Biochemical and Biophysical Analyses of Recombinant Forms of Human Topoisomerase I*

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Amino acid sequence comparisons of human topoisomerase I (Topo I) with seven other cellular Topo I enzymes reveal that the enzyme can be divided into four major domains: the unconserved NH2-terminal domain (24 kDa), the conserved core domain (54 kDa), a poorly conserved linker region (5 kDa), and the highly conserved COOH-terminal domain (8 kDa), which contains the active site tyrosine. To investigate this predicted domain organization, recombinant baculoviruses were engineered to express the 91-kDa full-length enzyme, a 70-kDa NH2-terminally truncated enzyme that is missing the first 174 residues, and a 58-kDa NH2- and COOH-terminally truncated core fragment encompassing residues 175–659. The specific activity of the full-length and Topo70 enzymes are indistinguishable from the native human Topo I purified from HeLa cells. Each protein is inhibited by camptothecin, topotecan, and 9-amino-campothecin, but not by ATP. Activity is stimulated by Mg2+, Ba2+, Ca2+, Mn2+, spermine, and spermidine. The magnitude of the stimulatory effect of Mg2+ is inversely proportional to the salt concentration. Furthermore, at KCl concentrations of 300 mM or greater, the addition of Mg2+ is inhibitory. The effects of Mg2+ and the polycations spermine and spermidine are partially additive, an indication that the stimulatory mechanisms of the two substances are different. Activity was strongly inhibited or abolished by Ni2+, Zn2+, Cu2+, Cd2+, and Co2+. An examination of the hydrodynamic properties of full-length Topo I, Topo70, and Topo58 demonstrates that the core, linker, and COOH-terminal domains fold into a globular structure, while the NH2-terminal domain is highly extended. A comparison of the circular dichroism spectra of full-length Topo I and Topo70 demonstrates that residues 1–174 (21 kDa) of Topo I are largely if not completely unfolded. This observation is consistent with the fact that the NH2-terminal domain is dispensable for activity.

Eukaryotic topoisomerase I (Topo I) is capable of relaxing both negatively and positively supercoiled DNA. The enzyme catalyzes changes in the superhelical state of duplex DNA by transiently breaking a single strand, allowing for unwinding of positively supercoiled DNA or rewinding of negatively supercoiled DNA (reviewed in Ref. 1). No metal cation or energy cofactor is required for Topo I activity, although Mg2+ and Ca2+, as well as the polycation spermidine, have been shown to stimulate activity (2–5). Phosphodiester bond energy is preserved during the nicking-closing cycle by the formation of a phosphotyrosine bond between the active-site tyrosine and the 3'-end of the broken strand (6–8). This covalent intermediate can be trapped by denaturing the enzyme during catalysis with either SDS or alkali (9–11). Sequence analyses of a large number of SDS-induced breakage sites indicated that the cellular Topo I enzymes will cleave at specific sequences (9, 12, 13), but there is only limited sequence similarity between such sites (9, 12–17). The SDS-induced cleavage at many breakage sites is enhanced by camptothecin, a plant alkaloid that inhibits the cellular enzymes by reversibly binding to the covalent Topo I–DNA intermediate in a manner that slows the relaxation step of catalysis (17–22).

The human Topo I is composed of 765 residues with a predicted molecular mass of 91 kDa. Sequence comparisons of cellular eukaryotic Topo I proteins demonstrate that the human Topo I can be divided into four domains (Fig. 1).2 Residues Met1–Lys197 (24 kDa) comprise the unconserved NH2-terminal domain, which is highly charged (Asp + Glu = 27%; His + Lys + Arg = 68%) and contains four putative nuclear localization signals (24). Residues Glu198–Ile561 (54 kDa) form the conserved core domain, which is followed by a short positively charged linker domain of unconserved residues Asp562–Glu696 (5 kDa). Finally, residues Glu697–Phe760 (8 kDa) make up the highly conserved COOH-terminal domain, which contains the active site tyrosine at position 723 (25, 26).2 Previous reports have demonstrated that the NH2-terminal domain is sensitive to proteolysis (3) and that residues 1–230 can be removed with little if any consequence for Topo I activity (25, 27). In contrast, a 5-amino acid deletion from the COOH terminus abolishes activity.3

We have used the baculovirus-infected insect cell system to overproduce full-length, as well as NH2- and COOH-terminal deletions of human topoisomerase I. The purified recombinant Topo I is by all biochemical criteria identical to the native enzyme purified from human cells. Furthermore, we find the activities of the full-length protein and an amino-terminally deleted enzyme (Topo70, missing residues 1–174)
Recombinant Forms of Human Topo I

**Plasmid Relaxation Assays**

Unless stated otherwise, stocks of protein were serially diluted 2-fold in standard buffer (150 mM KCl, 10 mM Tris-hydrochloride, pH 7.5, 1 mM DTt, 1 mM EDTA, 0.1 mM BSA), and reactions were initiated by the addition of 5 \( \mu l \) of the diluted enzyme to 15 \( \mu l \) of the appropriate buffer containing 0.5 \( \mu g \) of supercoiled pKSI1 plasmid substrate (Strategene). The final reaction conditions are indicated in the figure legends. The reactions were incubated at 37°C for 10 min and terminated with 5 \( \mu l \) of a stop mix containing 2.5% SDS, 15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, and 25 \( \mu l \) EDTA. The products were fractionated by 0.8% agarose gel electrophoresis and visualized by ethidium bromide staining. The inhibitors' camptothecin (Sigma), topotecan (NCI, National Institutes of Health), and 9-amino-camptothecin (NCI) were dissolved in Me2SO and stored at -20°C.

**Isolation of Recombinant Baculoviruses and Large Scale Sf9 Infeciton**—Recombinant baculoviruses were generated by cotransfecting Spodoptera frugiperda (Sf9) cells with linearized wild type Autographica californica multiple nucleocapsid nuclear polyhedrosis virus DNA (Invitrogen) together with transfer vector DNA, and plaque purified according to standard procedures provided by Invitrogen. In order to confirm that the recombinant viruses were expressing the appropriate protein, infected cells were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Sf9 cells were maintained in TC100 medium (Life Technologies, Inc.) supplemented with 10% Fetal calf serum, yeastolate (3.3 g/l), lactalbumin hydrolysate (3.3 g/l), 100 units/ml of penicillin, 100 \( \mu g/ml \) of streptomycin, and 100 units/ml of nystatin. Cells were cultured in 100 ml and 1-liter spinner flasks (Becton) and stirred at a rate of 60 rpm in an atmosphere of 50% \( O_2 \), 50% air at 27°C. The 1-liter flasks were assembled with bench-top impellers (Becton) that were adjustable to maintain a 1:1 air-to-solution interface.4 The maximum volume of medium used in the spinner flasks was 80 and 500 ml for the 100-ml and 1-liter flasks, respectively. Typically, cells were seeded at 0.5-0.8 \( \times 10^6 \) cells/ml and cultured until the density reached 3-3.5 \( \times 10^6 \) cells/ml and then resupplied in fresh medium at a density of 1-1.5 \( \times 10^6 \) cells/ml. The appropriate volume of virus stock was added to ensure a multiplicity of infection of approximately 10 plaque forming units/cell. After stirring for 1 h at room temperature, fresh medium was added to the cells such that the final density was 3 \( \times 10^6 \) cells/ml.

**Protein Purification**

Purification of Topo I from Baculovirus-infected Insect Cells—All purification steps except those involving room temperature high pressure liquid chromatography (Mono Q, Mono S, and POROs columns) were carried out at 4°C. At 48 h postinfeciton, approximately 3 \( \times 10^6 \) Sf9 cells were harvested by centrifugation for 5 min at 400 \( \times \) g. The cells were resuspended in 1 liter of ice-cold phosphate-buffered saline and centrifuged for 5 min at 400 \( \times \) g. The wash procedure was repeated twice with 250 ml of phosphate-buffered saline. The washed cells were resuspended by vigorous shaking in 40 ml of lysate buffer (50 mM KCl, 10 mM Tris-hydrochloride, pH 7.5, 2 mM MgCl2, 1% Triton X-100, 15 mM DTT, 0.15 mM phenylmethylsulfonil fluoride, 0.05 mM aprotinin).

S. G. Graber, personal communication.
The nuclei were washed twice in 80 ml of lysis buffer minus Triton X-100 and resuspended in 40 ml of resuspension buffer (50 mM KCl, 10 mM Tris-hydrochloride, pH 7.5, 2 mM MgCl₂, 25 mM DTT, 0.4 mg/ml phenylmethylsulfonyl fluoride, 0.12 mg/ml aprotinin). The nuclei were adjusted to 10 mg/ml EDTA and then lyzed by the addition of 50 ml of 20 mM Tris-hydrochloride, pH 7.5, 200 mM NaCl, 500 mM KPO₄, pH 7.4 (PPB). The nuclear extract was stirred at 4°C for 30 min. With continued stirring, 50 ml of polyethylene glycol (PEG) buffer (18% PEG 8000, 1 mM NaCl, 10% glycerol) was added dropwise in order to precipitate the DNA (30). After stirring for 40 min, the PEG precipitate was pelleted by centrifugation at 10,000 × g for 10 min. The resulting PEG supernatant was dialyzed against 4 liters of potassium phosphate buffer (400 mM NaCl, 200 mM KPO₄, pH 7.4) and loaded onto a Mono S column that was eluted with a 25-ml 50–200 mM KPO₄, pH 7.4 gradient. The Topo I flowed through the Mono Q column and was loaded onto a Mono S column (5H/R) that was equilibrated with K100 buffer (100 mM KPO₄, pH 7.4, 1 mM EDTA, 1 mM DTT) and was eluted with a 25-ml 50–200 mM KPO₄, pH 7.4, gradient. Topo I eluted as a single major peak at ~150 mM KPO₄. The peak Mono S fractions were pooled, concentrated with an Amicon ultrafiltration cell, dialyzed into storage buffer (50 mM KCl, 10 mM Tris-hydrochloride, pH 7.5, 1 mM DTT, 1 mM EDTA), and stored at ~20°C. The high level expression achieved in the baculovirus-infected insect cell system yielded ~10 mg of protein from 3 × 10⁹ HeLa S3 cells that were doubling every 20–24 h in S-modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 50 µunits/ml of nystatin. The initial steps in purification of HeLa Topo I, up to the point of isolating the clarified dialyzed PEG supernatant, were identical to that described above for the recombinant enzyme. The dialyzed PEG supernatant was loaded with an equal volume of water, filtered through a 0.45-μm syringe filter, and loaded onto a POROS SP20 (4.6/100) column (PerSeptive Biosystems) that was equilibrated with K100 buffer (100 mM KPO₄, pH 7.4, 1 mM EDTA, 1 mM DTT) and was eluted with a linear 50-ml NaCl gradient from 400 mM to 1 M. The f-Topo70 and f-Topo75 co-eluted at 140 mM KPO₄, while the f-Topo75 eluted at 180 mM KPO₄. The peak fractions of each protein were pooled and dialyzed against 100 mM NaCl, 20 mM Tris-hydrochloride, pH 7.5), and passed through a Mono Q column that was equilibrated with Mono Q buffer. Finally, the purified f-Topo70 and f-Topo75 fractions were dialyzed into storage buffer and maintained at ~20°C.

**Amino-terminal Sequencing**

The f-Topo75 and f-Topo70 proteins were fractionated by SDS-PAGE and then transferred to Immobilon-P (Millipore) membranes in 10 mM CAPS, pH 10. The proteins were visualized by staining the membranes with Coomassie Blue. The appropriate bands were excised and sent to DNA Express (Fort Collins, CO) for amino-terminal sequencing.

**Gel Filtration and Glyceral Gradient Sedimentation**

Fast protein liquid chromatography (Pharmacia) gel filtration analyses were performed at 25°C with a flow rate of 0.75 ml/min using a Superose 12 (Pharmacia) column that was equilibrated with gel filtration buffer (200 mM KPO₄, pH 7.4, 1 mM DTT, 1 mM EDTA). Purified protein samples of 10–100 μg were diluted into gel filtration buffer and injected in a total volume of 200 μl. Elution profiles of both Topo I constructs and molecular weight markers were monitored by UV absorbance at 280 nm and analyzed by SDS-PAGE. Glyceral gradient sedimentation analyses were performed by layering 250-μl samples of protein in 200 mM KPO₄, pH 7.4, onto a 3.8-ml linear 10–30% glyceral gradient containing gel filtration buffer. The gradients were centrifuged at 50,000 rpm in an SW60 rotor (Beckman) for 16 h at 25°C. Fractions of 300 μl were collected from the bottom of the gradient tube through a small puncture and analyzed by SDS-PAGE and silver staining. The experimental gradients each included carbonic anhydrase as an internal standard. Parallel gradients were used to determine the sedimentation profiles of the marker proteins.

**Circular Dichroism**

Proteins were extensively dialyzed into 10 mM KPO₄, pH 7.4. Exact molar concentrations of each protein were calculated from the A₂₈₀ values of fully denatured protein in 6 M guanidine hydrochloride using molar extinction coefficients that were calculated by the Genetics Computer Group (GCG) software. CD spectra were obtained at room temperature, using a Jasco 3000 spectropolarimeter with a 0.1-mm path length cell. The molar ellipticity spectrum for each sample was taken as the average of 8–12 individual scans. To eliminate the dichroism that is contributed by the sample buffer, the molar ellipticity spectrum for the dialysis buffer was subtracted from the molar ellipticity spectrum for each sample. The molar ellipticity values were normalized for the concentration of amide bonds in each sample and then converted to δ values (31).

**SDS-Polyacrylamide Gel Electrophoresis and Autoradiography**

SDS-PAGE was performed according to Laemmli (32). Proteins were visualized by Coomassie Blue or silver staining (33). Autoradiography was performed by exposing dried gels to Kodak XAR film.

**RESULTS**

Expression and Purification of Recombinant Forms of Human Topo I—With the long term goal of investigating the domain structure of human Topo I, we developed procedures to obtain large quantities of purified enzyme. This was achieved by the generation of recombinant baculoviruses that express wild type and active-site mutant (Y723F) versions of the full-length human Topo I and a 70-kDa protein (Topo70) which is missing the first 174 NH₂-terminal amino acids (Fig. 2A). The recombinant Topo70 retains at least one (residues 192–198) of the four potential nuclear localization signals that reside in the

**Recombinant Forms of Human Topo I**
Recombinant Forms of Human Topo I

A

| Name       | Primary Structure | MW (kDa) |
|------------|-------------------|----------|
| F.L. topo I|                   | 91       |
| topo70    |                   | 70       |
| topo58    |                   | 55       |

Fig. 2. Recombinant proteins. Panel A, baculoviruses were engineered to express the following proteins: wild type and active-site mutant (Y723F) full-length human Topo I (F.L. topo I), wild type and Y723F mutant versions of a 70-kDa NH2-terminally truncated Topo I (Topo70), which initiates translation with an engineered methionine immediately upstream of Lys135, and an NH2- and COOH-terminally truncated 58-kDa form of Topo I (Topo58), which has the same initiating methionine as Topo70 but is terminated after residue Ala659. The predicted molecular mass (kDa) for each protein is indicated at the right. Panel B, purified proteins (5 μg each) were fractionated by 9-17% SDS-PAGE and visualized by Coomassie Blue staining. Lane 1 contained molecular mass markers (Bio-Rad) myosin (200 kDa), β-galactosidase (116 kDa), phosphohexose isomerase (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), lysozyme (14.4 kDa), and apotinin (6.3 kDa). Lane 2, HeLa Topo I. Lane 3, recombinant Y723F full-length Topo I. Lane 4, Y723F proteolytic fragment of 75 kDa (f-Topo75). Lane 5, Y723F proteolytic fragment of 70 kDa (f-Topo70). Lane 6, Y723F recombinant Topo70. Lane 7, recombinant Topo58.

B

NH2-terminal domain (24). We also generated a recombinant baculovirus that expresses a NH2- and COOH-terminally truncated 58-kDa protein (Topo58), which encompasses the conserved core domain (Fig. 2A). The recombinant proteins have been purified to apparent homogeneity (Fig. 2B). The wild type full-length Topo I and Topo70 proteins have the same specific activity as the native HeLa enzyme (Table I, line 1). The active site Y723F mutant protein and Topo58 are at least 5000-fold less active than the wild type enzyme. The very low level of activity present in the mutant preparations is due to trace amounts of contaminating insect cell Topo I.

The recombinant full-length Topo I and HeLa cell Topo I co-migrate in SDS-PAGE analyses (Fig. 2B, compare lanes 2 and 3). Although the full-length proteins are predicted to be 91-kDa (25), they migrate anomalously in SDS gels with an apparent molecular mass of ~100 kDa. In contrast, the recombinant Topo70 migrates appropriately with an apparent molecular mass of ~70 kDa (Fig. 2B, lane 6). Since it has previously been observed that negatively charged residues retard the migration of proteins in SDS gels (34), it is likely that the 48 negatively charged residues in the first 174 NH2-terminal amino acids are responsible for the slow migration of the full-length protein.

While designing the purification scheme, we noticed that long term storage of nuclear extracts leads to proteolysis of the full-length enzyme into a fragments of 75 and 70 kDa, designated f-Topo75 and f-Topo70, respectively (Fig. 2B, lanes 4 and 5). Both of these fragments were purified and found to display activity equal to that of the full-length protein (Table I, line 1), indicating that they retain the active site tyrosine, which is very close to the COOH terminus. This observation, together with the fact that amino acids downstream of the active site are essential for activity (27), indicated that f-Topo75 and f-Topo70 represent NH2-terminally deleted forms of Topo I.

Amino terminal sequencing confirmed that f-Topo75 was missing the first 137 residues while f-Topo70 was missing the first 174 residues.

The Effect of Divalent Metal Cations and Polycations on Topo I Activity—Although not required for Topo I activity, divalent metal cations are known to stimulate the activity as much as 25-fold (2, 3). Therefore to further characterize the recombinant full-length Topo I and Topo70, we examined the effect of a variety of divalent cations on the activity of the two proteins. In the standard relaxation assay buffer (which contains 150 mM KCl), Mg2+, Mn2+, Ba2+, and Ca2+ were all found to stimulate Topo I activity approximately 16-fold (Table I, lines 2, 10, 19, 20, and 21). In contrast, Cd2+, Zn2+, Co2+, and Cu2+ completely inhibit Topo I (Table I, lines 22-25), while Ni2+ was found to inhibit activity 16-fold at 5 mM (Table I, line 26).

To further investigate the stimulatory effect of Mg2+, we thought it would be informative to determine the level of Mg2+ stimulation obtained over a range of salt concentrations (Fig. 3). Initially, we varied the salt concentration in the absence of Mg2+ and found that the optimal KCl concentration was 200-250 mM, with very low activity detected at KCl concentrations less than 10 mM or greater than 400 mM (Fig. 3A, and data not shown). We then assayed the effect of 10 mM Mg2+ over a range of KCl concentrations from 25 to 350 mM. This revealed that Mg2+ had its largest stimulatory effect (50-fold) at low salt concentrations (25 mM KCl). As the KCl concentration was increased, the stimulatory effect of Mg2+ steadily dropped, and at ~250 mM Mg2+ was slightly inhibitory. With higher salt concentrations, 300 and 350 mM, the addition of Mg2+ was 3- and 7-fold inhibitory, respectively. The full-length and NH2-terminally truncated Topo70 enzymes behaved identically in their responsiveness to salt and Mg2+ (data not shown).

For both forms of Topo I, there was an inverse relationship between the fold-effect of Mg2+ on activity and the KCl concentration. This is best depicted graphically as a logarithmic plot of the ratio of enzyme activity with and without Mg2+ versus the KCl concentration (Fig. 3B).

Like divalent metal cations, polycations such as spermine and spermidine have also been shown to stimulate Topo I activity (5, 35). However, previous reports did not describe the effect of a combination of Mg2+ and a polycation, which might shed light on the stimulatory mechanism of each alone. For example, if the Mg2+ and spermidine effects were additive, then this might suggest separate mechanisms for activation.

Thus, we examined the ability of spermidine and spermine to stimulate Topo I activity in the presence and absence of 10 mM MgCl2 (Table I). In the absence of Mg2+, spermine (1 mM) and spermidine (5 mM) were found to stimulate Topo I activity 8-fold at 150 mM KCl. However, the combination of 10 mM Mg2+ and 1 mM spermidine stimulated the activity 64-fold. This
suggests that the effects of polycations and Mg2⁺ are at least partially additive and that the two substances may stimulate Topo I activity by different mechanisms.

Effect of ATP on Topo I Activity—Topo I does not require ATP or any other energy source for activity. However, it has been reported that physiological concentrations of ATP (~2 mM) can inhibit human Topo I (36). In another report, ATP was shown to inhibit human Topo I only in the presence of 1 mM KPO₄ (37). These conflicting reports prompted us to examine the effect of ATP and KPO₄ on the activity of HeLa Topo I in the presence or absence of 10 mM MgCl₂ (Table I, lines 15-18). Topo I and 9-amino-camptothecin were also found to inhibit relaxation 8–16-fold in the presence or absence of Mg2⁺ (Table I, lines 15-18).

Hydrodynamic Properties of Full-length Topo I and Topo70—The sensitivity of the NH₂-terminal one-fourth of Topo I to proteolysis suggested that it might be in an extended conformation, while the remaining three-quarters of the protein, which is more resistant to proteolysis, might be more globular. To test this notion, we subjected full-length Topo I, Topo70, and Topo58 to gel filtration and glycerol gradient sedimentation analyses (Fig. 4). As previously reported (38), human Topo I chromatographed through gel filtration with an apparent molecular mass of ~300 kDa (Fig. 4A). In contrast, the same protein sedimented in a glycerol gradient with an apparent molecular mass of ~66 kDa (Fig. 4B). The discrepancy in the molecular mass estimates by the two methods suggests that Topo I has an extended shape and thus a larger frictional coefficient than would be expected for a globular protein of 91 kDa. In contrast, the NH₂-terminally truncated Topo70 was found to have an apparent molecular mass of ~66 kDa by sedimentation and ~96 kDa by gel filtration, indicating that the NH₂-terminal region is largely responsible for the asymmetric shape of the protein.

Since full-length Topo I and Topo70 both co-sediment with BSA in a glycerol gradient, we can assume that the sedimentation coefficients for the two proteins are approximately equal to that of BSA (s²₀,ₘ = 4.55) (39). Thus, the frictional coefficient (f) for each enzyme can be estimated from the formula f = m(1

The assay buffer contained 40 mM Tris-hydrochloride, pH 7.5, to ensure proper pH in certain reaction conditions that contained divalent metal ions.

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**Table I**

| Additives | Full-length Topo I | HeLa Topo I | Topo70 | f-Topo70 | f-Topo75 |
|-----------|--------------------|-------------|--------|---------|---------|
| 1. No additive | 1 | 1 | 1 | 1 | 1 |
| 2. 10 mM MgCl₂ | 16 | 16 | 16 | 16 | 16 |
| 3. 5 mM spermine | 8 | 8 | 8 | 8 | 8 |
| 4. 1 mM spermine + 10 mM MgCl₂ | 64 | 64 | 64 | 64 | 64 |
| 5. 1 mM spermine | 8 | 8 | 8 | 8 | 8 |
| 6. 4 mM ATP | 1 | 1 | 1 | 1 | 1 |
| 7. 4 mM ATP + 3 mM KPO₄ | 1 | 1 | 1 | 1 | 1 |
| 8. 4 mM ATP + 10 mM MgCl₂ | 8 | 8 | 8 | 8 | 8 |
| 9. 4 mM ATP + 10 mM MgCl₂ + 3 mM KPO₄ | 8 | 8 | 8 | 8 | 8 |
| 10. 6 mM MgCl₂ | 8 | 8 | 8 | 8 | 8 |
| 11. 50 μM camptothecin | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| 12. 50 μM camptothecin + 10 mM MgCl₂ | 1 | 1 | 1 | 1 | 1 |
| 13. 50 μM camptothecin + 5 mM spermidine | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 14. 50 μM camptothecin + 1 mM spermine | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 15. 50 μM topotecan | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 |
| 16. 50 μM topotecan + 10 mM MgCl₂ | 1 | 1 | 1 | 1 | 1 |
| 17. 50 μM 9-amino-camptothecin | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| 18. 50 μM 9-amino-camptothecin + 10 mM MgCl₂ | 1 | 1 | 1 | 1 | 1 |
| 19. 10 mM MnCl₂ | 16 | 16 | 16 | 16 | 16 |
| 20. 10 mM CaCl₂ | 16 | 16 | 16 | 16 | 16 |
| 21. 10 mM BaCl₂ | 16 | 16 | 16 | 16 | 16 |
| 22. 5 mM CdCl₂ | <0.005* | <0.005* | <0.005* | <0.005* | <0.005* |
| 23. 5 mM ZnCl₂ | <0.005* | <0.005* | <0.005* | <0.005* | <0.005* |
| 24. 5 mM CuSO₄ | <0.005* | <0.005* | <0.005* | <0.005* | <0.005* |
| 25. 5 mM CoCl₂ | <0.005* | <0.005* | <0.005* | <0.005* | <0.005* |
| 26. 5 mM NiCl₂ | 0.07* | 0.07* | 0.07* | 0.07* | 0.07* |

*The assay buffer contained 40 mM Tris-hydrochloride, pH 7.5, to ensure proper pH in certain reaction conditions that contained divalent metal cations.
The partial specific volume, $v$, is the partial specific volume, $r$ is the density of the solvent, and $s$ is the sedimentation coefficient (39). The partial specific volumes for full-length Topo I and Topo70 are both 0.74, as predicted from the amino acid content of each protein (39). Thus, taking $r$ as equal to 1.0 g/cm$^3$ (the density of water at 20°C), the frictional coefficients for the full-length protein and Topo70 are calculated to be $8.7 \times 10^{-8}$ and $6.6 \times 10^{-8}$ (g x s$^{-1}$), respectively. Given these frictional coefficients, the axial ratio (39) of the full-length protein is approximately 10, as compared with a value of 5 for Topo70 (BSA is 6), confirming the elongated shape for full-length Topo I.

Topo58 and Topo70 eluted from a gel filtration column with similar apparent molecular masses of ~83 and ~96 kDa, respectively. However, they sedimented with apparent molecular masses of ~59 and ~66 kDa. The ~30-kDa disparity between molecular mass estimates by the two methods indicates that the shared 58-kDa domain of the two proteins either contains a small highly extended region or is by itself somewhat elongated. Evidence from limited proteolysis studies (see accompanying paper (40)) indicates that the former possibility is more
likely to be correct. For example limited trypsin digestion of full-length Topo I generates a proteolytically resistant 55-kDa fragment that starts at residue Lys 197 and ends somewhere very close to residue Lys 654 (Fig. 1A in Ref. 40). The sensitivity to proteolysis strongly suggests that the NH2-terminal segment of the Topo58 protein is likely extended (residues Lys174–Lys197), providing the hydrodynamic feature that leads to the ~30-kDa discrepancy.

CD studies of Full-length Topo I and Topo70—The gel filtration and sedimentation analyses indicated that human Topo I has a highly extended NH2 terminus, which minimally includes the first 174 residues but probably extends up to residue 197. To further investigate this structural feature, we obtained the far UV CD spectra for both the wild type and active-site mutant (Y723F) versions of full-length Topo I and Topo70 (Fig. 5). Within experimental error, the CD spectra for the wild type and mutant versions of the two proteins are identical (Fig. 5, A and B). Hence, as expected the active-site mutation Y723F does not appreciably alter the structure of the enzyme as measured in this way. In contrast, the averaged CD spectra of Topo70 and full-length Topo I are different (Fig. 5C). To minimize experimental error in this comparison, we averaged the mutant and wild type spectra obtained for two different preparations of each protein. Topo70 has a significantly stronger polarization in the 190-nm range than the full-length protein. Since polarization at 190 nm is a very reliable predictor of α-helical content in proteins (31, 41), it is immediately apparent that the Topo70 protein has a greater percentage of α-helical content than the full-length protein. To confirm this notion, we compared the percentage secondary structures of the two proteins as predicted by the program VARSCLC1 (41). The predicted molecular mass of α-helix (~28 kDa) and parallel β-sheet (~3 kDa) are very similar for the two proteins. However, the full-length protein is predicted to contain an extra 10 kDa of unfolded regions, 8 kDa of turns, and 4 kDa of antiparallel β-sheet. Hence, while the NH2 terminus appears to be largely unfolded, it also appears to have a substantial quantity of turns and antiparallel β-sheet. The turns and antiparallel β-sheet could either be folded into a stable contiguous domain or instead could be a reflection of transiently formed secondary structure in an otherwise random coil (41). To assess which of the explanations is more likely, we analyzed the first 174 residues Topo I with the PEPTIDESTRUCTURE function of the GCG software package (Fig. 5D). This program predicts only very short regions of secondary structure for the NH2-terminal domain. Taking all of the information into account, gel filtration, sedimentation, and CD analyses, we conclude that the amino-terminal 174 residues of Topo I are largely unfolded and are comprised of very little if any extended secondary structure.

DISCUSSION

The Effects of Metal Cations and Polycations—The recombinant human Topo I produced in the baculovirus-infected insect cell system displays the same apparent molecular weight and specific activity as the native enzyme purified from HeLa cells. Consistent with earlier findings (3, 25, 27), the full-length Topo I and the NH2-terminally truncated Topo70 display identical activities. Both enzymes are inhibited by camptothecin, topotecan, and 9-amino-camptothecin but not by ATP. The activities of both are stimulated by Mg2+, Ba2+, Ca2+, and Mn2+, but are strongly inhibited by Ni2+, Zn2+, Cu2+, Cd2+, and Co2+. The stimulatory effect of Mg2+ was found to increase with decreasing salt concentration. Under low salt conditions of pH 7.5.

![Graphical representation of the Chou-Fasman peptide structure prediction of residues 1–174 made by the GCG software.](http://www.jbc.org/)

**Fig. 5. Circular dichroism.** CD spectra are presented graphically as δε values versus wavelength (nm). Panel A shows representative spectra for wild type (solid line) and active-site mutant (Y723F) (dashed line) versions of full-length Topo I. Panel B shows representative spectra for wild type (dashed line) and Y723F (solid line) versions of Topo70. Panel C depicts the average spectra for full-length Topo I (dashed line) and Topo70 (solid line), obtained by combining and then averaging mutant and wild type spectra (a total of seven spectra) for different preparations of each protein. Panel D is a graphical representation of the Chou-Fasman peptide structure prediction of residues 1–174 made by the GCG software. Sine waves, α-helix; sharp sawtooth, β-sheet; 180° change in direction, turns; dull sawtooth, random coil.
does not have any major effect on enzyme structure. For the 250 mM Mg\(^{2+}\) and 200 mM KCl. A higher KCl concentration (\(>250\) mM) also results in a final level of activity that was still 10-fold less than that achieved with the most favorable condition, a combination of 10 mM Mg\(^{2+}\) and 200 mM KCl. This suggests that Mg\(^{2+}\) may stimulate the activity by two mechanisms, one similar to that achieved by monovalent cations alone and another that further stimulates activity in the presence of monovalent cations up to 200 mM KCl. At higher KCl concentrations (\(>250\) mM) Mg\(^{2+}\) was found to be slightly inhibitory, as has been observed for the rat liver and vaccinia Topo I enzymes (4, 35).

There are several potential mechanisms whereby a divalent cation such as Mg\(^{2+}\) could effect a large stimulation of Topo I activity. For example Mg\(^{2+}\) is known to effectively shield the negative charge of the phosphate backbone of duplex DNA, which in addition to allowing the two strands to wind tighter (42) also reduces the effective diameter of the double helix (43, 44), making it more favorable for two duplexes to lie on top of each other to form a node (45). Since it has been shown that Topo I has a preference for binding to nodes (46, 47), it could be envisioned that the presence of Mg\(^{2+}\)-facilitated nodes recruits Topo I to supercoiled DNA, thereby effectively increasing activity. Alternatively, Topo I may simply prefer to relax DNA with a Mg\(^{2+}\)-shielded phosphate backbone. Another possibility is that Mg\(^{2+}\) binds to the enzyme effecting some allosteric activation. However, the fact that Mg\(^{2+}\) does not influence the patterns of limited proteolysis of either the free or DNA-bound protein (see accompanying paper (40)), suggests that Mg\(^{2+}\) does not have any major effect on enzyme structure. For the vaccinia Topo I, Mg\(^{2+}\) has been shown to stimulate activity by accelerating the release of DNA substrate following topoisomerization, which is the rate-limiting step in steady state catalysis under low salt conditions (48). Since the viral enzyme has no detectable divalent metal cation binding site (48), this effect is presumably mediated by metal cation binding to DNA. Given the known effects of monovalent cations on processivity (4) and the similarity between the stimulatory effects of monovalent cations and Mg\(^{2+}\), it seems likely that the cellular enzymes are stimulated by Mg\(^{2+}\) in a manner similar to the viral enzyme. The possibility of direct participation of a divalent metal cation in phosphodiester bond cleavage would seem to be excluded by the fact that metal cations are not required for Topo I activity.

The stimulatory effects of Mg\(^{2+}\) and the polycations (spermine and spermidine) were found to be at least partially additive, suggesting that the two substances may influence Topo I activity by separate mechanisms. Spermine and spermidine can effectively shield the phosphate backbone of duplex DNA by binding in the minor groove (49). This information and the fact that certain minor groove binding compounds are known to be inhibitors of Topo I (50) can be taken to suggest that the modification of the minor groove can influence Topo I activity. This hypothesis is further supported by the observed weak consensus sequence for Topo I cleavage (5’ -(A/T)(G/C)(A/T)-(3’), which is suggestive of protein contacts within the minor groove (9, 16). Alternatively, spermine and/or spermidine may have an allosteric effect on Topo I. Further experiments are needed to better define the effects of both divalent and polycationic cations on Topo I activity.

The NH\(_2\)-terminal Domain—The role of the unconserved NH\(_2\)-terminal domain remains elusive. All cellular eukaryotic topoisomerase I enzymes have this highly charged domain, which in every case examined is dispensable for in vivo activity (3, 24, 27, 30, 51). However, since this domain contains nuclear localization signals, it is nevertheless required for the in vivo function of Topo I (24). Of the four putative nuclear localization signals residing in NH\(_2\)-terminal domain of the human enzyme, residues Lys\(^{150}\)-Asp\(^{156}\) have been suggested to be the most important for nuclear localization based on sequence comparisons with other cellular eukaryotic Topo I enzymes (24). However, we find that Topo70, which starts at a methionine immediately 5’ to residue Lys\(^{175}\), is transported to the nucleus of insect cells. Hence, the single remaining intact nuclear localization signal (Lys\(^{192}\)-Glu\(^{198}\)) must be sufficient for nuclear transport, at least in insect cells.

Aside from its role in nuclear localization, what is the function of the NH\(_2\)-terminal? In the human enzyme, we find that residues 1-174 of the NH\(_2\)-terminal domain are largely if not completely unfolded and that the activity of the Topo70 enzyme (which is missing these residues) is indistinguishable from that of the full-length enzyme by every criterion tested. The highly extended nature of the NH\(_2\)-terminal domain was observed using analytical techniques that involved conditions of both low salt (circular dichroism, 10 mM) and high salt (sedimentation and gel filtration, 200 mM). Hence, the extended nature of the NH\(_2\)-terminal persist under variable salt concentrations and is not an artifact of any single analytical technique. With its high density of both negatively and positively charged residues (67% charged residues), the NH\(_2\)-terminal is almost zwitterionic in nature. Accordingly, we have observed that the full-length enzyme can be concentrated to >50 mg/ml without any signs of precipitation. In contrast, Topo70 can only be concentrated to ~5 mg/ml before it begins to precipitate (data not shown).

Hence, the NH\(_2\)-terminal domain acts as a solubilizing element in vitro. A conservative estimate for the quantity of Topo I in HeLa cells can be made based on our recovery of 250 μg of purified Topo I from 10\(^7\) HeLa cells. Since greater than 95% of the cell protein is associated with the nuclear compartment (data not shown), which has an average free water volume of approximately 3000 μm\(^3\) for HeLa S3 cells (52), we estimate that the minimal nuclear concentration of Topo I is ~100 μg/ml. This concentration is 50-fold below the solubility limit of Topo70. However, Topo I is known to be highly concentrated in regions of chromatin that are undergoing high levels of transcription such as activated heat-shock loci and the nucleolus (28, 29, 53, 54). Furthermore, UV cross-linking and SDS-induced trapping of Topo I-DNA complexes has revealed that Topo I is enriched at least 20-fold on rDNA relative to total DNA (29) and is enriched 20-fold at induced heat shock loci relative to the uninduced loci (23). Thus, the possibility exists that local concentrations of Topo I could approach 5 mg/ml, which might necessitate a solubility factor such as the zwitterionic NH\(_2\)-terminus.

The Shape of Topo I—In addition to revealing the extended nature of the NH\(_2\)-terminal domain, the comparison of the hydrodynamic properties of full-length Topo I to those of Topo70 and Topo58 revealed that the core, linker, and COOH-terminal domains fold into a globular structure. Hence, the catalytically active domain of Topo I is largely globular, while the NH\(_2\)-terminal domain is largely unfolded, consistent with it being dispensable for activity. Since the Topo58 core domain was capable of folding into a well behaved globular molecule, it probably represents a subdomain of Topo I that is capable of folding independent of the amino-terminal, linker, or core domains. Finally, the availability of milligram quantities of purified full-length Topo I, Topo70, and Topo58 greatly facilitated further studies of the structure-function relationships of Topo I. In the accompanying paper, we describe the use of limited proteolysis to further characterize the domain structure of the protein (40).

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