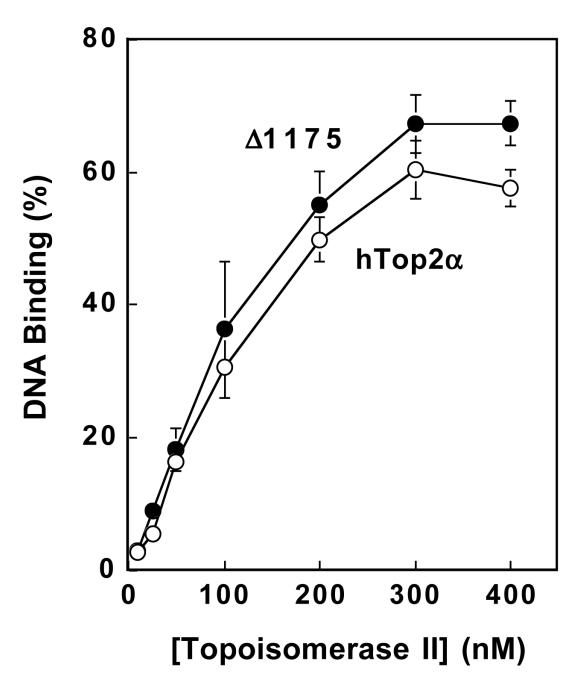
Figure 5.
Binding of topoisomerase IIα and hTop2αΔ1175 to DNA. The percent binding of radiolabeled linear pBR322 DNA to human topoisomerase IIα (hTop2α, open circles) or hTop2αΔ1175 (Δ1175, closed circles) was determined by the ratio of radioactivity retained on a nitrocellulose filter vs. the input amount of radioactivity. A range of 0-400 nM enzyme was examined at a constant concentration of 5 nM linear pBR322. Error bars represent the standard deviation of three independent experiments.
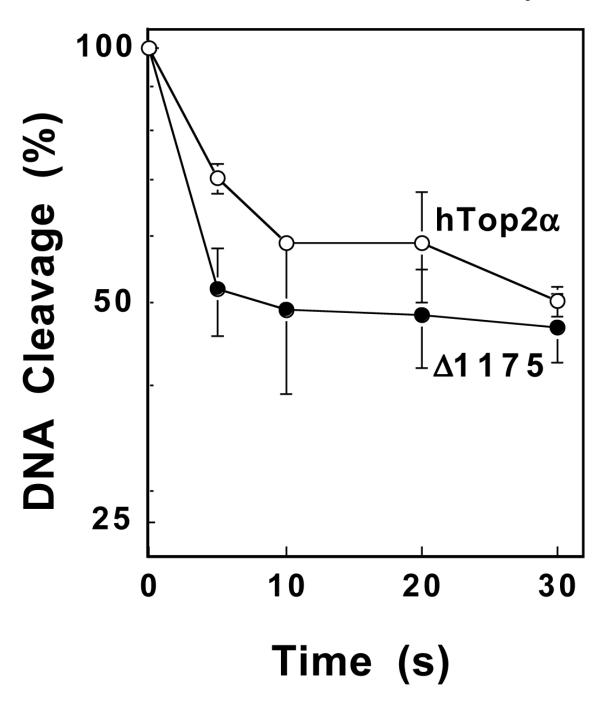
Figure 6.
DNA religation mediated by topoisomerase IIα and hTop2αΔ1175. DNA religation mediated by human topoisomerase IIα (hTop2α, open circles) or hTop2αΔ1175 (Δ1175, closed circles) is shown. The DNA cleavage/religation equilibrium was established at 37°C and religation was initiated by shifting samples to 0°C. The DNA cleavage observed at equilibrium for each enzyme was set to 100% at time zero. Error bars represent the standard deviation of three independent experiments.
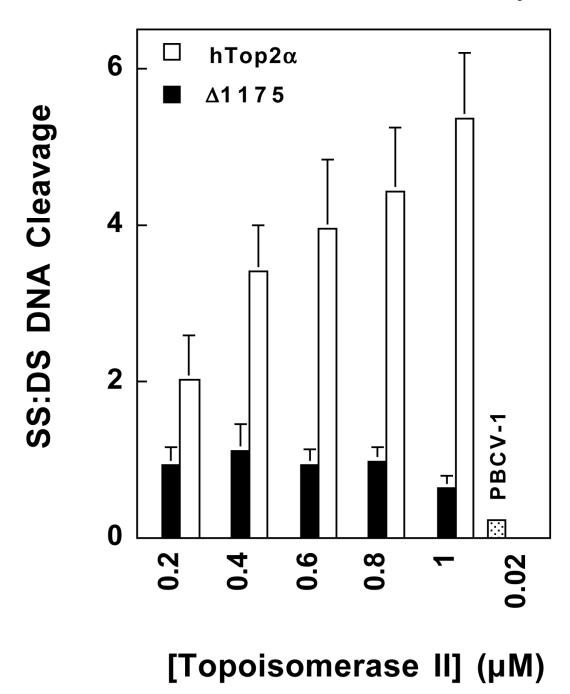
Figure 7.
The ratio of single-stranded to double-stranded DNA breaks generated by type II topoisomerases. The ratio of single-stranded DNA breaks to double-stranded DNA breaks generated by human topoisomerase IIα (hTop2α, open bars), hTop2αΔ1175 (Δ1175, closed bars), or PBCV-1 topoisomerase II (PBCV-1, shaded bar) is shown. A range of 0.2-1 μM enzyme was examined for the human enzymes, and the ratio of breaks produced by 0.02 μM PBCV-1 topoisomerase II is shown for comparison. Double-stranded and single-stranded DNA cleavage is expressed as the percentage of supercoiled substrate that is converted to linear or nicked circular molecules, respectively. Error bars represent the standard deviation of 3 independent experiments.
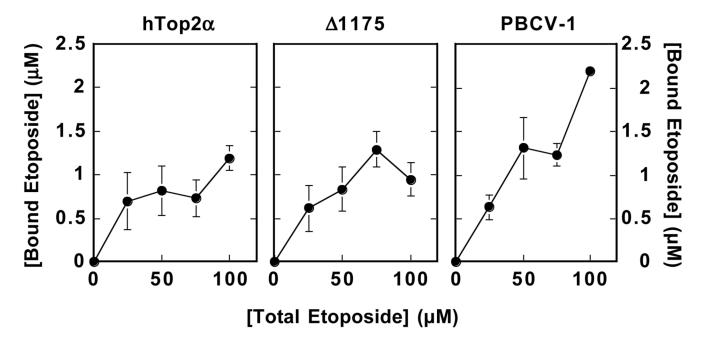
Figure 8.
Etoposide binding to type II topoisomerases. The ability of 3 μM human topoisomerase IIα (hTop2α, left panel), hTop2αΔ1175 (Δ1175, middle panel) or PBCV-1 topoisomerase II (PBCV-1, right panel) to bind [³H]etoposide was examined. The free and bound fractions of etoposide were separated by filtration through nitrocellulose membranes, and the binding of etoposide to the filter in the absence of enzyme was subtracted as background. Error bars represent the standard deviation of at least 3 independent experiments.
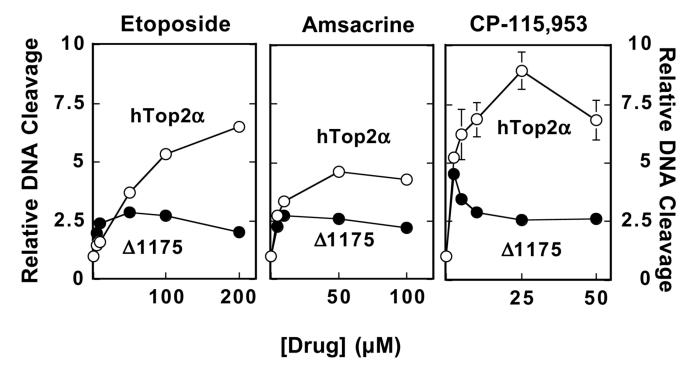
Figure 9.
Double-stranded DNA cleavage by type II enzymes in the presence of topoisomerase II poisons. The ability of human topoisomerase IIα (hTop2α, open circles) and hTop2αΔ1175 (Δ1175, closed circles) to cleave negatively supercoiled pBR322 plasmid was determined in the presence of three topoisomerase II-targeted anticancer drugs, etoposide, amsacrine, and CP-115,953. Relative DNA cleavage is based on levels of double-stranded DNA breaks generated by the respective enzymes in the absence of drugs. For each individual enzyme, DNA cleavage in the absence of drugs was set to 1. Error bars represent the standard deviation of three independent experiments.
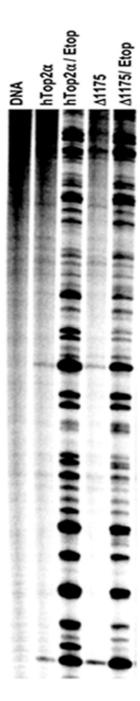
Figure 10.
Distribution of topoisomerase II-mediated DNA cleavage sites on a linear DNA substrate. A singly end-labeled 4330 bp fragment of pBR322 was employed. The autoradiogram shows a DNA control (DNA) and cleavage in the presence of human topoisomerase IIα (hTop2α), topoisomerase IIα plus 50 μM etoposide (hTop2α/Etop), hTop2αΔ1175 (Δ1175), or hTop2αΔ1175 plus 50 μM etoposide (Δ1175/Etop). Data are representative of three independent experiments.