Reconstitution of Enzymatic Activity by the Association of the Cap and Catalytic Domains of Human Topoisomerase I*

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When human topoisomerase I binds DNA, two opposing lobes in the enzyme, the cap region (amino acid residues 175–433) and the catalytic domain (Δcap, residues 433 to the COOH terminus) clamp tightly around the DNA helix to form the precleavage complex. Although Δcap contains all of the residues known to be important for catalysis and binds DNA with an affinity similar to that of the intact enzyme, this fragment lacks catalytic activity. However, a mixture of Δcap and topo31 (residues 175–433) reconstitutes enzymatic activity as measured by plasmid DNA relaxation and suicide cleavage assays. Although the formation of an active complex between topo31 and Δcap is too unstable to be detected by pull-down experiments even in the presence of DNA, the association of topo31 with Δcap persists and is detectable after the complex catalyzes the covalent attachment of the DNA to Δcap by suicide cleavage. Removal of topo31 from Δcap-DNA after suicide cleavage reveals that, unlike the cleavage reaction, religation does not require the cap region of the protein. These results suggest that activation of the catalytic domain of the enzyme for cleavage requires both DNA binding and the presence of the cap region of the protein.

Eukaryotic type I topoisomerases promote the relaxation of supercoiled DNA by nicking and rejoining one of the strands of the DNA. These enzymes are important for many biological processes including DNA replication, transcription, and recombination (1, 2). Eukaryotic topoisomerase I, the poxviral topoisomerases, and some bacterial topoisomerases belong to the type IB subfamily of topoisomerases (1, 3). The type IB subfamily members bind to double-stranded DNA and can relax either positive or negative supercoils in the absence of energy cofactors or divalent cations. DNA cleavage is initiated by the nucleophilic attack of the O-4 atom of the active site tyrosine on the scissile phosphate with the resultant covalent attachment of the DNA to Δcap by suicide cleavage. Allosteric effects from residues 433 to the COOH terminus (Ile215–Ala635), a linker domain (Pro636–Lys712), and a COOH-terminal domain (Gln713–Phe865) (Fig. 1A) (5–7). The highly charged NH2-terminal domain is dispensable for enzymatic activity in vitro (5) and contains nuclear targeting signals and binding sites for other proteins, such as nucleolin and SV40 large T antigen (8–10). Topo70 is a truncated form of human topoisomerase I with a molecular mass of 70 kDa that lacks residues 1–174 of the NH2-terminal domain but retains full enzyme activity in vitro (11, 12). The core domain is highly conserved and contains all of the residues directly implicated in catalysis except the active site Tyr723. The COOH-terminal domain is also highly conserved and contains the active site Tyr723. Separately purified COOH-terminal and core domains can interact with each other and reconstitute topoisomerase I activity in vitro (5, 13). The linker region that connects the core domain to the COOH-terminal domain is not conserved and is dispensable for activity in vitro, although without the linker the enzyme has a reduced processivity (12).

In the crystal structure of the human topoisomerase I complexed with DNA, the protein clamps around the DNA with most of the protein-DNA contacts involving the core and COOH-terminal domains (6, 14). The core domain can be further divided into three subdomains: 1) core subdomain I (residues 215–232, 320–433), 2) core subdomain II (residues 233–319), and 3) core subdomain III (residues 434–635). Core subdomains I and II form the top lobe or “cap” of the enzyme and cover the top of the DNA as the structure is usually oriented (6, 14). Core subdomain II does not come in contact with the DNA in the structure, but the folding of core subdomain II is similar to that of the homeodomain region found in a family of DNA-binding proteins (6, 14). Two long positively charged α helices (α6 from core subdomain I and α5 from core subdomain II) form a “V”-shaped structure on the front end of the cap that may contact the DNA during the rotation process (6, 14). Core subdomain III and the COOH-terminal domain form the bottom lobe of the protein. This region of the protein is homologous to the catalytic domain of the site-specific recombinases that include HP1 integrase, λ integrase, and Cre recombinase, and also to the catalytic domain of vaccinia topoisomerase (6, 15). The bottom lobe of the protein is attached to the cap region through a long α helix extending upwards from core subdomain III on one side of the bound DNA and by a salt bridge on the other side of the DNA formed by amino acid side chains extending from a pair of loops in core subdomains I and III (6, 14).

To dissect the function of the core domain and explore the functional relationship between the cap and the core subdomain III, we have studied the properties of three fragments of human topoisomerase I either alone or in pairwise combinations (Fig. 1A). Topo31 (residues 175–433), a 31-kDa fragment that consists of the cap region of the protein, binds DNA well,
whereas topo17 (residues 175–320), a 17-kDa fragment that mainly comprises subdomain II (the homeodomain-like region), cannot bind DNA. The third fragment corresponds to the catalytic domain of the enzyme and contains core subdomain III, the linker region, and the COOH-terminal domain (residues 433–765), and the fragment is referred to as Δcap. Although Δcap contains all of the elements required for catalysis and can bind DNA, it is catalytically inactive. However, when topo31 and Δcap are combined, they reconstitute enzymatic activity.

EXPERIMENTAL PROCEDURES

Generation of Truncation Mutants—All of the truncation mutants were generated using standard PCR methodology. To generate topo17, pGEX-topo70 DNA (13) was used as the PCR template, in combination with a plus-sense primer that starts at position 852 of the topoisomerase I cDNA sequence (16) and a minus-sense primer that contained two stop codons followed by an AucII restriction site immediately after the codon for residue 433 of the protein. The PCR products were purified and digested with NdeI and AucII and ligated to pGEX-topo70-WT DNA that had been cleaved with the same two restriction enzymes. To generate topo31, we used the same plus-sense primer and a minus primer that contains two stop codons followed by an AucII restriction site after the codon for residue 433 of the protein. The PCR fragment was purified and digested with SpolI and AucII and ligated to the pGEX-topo70-WT DNA that had been cut with the same two restriction enzymes. The Δcap mutant was generated using a plus-sense primer containing a BamHI site followed by an AUG codon that annealed upstream of residue 433 and a minus-sense primer that annealed downstream of position 2392 of the topoisomerase I cDNA sequence. The PCR fragment was purified and digested with BamHI and NheI and ligated to pFASTBAC1 topo70 (17) that had been cut with the same two restriction enzymes. All of the mutations were confirmed by dideoxy sequencing.

Protein Purification—GST-topo17 and GST-topo31 were expressed and purified from Escherichia coli TOP10F (Invitrogen) cells containing the pGEX-topo17 and pGEX-topo31 plasmids, respectively, as described previously for GST-topo12 (18). The GST-topo17 and GST-topo31 fusion proteins were either stored bound to the glutathione-Sepharose 4B beads in 100 mM KCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT at 4 °C or removed from the beads with Factor Xa, followed by dialysis in storage buffer (50% glycerol, 10 mM Tris-HCl, pH 7.4, 2 mM DTT, 1 mM EDTA) and storage at −20 °C. Δcap was purified from the recombinant baculovirus-infected SF-9 insect cells as described previously for topo70 (12).

Plasmid Relaxation Assays—Each reaction contained 500 ng of supercoiled pBluescript KSKII (+) DNA and a 2-fold serial dilution of topo31 (1, 2, 4, 8, 16, 32, and 64 pmol) either with or without 4 pmol of Δcap in 20 µl of reaction buffer (100 mM KCl, 10 mM Tris-HCl, pH 7.4, 1 mM DTT, 1 mM EDTA). The reactions were incubated at 37 °C for 1 h and then stopped by the addition of 5 µl of 5× stop buffer (2.5% SDS, 25 mM EDTA, 25% Ficoll 40, 0.08% bromphenol blue, 0.08% xylene). The products were analyzed by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized with a UV illuminator.

Suicide Cleavage Reactions—The duplex oligonucleotide suicide cleavage substrate CL14/CP25 was labeled and annealed as described previously (18). The suicide cleavage reactions were carried out by incubating 2 µg of topo17, topo31, or Δcap alone or in the indicated combinations in 20 µl of reaction buffer with 5 ng of the suicide cleavage substrate at 23 °C for 3 h. Topo70 (0.5 µg) was used as a positive control.
for suicide cleavage. 5 μl of 5× SDS loading buffer (5% SDS, 20% glycerol, 100 mM Tris-HCl, pH 8.0, 5% 2-mercaptoethanol, 0.12% bromphenol blue) was added to quench the reactions. The samples were boiled for 5 min and analyzed by 10% SDS-PAGE. The gel was stained with Coomassie Blue to visualize the protein bands and dried before exposure to film to compare the amount of labeled DNA present in the beads with the amount of DNA released from the supernatant.

Religation Kinetics—Covalent complexes used as substrates for the reigation reaction were generated by suicide cleavage as described previously (18). Suicide cleavage was carried out in 100 μl of reaction buffer containing 20 mM labeled suicide substrate and 0.5 μM topo70 for 1 h or 2.5 μl topo31 plus 2.5 μl Δcap for 6 h at 23 °C. The suicide cleavage reaction with topo70 was centrifuged at 15,000 × g for 5 min, 2 μl of 0.5 M KCl to prevent further cleavage during religation. High salt inactivation of the reaction with the reconstituted enzyme was unnecessary owing to the slow cleavage rate, and furthermore, high salt was found to dissociate the topo31-Δcap complex (see Fig. 7). The reactions were transferred to 37 °C and preincubitated for 2 min. Religation was initiated by the addition of a 300-fold molar excess of the 11-mer religation acceptor oligonucleotide (R11) that is complementary to the region downstream of the cleavage site (18). Aliquots of 10 μl were removed at different time points (5 s, 15 s, 30 s, 2 min, 5 min, and 60 min), and the reactions were stopped by the addition of an equal volume of 1% SDS. Religation was complete by the 60-min time point in both cases. Samples were ethanol-precipitated and dissolved in 10 μl of 1% SDS. Religation was complete by the 60-min time point in both cases. Samples were ethanol-precipitated and dissolved in 10 μl of 1% SDS. Religation was complete by the 60-min time point in both cases. Samples were ethanol-precipitated and dissolved in 10 μl of 1% SDS. Religation was complete by the 60-min time point in both cases.

To test the effects of salt on the GST-topo31 interaction with Δcap in the presence of 20 nM labeled suicide substrate in reaction buffer in a total volume of 100 μl at 23 °C for 6 h to allow suicide cleavage to occur. The beads were washed twice with 100 μl of 300 mM KCl and 100 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, and rotated at 23 °C for 5 min to elute the Δcap-DNA from the beads. The beads were removed by centrifugation at 10,000 rpm for 2 min. To eliminate all traces of GST-topo31, 5 μl of pre-equilibrated glutathione-Sepharose 4B beads were added to the supernatant and rotated at 23 °C for 30 min before removal of the beads by centrifugation. This step was repeated three times. The supernatant that contains the Δcap-DNA was divided into four 25-μl aliquots prior to preparing the samples for the religation assays. 50 μl of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT was added to two of the aliquots to make 75-μl reactions with a final KCl concentration of 100 mM. 3 μl of topo31 (0.7 μg/μl) was added to the first 75-μl reaction (topo31), and an equal volume of storage buffer was used to prepare two additional reactions for religation except the KCl concentration was adjusted to 800 mM. All the reactions were incubated at 37 °C for 2 min before religation was initiated by the addition of 5 μl of the R11 religation oligonucleotide (final concentration, 2 μM). A 10-μl aliquot was removed prior to the addition of R11 for the zero time point analyses. 10-μl aliquots were removed at the indicated times and quenched by adding an equal volume of 1% SDS. All the reactions were ethanol-precipitated and digested with 10 μl of 1 mg/ml of trypsin at 37 °C for 1 h. The samples were analyzed on a 20% sequencing gel and subjected to phosphorimager analysis using ImageQuant software.

Expression and Purification of Topoisomerase I Fragments—The functional association of human topoisomerase I fragments employed in this study are shown schematically in Fig. 1. Both topo17 (residues 175–320) and topo31 have the same NH2 terminus as topo70, but topo17 extends to the end of core subdomain II, whereas topo31 (residues 175–433) includes all of the cap region of the protein. The Δcap fragment corresponding to the catalytic domain begins where topo31 ends and extends through to the COOH terminus of the protein. Fusion constructs of topo17 and topo31 containing NH2-terminal GST were expressed and purified from E. coli. The fusion proteins were either used directly or the GST portion was removed by Factor Xa prior to use. Topo58 (residues 175–659) and the Δcap fragment (residues 433–765) were purified from recombinant baculovirus-infected insect cells. Analysis of the purified proteins by SDS-PAGE (Fig. 1, panel B) showed that all four fragments were essentially homogenous.

The Reconstitution of Relaxation Activity by a Mixture of Topo31 and Δcap—Although the Δcap fragment contains all of the amino acids that constitute the active site of the enzyme, no plasmid DNA relaxation activity was detectable in the purified fragment (Fig. 2, lane 9). As expected, no relaxation activity was associated with topo31 alone (Fig. 2, lanes 2–8). However, relaxation activity could be reconstituted by the addition of topo31 to Δcap. Under these conditions (1-h incubation), activity was first detectable at approximately a 2:1 molar ratio of topo31 to Δcap, and essentially complete relaxation was achieved at a molar ratio of 4 (Fig. 2, lanes 13 and 14, respectively). Freshly prepared Sepharose 4B beads were added to the labeled Δcap-DNA and the mixture was rotated at 23 °C for 2.5 h to test whether Δcap-DNA associates nonspecifically with the beads.

To test the effects of salt on the GST-topo31 interaction with Δcap-DNA, bead-bound covalent complexes were prepared as described previously and incubated for 5 min in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, and KCl concentrations ranging from 100 to 400 mM. The beads were collected by centrifugation and resuspended in 15 μl of 1× SDS loading buffer and analyzed by SDS-PAGE. 5 μl of 5× SDS loading buffer was added to each supernatant and similarly analyzed. The gel was stained with Coomassie Blue and dried before exposure to film to compare the amount of labeled DNA present in the beads with the amount of DNA released from the supernatant.
topo31
Δcap

Form II
Form I

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIG. 2. Plasmid relaxation assays for topo31-Δcap complexes. Topo31 was 2-fold serially diluted and incubated with a supercoiled DNA substrate in 20 μl of relaxation buffer either in the presence or absence of a constant amount of Δcap (4 pmol). Lane 1 contains the supercoiled DNA substrate only; lanes 2–8 show relaxation assays with increasing amounts of topo31 (1, 2, 4, 8, 16, 32, and 64 pmol) in the absence of Δcap; lane 9 contains 4 pmol of Δcap and the supercoiled substrate DNA; lanes 10–16 show relaxation assays in the presence of 4 pmol of Δcap with increasing amounts of topo31 (1, 2, 4, 8, 16, 32, and 64 pmol). The mobilities of the supercoiled (Form I) and nicked forms (Form II) of the substrate DNA are shown on the left.

Δcap contains all of the critical residues involved in catalysis yet it lacks enzymatic activity. This lack of catalytic activity could result from a reduced affinity of Δcap for DNA. To test this possibility a native gel mobility shift assay was used to measure the DNA binding properties of the various topoisomerase-derived fragments. Similar to topo70, topo31, topo17, and Δcap are positively charged, and because a covalently bound oligonucleotide only partially neutralizes the positive charge, the protein-DNA complexes fail to enter the native gel. Under these conditions, Kd is equal to the protein concentration at which the amount of unbound oligonucleotide observed in the gel has been reduced by a factor of 2 (19). Lysozyme has a similar pi value and was therefore used as a negative control for DNA binding by topo31, topo17, and Δcap. The binding assays showed that the affinity of the topo17 protein for the DNA was about the same as that of the lysozyme control, indicating that the binding is relatively nonspecific (Kd of ~5 × 10^{-6} M) (Fig. 5, insert). Topo31 bound DNA with a Kd of ~4 × 10^{-7} M, whereas Δcap bound the substrate DNA with a higher affinity (~1 × 10^{-7} M), which is only 2-fold lower than that of topo70 (~5 × 10^{-8} M). These results showed that topo31 binds DNA with a somewhat reduced affinity compared with topo70 and that the absence of activity for Δcap is not due to a failure to bind DNA.

GST-topo31 Remains Associated with Δcap After Suicide Cleavage—Topo31 and Δcap together can reconstitute complete topoisomerase I activity, indicating that topo31 interacts with and activates Δcap either before or after the addition of DNA. However GST-topo31 bound on the glutathione-Sepharose 4B beads failed to pull down a detectable quantity of Δcap after incubating the two proteins together either in the presence or absence of DNA (data not shown). This result could indicate that the interaction between Δcap and GST-topo31 is too weak to form a stable complex even in the presence of DNA, or that only a small fraction of the proteins interact to form the complex.

An alternative and more sensitive approach to detect the existence of the complex is to ask whether radioactively labeled Δcap-DNA and GST-topo31 remain associated after suicide cleavage. GST-topo31 bound to glutathione-Sepharose 4B beads was incubated with Δcap and labeled suicide substrate to form the covalent complex. The beads were collected by centrifugation to eliminate uncleaved substrate DNA and free Δcap. The bead-associated material was analyzed by SDS-PAGE (Fig. 6). The results showed that most of the radiolabeled Δcap-DNA covalent complex was associated with the beads (96%) (Fig. 6B, lanes 1 and 2), indicating that Δcap-DNA forms a complex with GST-topo31. However, it should be noted that the amount of Δcap containing covalently attached DNA was too small to be detected in the Coomassie Blue-stained gel (Fig. 6A, lane 1). The control analysis with isolated Δcap-DNA showed that the covalent complex did not associate nonspecifically with the glutathione-Sepharose 4B beads (Fig. 6B, lanes 4 and 5), indicating that GST-topo31 was responsible for mediating the association of Δcap-DNA with the beads. These results demonstrated that GST-topo31 activates Δcap and remains associated with Δcap after suicide cleavage.

Salt Effect on the Association of GST-topo31 and Δcap-DNA—To evaluate the stability of the Δcap-DNA/GST-topo31 complex, we tested the sensitivity of the complex to salt. The covalent Δcap-DNA complex that bound to the GST-topo31 on glutathione-Sepharose 4B beads was generated as described under “Experimental Procedures.” The beads were divided into several aliquots and washed with buffers at different KCl concentrations (Fig. 7). Whereas only a trace amount of Δcap-DNA (2.9%) was released from the beads at 100 mM KCl, most of the

Functional Association of Human Topoisomerase I Fragments
labeled material was released into the supernatant (89%) at 300 mM KCl (Fig. 7, panels B and C, lanes 2–4). Over the range of KCl concentrations tested, the association of GST-topo31 with the beads was stable (Fig. 7, panel A). Washing with KCl concentrations of 400 mM or higher did not significantly increase the amount of label released from the beads (Fig. 7, panels B and C, lane 5 and data not shown). These results indicated that the association of Δcap-DNA with GST-topo31 was disrupted between 200 and 300 mM KCl.

**Religation by Δcap Alone**—The salt sensitivity of the GST-topo31·Δcap-DNA interaction facilitated the separation of the Δcap-DNA covalent complex from GST-topo31. We purified the radiolabeled Δcap-DNA complex away from GST-topo31 in the presence of high salt and then carried out a religation reaction to test whether the covalent Δcap-DNA complex remains active and competent for religation. The religation reactions were carried out at 100 and 800 mM KCl in the presence or absence of added topo31 (Fig. 8). The results at 100 mM KCl showed that Δcap could carry out religation in the absence of added topo31, but the religation rate by Δcap-DNA in presence of added topo31 was at least 5-fold faster than the religation rate in the absence of topo31 (Fig. 8, panel B). Because topo31 can interact with Δcap at 100 mM KCl, we cannot exclude the possibility that the small amount of religation observed at this salt concentration in the absence of added topo31 was due to a trace amount of contaminating GST-topo31 undetected by Western blot analysis (data not shown). However, at 800 mM KCl, contaminating GST-topo31 should be unable to bind Δcap and consequently any observed religation should result solely from the activity of Δcap. As shown in Fig. 8, religation by Δcap readily occurred at 800 mM KCl in the absence of topo31. The lack of stimulation by exogenously added topo31 established that the observed religation was not mediated by contaminating GST-topo31. Thus the result showed that Δcap-DNA could carry out the religation reaction alone, although the religation rate at 800 mM KCl was much slower than the rate at 100 mM KCl in the absence of topo31.

**Discussion**

A functional analysis of fragments of human topoisomerase I shows that the cap region (topo31) of human topoisomerase I, which lacks catalytically important residues, can bind DNA although with a reduced affinity when compared with topo70. In an earlier study, a fragment corresponding to the same region of the *Saccharomyces cerevisiae* topoisomerase I was similarly shown to bind DNA (7). However, core subdomain II (topo17) does not bind DNA by itself despite displaying a structural homology to the homeodomain region found in a family of DNA-binding proteins (6). The Δcap fragment corresponds to the catalytic domain of the enzyme and contains all of the residues known to be directly involved in catalysis yet it is still enzymatically inactive. This lack of activity is due not to a defect in DNA binding because its affinity for DNA is only 2-fold lower than that of topo70. The absence of activity for Δcap is particularly surprising in view of the structural similarity of this portion of human topoisomerase I with that of the catalytic domains of λ integrase and vaccinia topoisomerase, both of which retain some catalytic activity as the isolated domains (14, 15, 21, 22).

The catalytically active domain of vaccinia topoisomerase I that lacks the NH₂-terminal region has been shown to bind DNA at specific sites with a lower affinity than the full-length enzyme and to exhibit a reduced catalytic activity. The missing NH₂-terminal domain contributes to DNA binding because point mutations at Tyr70 and Tyr72 in the NH₂-terminal domain that have been shown to contact the substrate near the scissile phosphate in the major groove cause a similar effect on the activity as removing the NH₂-terminal domain (23). When the crystal structures are compared, most of the active site residues of vaccinia topoisomerase spatially superimpose very
Fig. 4. Religation reactions by topo70 and the reconstituted topo31 plus Δcap. The religation reactions were carried out as described under "Experimental Procedures." Panel A, phosphorimager analysis of a sequencing gel showing the time course of appearance of the 23-mer religation product for topo70 and reconstituted Δcap plus topo31. The labeled band migrating between the oligonucleotide containing the trypsin-resistant peptide (Covalent complex) and the uncleaved oligonucleotide (Uncleaved oligo) resulted from suicide cleavage at an internal secondary cleavage site. Because this species does not participate in the religation reaction, it was ignored. Panel B, graphical analysis of the kinetics of appearance of religation products for topo70 (♦) and the topo31-Δcap complex (■).
well on the corresponding residues in human topoisomerase I except for the active site tyrosine (Tyr274), which is displaced away from the active site pocket and is not in a position to directly attack the scissile phosphate (15, 22, 24). This observation suggests that a precleavage conformational change in the catalytic domain is necessary to establish the correct position of the active site tyrosine for nucleophilic attack on the DNA. It has been suggested that DNA contacts by the NH2-terminal domain of the enzyme are involved in facilitating this precleavage conformational change (15, 23). In the crystal FIG. 5. DNA binding assays. The gel shift assay was carried out as described under “Experimental Procedures.” The percentage of unbound duplex oligonucleotide present in the gel was quantified using the phosphorimager and plotted against the protein concentration. Because lysozyme and topo17 have a lower affinity for DNA, the protein concentrations used in the gel shift assays were 10-fold higher than those used for topo70 (Δ), topo58 (○), topo31 (●), and Δcap (□). The binding profiles for topo17 (◆) and lysozyme (○) are shown in the inset.

FIG. 6. Association between Δcap-DNA and GST-topo31. GST-topo31 bound to glutathione-Sepharose 4B beads was mixed with Δcap, and suicide cleavage with labeled substrate DNA was carried out as described under “Experimental Procedures.” After incubation, the reactions were either loaded directly onto an SDS-polyacrylamide gel as a control for the total amount of Δcap-DNA formed (lane 3) or the beads (lane 1) and supernatant (lane 2) were separately analyzed to test whether Δcap-DNA remained associated with the GST-topo31 on the beads. As a control for nonspecific interactions with the beads, Δcap-DNA was eluted from the beads with high salt and then incubated with fresh glutathione-Sepharose 4B beads at 100 mM KCl to detect whether Δcap-DNA in the absence of GST-topo31 bound to beads (lane 4) or remained in the supernatant (lane 5). Panel A, the SDS-polyacrylamide protein gel stained with Coomassie Blue; the size markers are indicated along the left. Panel B, autoradiograph of the same gel.

FIG. 7. Salt effect on the association between Δcap-DNA and GST-topo31. GST-topo31 bound to glutathione-Sepharose 4B beads was incubated with labeled suicide DNA and Δcap to generate Δcap-DNA covalent complexes. Aliquots of the beads that contain the Δcap-DNA complexes were treated at different KCl concentrations, and the supernatants and the beads were separately analyzed by SDS-PAGE. Panel A, the Coomassie Blue-stained gel of the beads after salt treatment. Lane 1, prestained protein size markers; lanes 2–5, beads washed with 100, 200, 300, and 400 mM KCl, respectively. Panel B, autoradiograph of the SDS-polyacrylamide gel shown in panel A (Beads). Panel C, autoradiograph of the SDS-polyacrylamide gel of the supernatant after salt treatment.
structure of the λ integrase catalytic domain, the active site tyrosine is located on a flexible segment and is similarly distant from the other catalytic residues (21, 22, 25), again suggesting that a conformational change must precede cleavage. In both of these cases, the protein fragments that contain the displaced active site tyrosines retain some enzymatic activity, suggesting DNA binding alone is sufficient to induce the precleavage conformational change required for catalysis.

A possible explanation for the lack of activity of the isolated catalytic domain of human topoisomerase I (Δcap) is that, similar to vaccinia topoisomerase and λ integrase, the architecture of the active site is not properly assembled for catalysis. More-

FIG. 8. Religation by Δcap alone. Δcap-DNA was generated by suicide cleavage in the presence of GST-topo31 bound to glutathione-Sepharose 4B beads and eluted from the beads by a high salt wash. Religation was carried out at either low salt (100 mM KCl) or high salt (800 mM KCl) condition in the presence or absence of topo31 as described under “Experimental Procedures.” Panel A, phosphorimager analysis of sequencing gel for the kinetics of religation under the indicated conditions. Panel B, the percentage of religated oligonucleotide was quantified and plotted against time.
over, by analogy with the vaccinia topoisomerase and the tyrosine recombinases, it may be that the nucleophilic Tyr^{223} in the isolated catalytic domain is displaced from the proper position for cleavage. Because the isolated catalytic domain is inactive, we propose that DNA binding is not sufficient in this case to induce the precleavage conformational change that assembles the active site for catalysis, and instead the conformational change depends also on the presence of the cap region. A crystal structure of human topoisomerase I in the absence of DNA is required to test this conjecture. Nonetheless a 68-kDa recombinant human topoisomerase I has been shown by Raman and CD spectroscopy to undergo a conformational change after DNA binding and cleavage in solution (26). This transformation is mostly localized to the core and the COOH-terminal domains, and primarily reflects the relative movement of domains upon DNA binding. Whether the observed effects are related to a precleavage conformational change that assembles the proper active site remains to be determined.

Our results show that in human topoisomerase I, the cap region can bind DNA and is required to activate Δcap for cleavage. However, the cap region is not required for the religation reaction, and apparently once Δcap becomes covalently attached to the DNA after suicide cleavage, it is competent to carry out religation in the absence of the cap. What most clearly distinguishes religation from cleavage is the nature of the nucleophile. If indeed the cap is required to reorient the nucleophilic Tyr^{223} for the cleavage reaction, then it might not be required for the religation reaction where the nucleophilic 5′-hydroxyl is held in place by virtue of the base pairing between the religation oligonucleotide and the downstream non-scissile strand. Alternatively, if Δcap is inactive for reasons other than the positioning of Tyr^{223}, then the simple presence of the covalently bound DNA appears sufficient to maintain the appropriate activated conformation for religation.

The activation of Δcap by topo31 implies that there is an interaction between the two protein fragments at least when they are bound together on the DNA. Given that the only noncovalent contact between the cap and the remainder of the protein in the crystal structure involves a salt bridge between Lys^{369} and Glu^{497} within the “lips” region of the protein (6), it is not surprising that no interaction was detectable using a Coomassie Blue-stained SDS-polyacrylamide gel analysis in the absence of DNA. Although no interaction was seen in the presence of DNA using the same assay, the use of a much more sensitive assay involving cleavage of a radiolabeled suicide substrate did permit detection of a stable interaction. Our results do not allow us to address whether detection in this case was simply due to the increased sensitivity of the assay or whether the interaction between topo31 and the Δcap is stabilized by the formation of the covalent complex.

In the ternary topo31-Δcap-DNA complex, the most likely region for communication between the cap and the catalytic domains is within the lip region where the two lobes of the intact protein interact with each other. Two lines of evidence support the notion that interactions within this region could be transmitted to the active site region of the enzyme or to the bound substrate DNA. First, two residues located within the lips region, the side chain of His^{367} and a main chain nitrogen of Arg^{364}, both contact a phosphate located in the non-scissile strand one nucleotide away from the cleavage site (6). Second, it has been shown that the camptothecin-resistant mutation G363V in the lips region of human topoisomerase I can suppress the lethal phenotype of a T718A mutant that, by itself, mimics camptothecin treatment by stabilizing the covalent intermediate (27). Molecular modeling studies further substantiate that structural changes within the lips region of the protein could influence the architecture of the active site of the enzyme and therefore potentially have an effect on the chemistry of catalysis (27).

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