Analysis of a Core Domain in Drosophila DNA Topoisomerase II
TARGETING OF AN ANTITUMOR AGENT ICRF-159*

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To investigate the biochemical properties of individual domains of eukaryotic topoisomerase (topo) II, two truncation mutants of Drosophila topo II were generated, ND406 and core domain. Both mutants lack the ATPase domain, corresponding to the N-terminal 406 amino acid residues in Drosophila protein. The core domain also lacks 240 amino acid residues of the hydrophilic C-terminal region. The mutant proteins have lost DNA strand passage activity while retaining the ability to cleave the DNA and the sequence preference in protein/DNA interaction. The cleavage experiments carried out in the presence of several topo II poisons suggest that the core domain is the key target for these drugs. We have used glass-fiber filter binding assay and CsCl density gradient ultracentrifugation to monitor the formation of a salt-stable, protein-clamp complex. Both truncation mutant proteins can form a clamp complex in the presence of an antitumor agent, ICRF-159, suggesting that the drug targets the core domain of the enzyme and promotes the intradimeric closure at the N-terminal interface of the core domain. Furthermore, the salt stability of the closed protein clamp induced by ICRF-159 depends on the presence and closure of the N-terminal ATPase domain.

DNA topoisomerases are enzymes that catalyze changes in the topology of DNA via a mechanism involving the transient breakage and rejoining of phosphodiester bonds in the DNA backbone (for reviews, see Refs. 1–7). Their biological functions include the removal of DNA supercoils generated during various cellular processes, such as DNA replication and transcription, decatenation of the intertwined DNA duplexes for the segregation of chromosomes, and regulating the cellular level of DNA supercoiling.

DNA topoisomerases can be divided into three subfamilies, types IA, IB, and II, on the basis of sequence homology and mechanism of action. Type II enzymes (topo II)1 catalyze the ATP-dependent passage of a DNA segment through a transient double-strand break in another segment. In such a reaction, the enzyme-mediated reversible cleavage is generated by the transesterification reactions between a pair of active site tyrosines and two DNA phosphodiester bonds staggered 4 base pairs apart. Eukaryotic topo II has a dimeric structure, whereas bacterial topo IIIs, including gyrase and topo IV, are A2B2 heterotetramers. However, the N-terminal and the central part of eukaryotic topo II are homologous to the bacterial gyrB and gyrA subunits, respectively.

Recent biochemical and structural studies have provided further understandings on the mechanism of topo II-catalyzed reaction (Refs. 8–11; also reviewed in Ref. 12). The reaction starts with the enzyme binding to a segment (G-segment) of DNA and generating a double strand break to serve as a protein-mediated DNA gate. The ATPase domain of the enzyme, which is located at the N-terminal part of each subunit, can dimerize and close the N-terminal protein clamp (N-gate) upon the binding of ATP. This closure can capture another DNA segment (T-segment) and trigger a cascade of conformational changes in the enzyme-DNA complex, which results in the passage of T-segment through the double-strand DNA break in the G-segment, the resealing of the DNA gate, and the exit of transported T-segment through the C-terminal protein clamp (C-gate).

Results from the mapping of proteolytic cleavage sites revealed at least three domains within each subunit of eukaryotic topo II (Refs. 13–15; outlined in Fig. 1). The N-terminal domain is homologous to the N-terminal half of gyrB subunit, which possesses the ATPase activity (16, 17). The core domain includes the regions homologous to the C-terminal one-third of the gyrB subunit (B’) and N-terminal two-thirds of the gyrA subunit (A’). This part of the gyrA subunit contains the active site tyrosine and is involved in breaking and rejoining the DNA backbone (reviewed in Ref. 5). Recently, the crystal structure of the core domain of yeast topo II has been solved, showing a heart-shaped profile and two dimer contacts at the B’-B’ and A’-A’ interfaces (10). The hydrophilic C-terminal domain of eukaryotic topo II is less conserved between different species and has been shown to be dispensable for the biochemical activities of topo II (18–21).

Some of the antibiotics and antitumor drugs target the different conformations of the topo II-DNA complex during the catalytic cycle. Topo II poisons, such as amsacrine, etoposide, and quinolones, can stimulate the topo II-mediated DNA cleavage by either inhibiting the rate of DNA religation or enhancing the forward rate of cleavage (Ref. 22; also reviewed in Refs. 23 and 24). Other drugs such as bisdioxopiperazine derivatives (including ICRF-159 and ICRF-193), aclarubicin, fostriecin, and merbarone, belong to topo II inhibitors that do not stabilize topo II/DNA cleavable complex but instead inhibit the overall catalytic activities (25–28). Although the mechanisms of these structurally disparate drugs are unclear, recent studies on ICRF-193 have indicated that it targets topo II in yeast cells (29) and traps the enzyme in a closed clamp conformation (30).

To further investigate the biochemical properties of individual domains of eukaryotic topo II, two truncation mutants of
Drosophila topo II were generated, ND406 and core domain (Fig. 1). Both mutants lack the ATPase domain, corresponding to the N-terminal 406-amino acid residues in Drosophila topo II. The core domain also lacks 240 amino acid residues of the hydrophilic C-terminal region. Our studies have shown that in the presence of ICRF-159, both mutants could trap circular DNA in a topological link, suggesting that the drug targets the core domain of the enzyme and promotes the intradimeric closure at the N-terminal interface in this protein.

**EXPERIMENTAL PROCEDURES**

**Construction of Truncation Mutants—**Both ND406 and core constructs share the same 5'-end deletion point. We used polymerase chain reaction to remove the first 1477 base pairs from the top2 cDNA. The oligonucleotide primers used were as follows: 1) 5'-CCA GGG TCC ATG TCA ACT AAG GGC GGT CGC TGT AAT GAA CTC GAG 3' and 2) 5'-TGT AAT GAA CTC CTC GAG 3'. Primer 1 contains a BamHI site (underlined) and the first three codons of yeast topo II (italicized), followed by nucleotides 1478–1495 of Drosophila top2 cDNA. Primer 2 contains nucleotides 1910–2033 of Drosophila topo2 cDNA. Primer 3 contains nucleotides 1910–1927 of the Drosophila top2 cDNA with an XhoI site (underlined) at its 3'-end. The PCR fragment, after cleavage with BamHI and XhoI, was cloned into yeast expression vector YEp24 with the restriction enzyme EcoRI. Two constructs previously generated to encode a truncated topo II mutant lacking C-terminal 240 amino acids (20). These two constructs were used for the expression of ND406 and core domain protein.

**Purification of Wild-type and Mutant Topo II—**Drosophila topo II was overexpressed in yeast BCY123 (p4p4, prb1, bar1, lys2::GAL1-GAL4, ura3) as a generous gift from Dr. Janet Lindsey, University of Utah). The expression of Drosophila top2 constructs were induced in YEP medium containing 2% galactose. An 8-liter culture of yeast cells overproducing Drosophila topo II was harvested and lysed as described in Ref. 37. The cleared lysate was sequentially purified by chromatography on Bio-Rex70 (Bio-Rad), Poros HQ, and HE column (PerSeptive Biosystems, Framingham, MA) as described previously for the full-length protein (32). The chromatography profile of NO460 protein was the same as the wild-type protein. The peak fractions were eluted at 1.0 M NaCl from HQ column and 0.4 M NaCl from HE column. The core mutant did not bind to the HQ column. Thus, the flow-through of the HQ column was directly loaded onto the HE column, and the peak fractions were eluted at 0.4 M NaCl. The purified proteins were dialyzed against the storage buffer containing 15 mM NaPi, pH 7.0, 0.1 mM EDTA, 0.1 M NaCl, and 50% glycerol for 16 h. A mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 mM pepstatin) was included throughout the purification process.

**Cleavage Reaction—**DNA cleavage was performed as described by Lee and Hsieh (33). A 20-μM reaction mixture containing 0.3 μg (0.05 pmol) of pCAspeRhs3 supercoiled DNA, 1.5 pmol of topo II protein, 10 mM Tris-HCl (pH 7.9), 50 mM KCl, 100 mM NaCl, 0.1 mM EDTA, 10 mM MgCl2, and 50 μg/ml bovine serum albumin. 1 mM ATP and 5 μCi of VM26 or amonafide were included as one of the reactions. Reactions were started by adding 1 mM ATP to a final concentration of 5 μM or 4 M.50 μCi of the mixture was then filtered and washed four times with 0.1 ml of reaction containing the buffer and was washed four times with 0.1 ml of the reaction buffer plus either 1 μl or 4 μl NaCl. To quantify the filter-precipitated DNA, the filter was dried at room temperature and then put in 10 ml of a solution containing 0.1% SDS and 100 μg/ml proteinase K (Life Technologies, Inc.). The samples from high salt wash and SDS elution were then analyzed by 1.6% agarose gel electrophoresis in the presence of 1 μg/ml ethidium bromide.

**Other Methods—**Topo II strand passage activities were tested by the unknotted of P4 knotted DNA, supercoiled relaxation, and decatenation assays as described in Ref. 37. CaCl2 density gradient ultracentrifugation was done as described in Ref. 32.

**RESULTS**

**Expression of the N-terminal Deletion Mutant and Core Domain of Drosophila Topoisomerase II—**In order to study the functions of the core domain of eukaryotic topoisomerase II, we constructed two truncation mutants of Drosophila topoisomerase II (Fig. 1). The truncation end points were determined on the basis of our previous analysis of the domain structure using protease cleavage and linker insertion mutagenesis. Two major trypsin cleavage sites were determined to be located at residues 406 and 1200 (Fig. 1A). The mutant lacking the N-terminal 406 amino acids of the full-length topo II is termed ND406 (Fig. 1B). The fragment containing amino acid 407–1207 is termed ND406A (Fig. 1C). In addition to the same N-terminal deletion as ND406, the core fragment lacks 240 amino acids at the C terminus, which has previously been determined to be a homologue of the heat shock gene HSP70 was used to map the topo II cleavage sites as described earlier (34). Plasmid DNA 6/122, a derivative of pBR322 with an insertion of 1.7 kb SalI fragment from the intergenic region of Drosophila heat shock gene HSP70, was used to prepare the uniquely end-labeled linear DNA. 6/122 was linearized by HindIII, radiolabeled at its 5'-ends, and treated with EcoRI to remove one of the end labels. 0.5 μl of labeled DNA substrate and 75 μl of topo II proteins were included in this cleavage assay and carried out in a condition identical to that of the plasmid cleavage.

**Protein-mediated Filter Binding Assay—**Topo II-mediated binding of DNA to GFP glass-fiber filters (35) was done as described by Roca and Wang (36). One radiolabeled DNA substrate used in the filter binding assays was generated by nick translation of nicked pH 624 (36) carried out in the presence of either [α-32P]dTTP (0.5 Ci/mmol) or [8-3H]dCTP (2.5 Ci/mmol) for 1 h at 14 °C, followed by ligation with T4 DNA ligase in the presence of 5 μl of ethidium bromide for 3 h at 25 °C. The mixture was then phenol extracted and run through a Sephadex G-50 spin column (Amersham Pharmacia Biotech) to remove the unincorporated nucleotides. The tritium-labeled DNA sample was linearized by restriction enzyme EcoRI. 25 μl of reaction mixture contained an equal molar mixture of linear and circular form of labeled DNA (0.05 pmol), 3 pmol of topo II protein in a solution of 10 mM Tris-HCl (pH 7.9), 50 mM KCl, 100 mM NaCl, 0.1 mM EDTA, 10 mM MgCl2, 50 μg/ml BSA. ICRF-159 and ATP were included in some of the reaction mixtures. The reaction mixture was incubated at 30 °C for 15 min and stopped by the addition of a chilled solution of 5 M NaCl to give a final concentration of 1 μl or 4 μl NaCl. To quantify the filter-retained DNA by scintillation counting, the filter was dried at room temperature and then put in 10 ml of a solution containing 0.1% SDS and 100 μg/ml proteinase K (Life Technologies, Inc.). The samples from high salt wash and SDS elution were then analyzed by 1.6% agarose gel electrophoresis in the presence of 1 μg/ml ethidium bromide.

**ICRF-159 Interacts with Topo II Core Domain**
not essential for the in vitro topo II activities (20). The ND406, core domain, and wild-type topo II were overexpressed in yeast cells and purified to over 90% in homogeneity based on the analysis of an SDS-polyacrylamide gel electrophoresis gel stained with Coomassie Blue dye (Fig. 2). An immunoblot of a similar gel using antibody against Drosophila topo II shows a single band at 170, 120, and 92 kDa, consistent with the sizes of full-length, ND406, and core topo II, respectively (data not shown). Additionally, a Western blot using antibody against yeast topo II was quantified to show that the endogenous yeast topo II was present in an amount less than 0.5% of the purified full-length and core protein and less than 2% of the protein in the purified ND406 samples (data not shown).

ND406 and Core Domain Retain Double-strand Cleavage Activity and Interaction with Topo II Targeting Antitumor Agents—Consistent with the fact that the strand passage activity of eukaryotic is ATP-dependent, both of our truncation mutants that lack the ATPase domain show no activity in the supercoil relaxation, unknotting, and decatenation assay (data not shown). In contrast, deletion of the ATPase domain and the C-terminal tail of topo II does not affect its DNA breakup and rejoining activity. Fig. 3 illustrates the cleavage of pCaSpeRhs83 plasmid DNA by wild-type and mutant topo II in the presence of two topo II targeting antitumor drugs, amonafide and teniposide VM26. These topo II poisons, though with distinct structures and abilities to intercalate DNA, have been shown to inhibit topo II activities by promoting the double-strand DNA cleavage as wild-type enzyme (Fig. 3, lanes 1–3). The DNA cleavage activity is assayed by the amount of linear DNA generated in the reaction. The presence of 5 μM VM26 or 5 μM amonafide significantly stimulates the DNA cleavage by both wild-type and mutant topo II (Fig. 3, lanes 4–9). In addition, we have tested the effect of two other topo II poisons, amsacrine and menadione, in DNA cleavage reactions and obtained similar results (data not shown). These data suggest that the key target for the above drugs resides in the core domain of topo II. For the wild-type enzyme, the cleavage of double strand DNA is also stimulated by the presence of ATP either in the absence of drugs or in their presence (compare Fig. 3, lanes 1, 4, and 7 with lanes 10, 13, and 16, respectively). In contrast with the wild-type enzyme, neither of the truncation mutants shows any ATP stimulation. This is as expected because the mutant proteins lack the ATP binding and hydrolysis domain.

Sequence Specificity in the Topo II-mediated DNA Cleavage Reaction—The DNA cleavage by topo II exhibits a moderate sequence specificity and provides a convenient assay to determine the sequence preference in the topo II/DNA interactions (40–42). We have used a unique end-labeled, linear DNA substrate to map the DNA cleavage sites for wild-type and mutant proteins (Fig. 4). The DNA substrate used here contains several clusters of strong topo II cleavage sites (marked as T1–T6, in the order of increasing cleavage) in the intergenic region of a heat shock gene HSP70. Tight topo II binding sites are located in these strong cleavage sites based on nuclease footprinting data (34). In the absence of any cofactors, nearly identical DNA cleavage patterns were observed for all three proteins except for the presence of a minor cleavage band by core protein between T5 and T6 (Fig. 4, lane 3, asterisk). The presence of VM26 and amonafide greatly stimulates the cleavage by wild-type and mutant proteins to a similar extent (Fig. 4, lanes 4–6). Interestingly, the addition of an intercalative topo II poison, amonafide, generate a different cleavage pattern, as evidenced by the appearance of two additional strong cleavage bands (Fig. 4, a and b). Similar to the data obtained from plasmid DNA cleavage, only the cleavage by the wild-type protein is stimulated by the presence of ATP (Fig. 4, compare lanes 10–18 to lanes 1–9). This is especially evident when we compare the amount of uncleaved DNA substrate left in each reaction. The above experiment therefore demonstrates that not only is the core domain of topo II the main target for topo II drugs but it also contains the essential element for the sequence preference in protein/DNA interaction. Neither the N-terminal ATPase domain nor the hydrophilic C terminus plays an important role in protein/drug or protein/DNA interaction for the in vitro topo II cleavage reaction.

Wild-type and Mutant Topo II Can Trap the Circular DNA in the Presence of Bisdioxopiperazine—Besides the topo II poisons, which function by promoting the cleavable complex between the double strand DNA and topo II, there are a number of topo II-targeting drugs that do not promote the topo II-mediated DNA cleavage. An antitumor agent, bisdioxopiperazine, has been shown to inhibit the topo II activities while compromising the effect of VM26 on DNA cleavage (27, 43). A member of this drug family, ICRF-193, can inhibit yeast topo II
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Fig. 4. Mutant and wild-type top II have similar DNA sequence specificity in the cleavage reaction. 75 nM of wild-type top II (labeled as W, lanes 1, 4, 7, 10, 13, and 16), ND406 (N, lanes 2, 5, 8, 11, 14, and 17), or core (C, lanes 3, 6, 9, 12, 15, and 18) were incubated at 30 °C for 30 min with 0.05 nM of 5’-end-labeled 6/122 DNA (2.5 x 10^6 cpm/pmol) either in the presence of 1 mM ATP (lanes 10–18) or in its absence (lanes 1–9). Some of the reactions included 5 μM of anticancer drug, VM26 (lanes 4 and 13–15) or amonafide (lanes 9 and 16–18). The strong top II binding and cleavage sites, marked as T1-T6 in the order of increasing intensities (34), two strong cleavage bands (a and b) stimulated in the presence of amonafide, and size markers (in base pairs) are shown on the sides of the autoradiogram. A minor cleavage site for the core domain protein is marked by an asterisk in lane 3.

activities by trapping the enzyme in the form of a closed clamp (30). To further investigate the interaction between eukaryotic top II and this family of drugs, we have used the glass-fiber filter binding assay to test the trapping of circular versus linear DNA by the top II proteins in the presence of ICRF-159 (Fig. 5). A mixture of radiolabeled negatively supercoiled DNA and its linear form was used in these assays (Fig. 5, lane 19). In the absence of ICRF-159, there is a trace amount of both circular and linear form of DNA being trapped (Fig. 5, lanes 2, 4, and 6). For all three proteins, the addition of ICRF-159 greatly stimulates the retention of the circular but not the linear DNA on the glass-fiber filter either in the presence of ATP (Fig. 5, lanes 14, 16, and 18) or in its absence (lanes 8, 10, and 12). Although ATP is not required for the retention of circular DNA on the glass-fiber filter, its presence allows the full-length top II to relax the supercoiled circular DNA. Both the monomeric and dimeric form of supercoiled circles are relaxed under such conditions (Fig. 5, lanes 13 and 14). The relaxed dimeric circle runs faster than its supercoiled form and apparently migrates with the linear DNA under such electrophoresis conditions. Therefore, we have used two-dimensional agarose electrophoresis to further analyze the filter-retained DNA sample identical to that run in Fig. 5, lane 14, and confirmed that the major DNA content in this sample was relaxed monomeric and dimeric circle (data not shown). These results demonstrate that the full-length top II can form an ICRF-159 induced clamp complex regardless of the presence of ATP. The closure of this protein clamp results in a salt-resistant capture of the DNA that is specific to the circular form.

The surprising result in this experiment is the formation of a clamp complex with the truncated proteins. The presence of a nonhydrolyzable ATP analog, AMPPNP, can promote the closure of the N-gate in gyrB domain, resulting in the formation of a salt-resistant clamp complex (8, 32, 44). Because the truncated proteins have their N-gate domain deleted, the clamp has to come from another protein/protein interface in the top II molecule. The formation of a different protein clamp is also supported by the result that in the presence of ICRF-159, the full-length top II can trap a circular DNA even without ATP. In this case, N-gate closure can not be responsible for the formation of clamp complex because its closure requires the binding of a nucleotide cofactor.

The Effect of ICRF-159 on the Formation of Salt-stable Complex between Topo II and Circular DNA—Because the clamp complex formed by the truncated protein might be distinct from that formed by the wild-type top II in the presence of AMP-PNP, we have extended this observation and tested the retention of circular and linear DNA on the glass-fiber filter at different drug concentrations. A filter binding assay with differentially labeled DNA substrate was developed to facilitate the quantification of the DNA retained on the filter. A mixture of 32P-labeled circular DNA and 3H-labeled linear DNA was used in these experiments. After being washed with 1 M NaCl solution, the filters were processed for liquid scintillation counting. Fig. 6 shows the results of such experiments. In the absence of ICRF-159, the trapping of circular DNA is about 5%, as indicated by the fraction of filter-retainable 32P counts (Fig. 6, solid columns). At ICRF-159 concentrations as low as 40 μM, significant trapping of circular DNA was observed for both wild-type and core fragment (Fig. 6, hatched columns). The presence of 1 mM ATP at this drug concentration further increases the trapping of circular DNA by wild-type protein to nearly 40% (Fig. 6, dotted column, set 2). When ICRF-159 was added to a concentration of 1 mM, a similar degree of trapping was achieved for both proteins, regardless of the presence of ATP (Fig. 6, dotted columns). Under all conditions tested, the retention of linear DNA on the glass-fiber filter was comparable, if not less, to the control without drug (Fig. 6, open columns). These results further demonstrate that bisdioxopiperazine is capable of trapping circular DNA by interacting with the core domain of top II. In addition, when the drug concentration is below saturation, ATP can promote the clamp complex formation between circular DNA and full-length top II, whereas it has no significant effect on the core domain.
The Effect of Salt on the ICRF-dependent Trapping of Circular DNA—The result of clamp complex formation by truncated proteins suggests the involvement of different intradimeric protein-protein interfaces other than that at the N-terminal ATPase domain. To examine the possible difference in biochemical properties between these two types of clamp complex, we have set out to test the salt stability of the ICRF-dependent complex between topo II and circular DNA. In the experiment shown in Fig. 7, the final salt concentration is 1 M. It has been demonstrated that the closure of N-terminal protein clamp in the presence of a nonhydrolyzable ATP analog, AMPPNP, can trap the circular DNA in a complex that is resistant to salt ranging from 0.5 M to a concentration as high as 3 M (8, 9, 32, 44, 45). Therefore, we have carried out similar filter binding experiments and stopped the reaction with a final concentration of 4 M NaCl. Although a previous study has demonstrated the nonspecific retention of protease-treated φ29 DNA in salt solutions above 3.5 M (35), we have found that without any bound protein, less than 5% of the labeled DNA substrate was retained on the GF/C filter in salt concentrations as high as 5 M (data not shown). In the absence of ATP, the ICRF-induced trapping of circular DNA by both wild-type and core fragment decreased by 50 and 70%, respectively, when the reactions are stopped at a 4 M NaCl concentration (Fig. 7, solid versus shaded, sets 1 and 3). In an interesting contrast, the ICRF-induced complex between wild-type topo II and circular DNA was resistant to 4 M salt when ATP was included in the initial reaction mixture (Fig. 7, solid versus shaded, set 2). As expected, the presence of ATP in the initial reaction mixture does not have any effect on the salt stability of the complex formed between core domain protein and circular DNA (Fig. 7, solid versus shaded, set 4). As monitored by the counting of 3H in the same experiments, linear DNA cannot form any filter-retainable complex with either protein under all conditions (data not shown). Therefore, ATP is only required for the full-length topo II to form an ICRF-induced clamp complex that is stable in high concentration of salt. This protein clamp is likely due to the closure of the N-gate after its binding to ATP. The dramatic difference in the salt stability between two types of clamp complex can also be demonstrated by the analytical CsCl density gradient ultracentrifugation. Here we monitor the complex formation between circular DNA and topo II proteins in 5 M CsCl (Fig. 8). In such experiments, the protein/DNA complex is distinguished from DNA by a reduced density and will band at distinct positions in the lighter range of CsCl gradient (46). Previously, we observed the generation of DNA species with multiply interlocked protein rings in the presence of AMPPNP (32). Under the reaction conditions similar to those described in filter binding experiments, we observed multiple peaks of protein/DNA complex between wild-type topo II and DNA in the presence of ATP (Fig. 8C). However, only a minor protein/DNA complex between full-length topo II and circular DNA was observed in the absence of ATP (Fig. 8B). No significant amount of salt-stable complex can form for core domain under all the conditions tested here (Fig. 8, D–F). The control experiments using linear DNA do not show any peaks corresponding to protein/DNA complex (data not shown). Taken together, these results suggest that whether the protein clamp induced by ICRF-159 can be resistant to a very high salt solution depends on the presence and closure of the N-terminal ATPase domain. Neither core domain protein nor full-length protein without ATP can form a complex stable in salt concentrations over 4 M.

**FIG. 6.** The effect of ICRF-159 on the topo II/DNA clamp complex. Filter binding experiments similar to those described in Fig. 5 were carried out in the reactions containing no ICRF-159 (solid columns), 40 μM ICRF-159 (hatched columns), or 1 mM ICRF-159 (dotted columns). The reaction mixtures contained both the circular form (32P labeled) and linear DNA (3H labeled), and either wild-type (sets 1 and 2) or core topo II (sets 3 and 4) protein was included in the reactions. The experiments shown in sets 2 and 4 were carried out in the presence of 1 mM ATP. After filtering the mixture through a GF/C filter, the amount of filter-retainable complex between topo II and circular DNA was quantified by the 32P counts from liquid scintillation counting. The fractions of the filter-retained linear DNA in the same reaction were quantified by 3H counts and are represented by the open columns, adjacent to each column on the right. For each reaction, at least three independent experiments were performed. The bar above each column shows the S.D. of these measurements.

**FIG. 7.** The effect of salt on the ICRF-dependent clamp complex. Filter binding experiments similar to those described in Fig. 5 were carried out in the reactions containing 1 mM of ICRF-159. The reactions were stopped by the addition of NaCl to a final concentration of either 1 M (solid columns) or 4 M (hatched columns). The mixture was further processed and analyzed as described in Fig. 6. Either wild-type (sets 1 and 2) or core topo II protein (sets 3 and 4) was included in the reactions. The experiments shown in sets 2 and 4 were carried out in the presence of an additional 1 mM ATP. For each reactions, at least three independent experiments were performed. The bar above each column shows the S.D. of these measurements.

DISCUSSION

The nature of a multi-domain structure for eukaryotic topo II is revealed by many lines of evidence, including sequence comparison, protease sensitivity, mutagenesis, and x-ray crystal structures (for examples, see Refs. 10, 13, 14, and 47). In this study, we have carried out the characterization of two truncation mutants of *Drosophila* topo II lacking the ATPase domain: a 120-kDa ND406, which is like full-length protein except for the deletion of N-terminal 406 residues to remove the ATPase domain, and a 97-kDa core domain, which has the hydrophilic C terminus also removed. Both truncated proteins have lost their DNA strand passage and ATPase activities but still retain the ability to cleave double-stranded DNA. An earlier study has shown that a similar core domain of yeast topo II also retains only the DNA cleavage ability (10). We have further tested the sensitivity of these truncation constructs towards a number of anticancer drugs and shown that, like the wild-type topo II, the DNA cleavage by the two truncated mutants can be stimulated...
by several topo II poisons. However, the stimulatory effect of ATP on the DNA cleavage has only been observed in the experiment containing wild-type enzyme. These results have confirmed that the ATPase domain and DNA cleavage domain are relatively independent in eukaryotic topo II structure.

Bis(2,6-)dioxopiperazine and its derivatives are antitumor drugs with topo II as their intracellular target in both yeast and mammalian systems (29, 48). They function as the inhibitory agents for the catalytic activities of topo II but do not stabilize the drug family inhibiting the covalent complex with DNA (27, 43). A recent study by Roca et al. (30) has indicated that a member of this drug family inhibits topo II (Ref. 30 and data not shown). When both ATP and ICRF are bound but not being hydrolyzed, similar to the situation when a nonhydrolyzable ATP analog is present. Thus, under such conditions (see Fig. 7, sets 1 and 4), we could not exclude the possibility that the addition of a high salt solution could irreversibly trap a fraction of protein/DNA clamp complex in a dead-end but still salt-resistant complex. Interestingly, our study has shown that in the presence of ATP, the ICRF-induced complex formed between full-length topo II and circular DNA is resistant to high-salt solutions over 4 m. ICRF drugs have been demonstrated to inhibit the ATPase activity of topo II (Ref. 30 and data not shown). When both ATP and ICRF drugs are present in the reaction mixture, it is likely that ATP is bound but not being hydrolyzed, similar to the situation when a nonhydrolyzable ATP analog is present. Thus, under such a condition, not only the B’ regions can interact with each other to form a closed conformation, but also the ATPase domain in the full-length topo II dimer. This would result in a clamp complex more stable than that formed due to the B’-B’ interaction alone. The notion that the closure of N-gate can further stabilize the ICRF-induced closure of B’-B’ interface is also supported by the fact that at a low drug concentration, the presence of ATP can increase the formation of full-length topo II/circular DNA complex (Fig. 6, set 2). In a previous study with full-length yeast topo II and ICRF-193, it was shown that ATP is required for the formation of the filter-retainable complex, although it is not required post-complex formation (30). In our study with Drosophila enzyme and ICRF-159, the presence of ATP can stimulate the filter-retainable complex but is not required for its formation. Because such a complex, formed in the absence of ATP has a reduced salt stability, it is possible that the difference between these observations is due to the assay conditions and the reagents like enzyme and ICRF con-
generators used in the reaction. We have used a filter binding experiment with either gel electrophoresis or liquid scintillation counting to monitor the clamp complex formation in the presence of ICRF-193. The results with the Droso phila enzyme are similar to that obtained with its congener ICRF-159 (data not shown). We can therefore rule out the possibility that ICRF-159 and ICRF-193 have significantly different mechanisms of action. It is possible that the clamp complex formation due to the B-B' interactions is less stable than the clamp closure of the gyrB N-terminal dimer interface and thus more sensitive to the experimental conditions. Under the conditions employed in our experiments, the B-B' interface is more sensitive to the experimental conditions. Under the conditions employed in our experiments, the B-B' interaction of the enzyme, it does not address how this closure occurs. The drug can function directly on the B-B' interface, or it can interact with other regions of the enzyme and initiate conformational changes to stabilize this interface. Further experiments are needed to determine the specific regions of the enzyme that interact with this family of drugs and to explore in more detail the mechanism of its inhibition.

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