The Domain Organization of Human Topoisomerase I*

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Using limited proteolysis, we show that the domain boundaries of human topoisomerase I closely parallel those predicted from sequence comparisons with other cellular Topo I enzymes. The enzyme is comprised of (i) an NH₂-terminal domain (~24 kDa), which is known to be dispensable for activity, (ii) the core domain (~54 kDa), (iii) a linker region (~3 kDa), and (iv) the COOH-terminal domain (~10 kDa), which contains the active site tyrosine. The highly conserved core and COOH-terminal domains are resistant to proteolysis, while the unconserved NH₂-terminal and linker domains are sensitive. Noncovalent binding of Topo I to plasmid DNA or to short duplex oligonucleotides decreases the sensitivity of the linker to proteolysis by approximately a factor of 10 but has no effect on proteolysis of the NH₂-terminal domain. When the enzyme is covalently complexed to an 18 base pair single-stranded oligonucleotide, the linker region is sensitive to proteolysis whether or not duplex DNA is present. The net positive charge of the linker domain suggests that at a certain point in catalysis the linker may bind directly to DNA. Further, we show that limited subtilisin cleavage can generate a mixture of 60-kDa core and ~10-kDa COOH-terminal fragments, which retain a level of topoisomerase activity that is nearly equal to undigested control samples, presumably because the two fragments remain associated after proteolytic cleavage. Thus, despite its potential role in DNA binding, the linker domain (in addition to the NH₂-terminal domain) appears to be dispensable for topoisomerase activity. Finally, the limited proteolysis pattern of the human enzyme differs substantially from the limited proteolysis pattern of the vaccinia viral Topo I, indicating that the two enzymes belong to separate eukaryotic topoisomerase I subfamilies.

Eukaryotic topoisomerase I (Topo I)1 reveals both negatively and positively supercoiled DNA by catalyzing the transient breakage of a phosphodiester bond in a single DNA strand (reviewed in Ref. 1). No metal cation or energy cofactor is required for Topo I activity. Cleavage of a phosphodiester bond in DNA involves a transesterification reaction in which the nucleophilic O-4 oxygen of the active site tyrosine (amino acid 723 in human Topo I (2, 3)) attacks the phosphodiester linkage. This results in the formation of a phosphotyrosine bond between the enzyme and the 3’ end of the broken strand. Reversal of the transesterification reaction restores the phosphodiester bond and liberates the enzyme. Two models, free rotation and enzyme-bridging, have been proposed to explain the mechanism by which Topo I promotes topoisomerization of the DNA (reviewed in Ref. 1). The free rotation model proposes that the 5’ end of the broken strand is released from the active site and is allowed to freely rotate about the unbroken strand. The enzyme-bridging model proposes that the unbroken strand is passed through an enzyme-bridged break, formed by covalent attachment to the 3’ end of the broken strand and noncovalent binding to the 5’ end of the broken strand.

Sequence comparisons of the cellular eukaryotic Topo I enzymes2 reveals the human enzyme (765 residues, 91 kDa) can be divided into four domains (see preceding paper (5)): the highly charged (Asp + Glu = 27%; His + Lys + Arg = 68%) unconserved NH₂-terminal domain (residues Met1–Lys197, 24 kDa), which contains four putative nuclear localization signals (6); the conserved core domain (Glu198–Ile651, 54 kDa); a short unconserved positively charged linker domain (Asp652–Glu696, 5 kDa); and the highly conserved COOH-terminal domain (Gln697–Phe765, 8 kDa) which contains the active site tyrosine at position 723 (Refs. 2, 3, and 7). In the accompanying paper (5) we demonstrate that the NH₂-terminal domain is mostly if not completely unstructured, an observation that is consistent with the fact that this domain is sensitive to proteolysis and is dispensable for activity (5–9).

Weak amino acid sequence homology exists between the cellular Topo I enzymes and the vaccinia viral Topo I (similarity ~ 43%, identity ~ 20%). Two segments of the human enzyme show similarity to the vaccinia Topo I. These are residues Pro357–His632 of the core domain and residues Gly723, Phe765 of the COOH-terminal domain, which includes the active site tyrosine.2 Like the cellular enzymes, vaccinia Topo I relaxes negatively and positively supercoiled DNA, does not require an energy cofactor or divalent cation, and is stimulated by Mg²⁺ (10). Despite these similarities, there are major differences between the two enzymes. First, the vaccinia enzyme is not inhibited by camptothecin (10), a plant alkaloid that inhibits the cellular enzymes by slowing the religation step of catalysis (11–14). Furthermore, the vaccinia enzyme cleaves DNA at a unique recognition sequence (15–17), while the cellular enzymes, although they cleave at specific sequences (18, 19), have only a limited sequence preference (14, 20–25).

To further examine the domain structure of human Topo I, we subjected the recombinant enzyme to limited proteolysis with trypsin and subtilisin. The digestion patterns show that...
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the conserved core and COOH-terminal domains are globular, tightly folded segments of the protein, while the NH2-terminal and the linker domain are extremely sensitive to proteolysis. We also find that noncovalent binding of duplex DNA results in protection of the linker domain, but not the NH2-terminal domain, from proteolysis. When the enzyme is permanently trapped in a covalent complex with a single-stranded oligonucleotide, the linker region is rendered sensitive to proteolysis whether duplex DNA is present or not. Taken together, our results provide the first structural model for human Topo I and provide important insights into the nature of its interaction with DNA. A comparison of this model with that recently proposed for the vaccinia viral Topo I suggests that the cellular and viral enzymes belong to different Topo I families.

EXPERIMENTAL PROCEDURES

General—The recombinant proteins including (i) the wild type and Y723F forms of full-length human Topo I (91 kDa), (ii) the wild type and Y723F forms of the NH2-terminally deleted Topo70, which starts with an engineered methionine immediately adjacent to residue Lys151 (70 kDa), and (iii) the core fragment Topo58, which has the same NH2-terminally deleted Topo70 NADH-oxidase activity (88 kDa) were generated and purified as described in the accompanying paper (see Fig. 1B in Ref. 5). Long-term storage of crude insect cell nuclear extracts containing recombinant full-length Topo I resulted in proteolytic breakdown of the full-length protein into fragments of 70 and 75 kDa, designated f-Topo70 and f-Topo75 (8). Both fragments were purified by Ni2+-NTA affinity chromatography, and amino-terminal deletion analyses were carried out as described in the accompanying paper (5).

Antiserum and Immunoblotting—Polyclonal anti-Topo I serum was raised in a rabbit that had been injected with purified recombinant human Topo I. Antiserum for affinity chromatography purification was achieved by incubation of the crude serum with Affi-10 agarose beads (Bio-Rad) to which Topo I had been conjugated. The affinity beads were washed extensively with 100 mM KPO4, pH 7.4, and eluted with 100 mM NaCl, 100 mM NaOH, and 1 mM EDTA. The column was eluted with 100 mM KPO4, pH 7.4, 1 mM EDTA, 1 mM DTT (KDB) (170 mM KPO4, pH 7.4, 1 mM EDTA, 1 mM DTT, 10 mM MgCl2), and terminated by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mM. To assess the effect of DNA on the activity of trypsin and subtilisin, resorufin-labeled casein was subjected to digestion in the presence and absence of DNA. Digestions were carried out in SDB with or without 10 mM MgCl2, and in the presence or absence of 80 μg/ml of plasmid DNA. The reactions were terminated, and the undigested protein was precipitated by the addition of 6 volumes of 0.5 M Tris-hydrochloride, pH 8.5, and subjected to spectrofluorometric analysis with an excitation wavelength of 574 nm and a detector wavelength of 584 nm. In this way, proteolysis of the fluorescent substrate was assessed by measuring the quantity of trichloroacetic acid-soluble chromophore following digestion. This assay demonstrated that DNA has little if any effect on the activity of either trypsin or subtilisin in either the presence or absence of Mg2+ (data not shown).

Purification of Radiolabeled Topo I-Oligonucleotide Covalent Complexes—Radiolabeled Topo I-oligonucleotide complexes were generated by incubating 40 μg of topoisomerase (full-length Topo I or Topo70) with 5 μg of 5'-32P-end-labeled suicide oligonucleotide (either 5'-AAAAAAGCTTAGAAAAATTTTT-3' or 5'-AAAAAGACTTAGAAAAATTTTT-3') in 100 μl of suicide buffer (100 mM NaCl, 10 mM Tris-hydrochloride, pH 7.5, 1 mM DTT, 1 mM EDTA, 10 mM MgCl2) for 3 h at room temperature. The unreacted protein and protein-DNA complexes were separated from excess suicide substrate by small scale SP-Sepharose (Pharmacia Biotech Inc.) chromatography. The reaction mixture was applied to a 0.25-ml bed volume of SP-Sepharose that was equilibrated with 100 mM KPO4, pH 7.4, 1 mM EDTA, 1 mM DTT. The column was washed with 20 volumes of the same buffer to remove excess oligonucleotide and then eluted with two 0.25-ml washes of 500 mM KPO4, pH 7.4, 1 mM EDTA, 1 mM DTT. The eluate, which contained a mixture of suicided complexes and unreacted enzyme, was stored at 4 °C.

RESULTS

Limited Proteolysis of Topo I—To examine the domain structure of Topo I, we subjected recombinant full-length Topo I to limited proteolytic digestion with a variety of proteases. Subtilisin and trypsin were found to produce the most informative limited proteolytic digestion with a variety of proteases. Subtilisin and trypsin were found to produce the most informative

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Limited subtilisin and trypsin digestion of Topo I. A, full-length Topo I (F.L. topo I) was digested with increasing concentrations of subtilisin (lanes 2–5) or trypsin (lanes 7–10). The 34-μl reactions contained 10 μg of Topo I plus the following quantities of protease; 0.2 μg (lanes 2 and 7), 0.1 μg (lanes 3 and 8), 0.05 μg (lanes 4 and 9), 0.025 μg (lanes 5 and 10), or no protease (lanes 6 and 11). Aliquots of the digestion products were fractionated by 5–20% SDS-PAGE and visualized by Coomassie Blue staining. Lanes 1 and 12 contained a mixture of 5 μg of f-Topo75 and f-Topo70 (see Ref. 5). Lane 13 contained 10 μg of Topo I that was not incubated under digestion conditions. Lane 14 contained the molecular mass markers (Sigma) bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), ß-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa). B, after the addition of PMSF, 3-μl aliquots of samples 2, 5, and 6 (displayed in panel A) were mixed with 100 μl of dilution buffer (10 mM Tris-hydrochloride, pH 7.5, 1 mM DTT, 1 mM EDTA, 0.1 mg/ml bovine serum albumin), serially diluted 3-fold in dilution buffer, and then subjected to plasmid relaxation assays. The left panel shows the assays of Topo I that was incubated with no subtilisin (sample 6). The first two lanes of the middle panel contained a sample of input plasmid and a 1-kilobase pair ladder (Life Technologies, Inc.), respectively. The remaining lanes of the middle panel show the assays of Topo I that was digested with 0.025 μg of subtilisin (sample 5). The left panel shows the assays of Topo I that was digested with 0.2 μg of subtilisin (sample 2). For each assay, the 3-fold dilutions are displayed from left to right; starting at ~30 ng/μl of input Topo I. Arrows above the panels are pointed in the direction of increasing dilution.

s-Topo60 fragment (sample 2), there was only a ~3-fold drop in enzyme activity. Since it has been shown previously that the smallest NH2-terminally deleted form of Topo I that retains activity is 63 kDa (starting at residue 231) (9), this result suggested that s-Topo60 (which lacks the COOH-terminal domain) may act in conjunction with a COOH-terminal fragment to achieve activity.

Identification of COOH-terminal Proteolytic Fragments—To determine the fate of the COOH-terminal domain during digestion, we radiolabeled the COOH terminus of recombinant Topo I by incubating the enzyme with a 32P-5’-end-labeled 22-base “suicide” oligonucleotide, which is capable of forming an intermolecular 8-base pair duplex at its 3’ end. Topo I cleaves one strand of this duplex region, and concomitantly becomes covalently linked to the 3’ end at the site of cleavage (data not shown). However, the topoisomerization reaction cannot be completed since the 3’ segment of the broken strand falls away from the cleaved complex, leaving it with no 5’ hydroxyl to attack the phosphotyrosine bond and reverse the reaction.

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This results in the formation of a stable radiolabeled covalent complex, which was separated from excess uncleaved oligonucleotide by SP-Sepharose chromatography. It is important to note that the SP-Sepharose eluate contains only a small fraction of radiolabeled molecules, while the bulk of the protein is unlabeled since it did not undergo suicidal cleavage. This mixture was then digested with increasing quantities of either subtilisin or trypsin. Fragments containing the COOH-terminal domain were identified by autoradiography following fractionation by SDS-PAGE (Fig. 2). This analysis revealed that the 67–75-kDa fragments each contain the COOH-terminal domain (subtilisin lanes 5–8, trypsin lanes 12–16; compare silver stained gels in panel A with the autoradiographs in panel B). As expected, 32P label is absent from the s-Topo60 and t-Topo55 fragments, confirming that they are missing the COOH-terminal domain. It can also be seen that with increasing levels of protease, a 32P-labeled COOH-terminal fragment of ~15 kDa was released from the larger fragments. This 32P-labeled COOH-terminal fragment is composed of a ~5-kDa oligonucleotide that is covalently attached to a COOH-terminal
we subjected both wild type and Y723F mutant full-length Topo I to digestion with increasing quantities of subtilisin in the presence or absence of plasmid DNA (Fig. 3). The DNA: protein mass ratio was set at 2:1 to ensure that protein-DNA complexes remained soluble (data not shown).

In the presence or absence of either DNA or Mg$^{2+}$, the full-length protein was nearly completely converted into a combination of 73- and 75-kDa fragments at low subtilisin concentrations (Fig. 3: lanes 4, 9, 14, and 19). Thus, the initial removal of the NH$_2$-terminal domain from the full-length protein is relatively unaffected by the presence of DNA. In contrast, proteolysis of the 73-kDa fragment to produce the s-Topo60 core is inhibited approximately 10-fold in the presence of DNA (for example, compare lanes 1–4 with lanes 6–9 of both panels A and B). Thus, cleavage between the s-Topo60 core domain and the −10-kDa COOH-terminal domain is inhibited by bound DNA. The block to proteolysis within this region was of the same magnitude in the presence or absence of Mg$^{2+}$ (compare lanes 1–10 with lanes 11–20 of both panels A and B) and was observed with wild-type and active-site mutant enzymes. In the case of the wild-type enzyme, the plasmid DNA is relaxed to completion before subtilisin is added. However, the Y723F protein contains less than 0.1 units (one unit is the amount of enzyme required to fully relax 1 μg of plasmid DNA in 10 min at 37°C) of endogenous insect cell topoisomerase/μg of recombinant enzyme. Therefore, the 20-min room temperature incubation with 5 μg of mutant enzyme results in very little relaxation of the 10 μg of plasmid DNA present during digestion. Thus, supercoiled (when the Y723F mutant is used) and relaxed plasmid DNAs (when the wild-type enzyme is used) are equally effective at blocking cleavage between the s-Topo60 core and the −10-kDa COOH-terminal domains.

The Effect of Duplex Oligonucleotides on Proteolysis of Full-length Topo I and Topo70—There is mounting evidence that Topo I has a preference for binding to DNA nodes, points at which two duplexes cross over (29, 30). Thus, it is possible that node binding causes resistance to proteolysis in the linker region between the core and COOH-terminal domains. To test this possibility, we examined the effect of a short duplex oligonucleotide on the kinetics of limited proteolysis. The rationale for this experiment lies in the fact that, while plasmid DNA molecules, and particularly supercoiled plasmid DNA, have a propensity to form intramolecular nodes (31), short oligonucleotides do not. In the same experiment, we also sought to examine the effect of removing the NH$_2$-terminal domain on the pattern of limited proteolysis. Full-length Topo I and the NH$_2$-terminally truncated Topo70 were subjected to digestion with increasing concentrations of subtilisin in the presence or absence of a 10-fold molar excess of a 22-base pair duplex oligonucleotide (Fig. 4). The oligonucleotide (Fig. 4C) contained the hexadecameric sequence from the rDNA of Tetrahymena plus upstream and downstream base pairs that are known to be important for maximal Topo I cleavage of this high affinity binding site (18, 19, 32–36). The results demonstrate that, as with the plasmid DNA, cleavage of full-length Topo I to generate the −73-kDa fragment was only slightly inhibited (<2-fold) by the presence of the duplex oligonucleotide (compare lanes 3–6 to lanes 12–15 of panel A). In contrast, the oligonucleotide inhibited by ~10-fold the cleavage of both Topo70 and the −73-kDa fragment that is generated by proteolytic removal of the amino terminus from full-length Topo I (compare lanes 3–9 to lanes 12–18 of panels A and B).

In general, the digestion patterns of the −73-kDa fragment and Topo70 were very similar (Fig. 4; compare lanes 5–9 and lanes 16–18 of panels A and B). Like the full-length protein, Topo70 appeared to be cleaved by subtilisin at or near Lys$^{554}$,

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since a 58-kDa (s-Topo58) fragment that migrated in SDS-PAGE slightly faster than the recombinant Topo58 was produced. Taken together, these results demonstrate that the lack of the NH2-terminal domain does not affect the digestion pattern of Topo I. It can also be concluded that a short duplex oligonucleotide inhibits cleavage between the core and the COOH-terminal domains to approximately the same extent as long plasmid DNA.

It should be noted that with high concentrations of subtilisin, the s-Topo60 core fragment is further digested into at least one fragment of 33 kDa (designated s-Topo33; Fig. 4A, lanes 8 and 9). Similarly, further digestion of s-Topo58 produces a fragment of 30 kDa, which migrates slightly slower than subtilisin (designated s-Topo30; Fig. 4B, lanes 8 and 9).

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Fig. 4. The effect of duplex oligonucleotide DNA on subtilisin digestion of full-length Topo I (F.L. topoI) and Topo70. A, active site mutant Y723F full-length Topo I was digested with 2-fold increasing concentrations of subtilisin (starting at 1 ng/μl) in SDB plus 10 mM MgCl₂. The digests were performed either in the absence or presence of a 22-base pair duplex oligonucleotide DNA (−DNA, lanes 2–9, +DNA, lanes 11–18). To ensure that protein-DNA complexes were soluble (data not shown), the DNA-topoisomerase mass ratio was set at 2:1 (2 μg of duplex-mer and 1 μg of enzyme in a 30-μl reaction). The digestion products were fractionated by 9% SDS-PAGE and visualized by silver staining. Lanes 1, 10, and 19, respectively, contained 1 μg of untreated full-length Topo I Y723F, Topo70 Y732F, and Topo58. Lanes 2 and 11 contained samples that were incubated under digestion conditions without any subtilisin (NO Subt.). B, Topo70 was digested under the same conditions described above for full-length Topo I. C, the duplex 22-mer used in the above experiments contains the hexadecameric sequence (underlined), which is known to be a high affinity binding site for mammalian topoisomerases (19, 35, 36). The site of Topo I cleavage is indicated with a small arrow.

Since a 58-kDa (s-Topo58) fragment that migrated in SDS-PAGE slightly faster than the recombinant Topo58 was produced. Taken together, these results demonstrate that the lack of the NH2-terminal domain does not affect the digestion pattern of Topo I. It can also be concluded that a short duplex oligonucleotide inhibits cleavage between the core and the COOH-terminal domains to approximately the same extent as long plasmid DNA.

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or absence of plasmid DNA (autoradiograph reveals that the radiolabeled Topo70-oligo subtilisin (data not shown).

The presence or absence of Mg\(^{2+}\) was found to have no effect on the patterns of limited proteolysis of either the free or DNA bound protein, suggesting that Mg\(^{2+}\) does not have any major effect on enzyme structure. An examination of the Y723F active site mutant enzyme revealed that the DNA-induced protection of the linker segment occurs in the absence of DNA strand breakage, when Topo I is noncovalently bound to DNA. In contrast, the linker domain of permanently trapped covalent complexes is sensitive to proteolysis whether or not DNA is present.

In principle, the effects of DNA binding on proteolysis of Topo I can be explained by either of two general models. The first model supposes that direct DNA binding to a given region blocks access of the protease, resulting in resistance to cleavage. The second model states that DNA binding to one region of the protein causes a conformational shift, rendering another region more or less sensitive to proteolysis. The relatively large number of positively charged residues in the linker domain (15 out of 45, residues Asp\(^{652}\)–Glu\(^{696}\)) suggests that it may bind directly to DNA. Thus, the first model would most easily explain our observation that the linker domain resists proteolysis when Topo I is noncovalently bound to duplex DNA. However, other than being positively charged, the linker region is not well conserved among the cellular topoisomerases. Therefore, it is involved in direct DNA contacts, one would have to suppose that the interactions would be somewhat flexible in their amino acid sequence requirements.

The observation that the linker domain of permanently trapped covalent complexes is sensitive to proteolysis both in the presence and absence of added duplex DNA is not necessarily inconsistent with the direct binding model if one assumes that the short segment of single-stranded DNA is insufficient to protect the linker from proteolysis. In this case, one

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**Fig. 6.** Domain structure of Topo I. As revealed by the biochemical analyses presented in this and the accompanying manuscript (5), the domain structure of human Topo I closely parallels that which is predicted from sequence comparisons. The NH\(_2\)-terminal domain (residues 1–197) is largely if not entirely unfolded and is dispensable for activity. The core domain (residues 198–651) is largely resistant to proteolysis, as is the COOH-terminal 10-kDa domain (residues 697–765). The linker region (residues 652–696) is sensitive to proteolysis in the absence of DNA, but in the presence of DNA it is 10-fold more resistant to proteolysis. In the suicided covalent complex, this same linker region is sensitive to proteolysis both in the absence and in the presence of excess duplex DNA. Arrows, sites of proteolytic cleavage within the NH\(_2\)-terminal domain that have been defined by amino-terminal sequencing. Broken arrows, predicted approximate sites of limited subtilisin and trypsin cleavage within the linker domain. Small arrow, a subtilisin cleavage site located somewhere close to the middle of the core domain (see Fig. 4). Filled circles, putative nuclear localization signals. Open circle, a single nuclear localization signal that is sufficient for nuclear transport.

**DISCUSSION**

Domain Organization of Human Topo I—Limited digestion with subtilisin and trypsin reveals that human Topo I is comprised of four major domains whose boundaries closely parallel those predicted from sequence comparisons of cellular Topo I enzymes (Fig. 6). The 24-kDa unconserved NH\(_2\)-terminal domain (residues Met\(^{1}\)–Lys\(^{197}\)) is highly sensitive to proteolysis and is largely if not completely unfolded (see accompanying paper (5)). It is cleaved at the carboxy side of residues Lys\(^{137}\) and Lys\(^{174}\) by an unidentified insect cell protease and at residues Lys\(^{150}\) and Lys\(^{196}\) by subtilisin and trypsin, respectively. The NH\(_2\)-terminal domain is followed by the conserved 54-kDa core domain (residues Glu\(^{198}\)–Ile\(^{651}\)), which is connected to the 8-kDa COOH-terminal domain (residues Gin\(^{697}\)–Phe\(^{765}\)) via a short linker region. In the absence of DNA, the linker region is sensitive to proteolysis and is cleaved in at least two positions, probably located at Lys\(^{654}\) and Lys\(^{688}\).

DNA Binding—Proteolysis of the linker segment located between the core and COOH-terminal domains is inhibited by either plasmid DNA or a 22-base pair duplex oligonucleotide. Since the length of DNA was not a factor in protecting the linker domain from proteolysis, we conclude that binding to DNA nodes, such as those formed when two duplexes cross within a supercoiled plasmid (29–31), is probably not responsible for protection of the linker domain. However, it is difficult to exclude the possibility that the enzyme binds two segments of a plasmid or two separate duplex oligonucleotides to form the equivalent of a node within the binding pocket of the enzyme. The presence or absence of Mg\(^{2+}\) was found to have no effect on DNA binding to oneregion of the protein causes a conformational shift, rendering another region more or less sensitive to proteolysis. The relatively large number of positively charged residues in the linker domain (15 out of 45, residues Asp\(^{652}\)–Glu\(^{696}\)) suggests that it may bind directly to DNA. Thus, the first model would most easily explain our observation that the linker domain resists proteolysis when Topo I is noncovalently bound to duplex DNA. However, other than being positively charged, the linker region is not well conserved among the cellular topoisomerases. Therefore, it is involved in direct DNA contacts, one would have to suppose that the interactions would be somewhat flexible in their amino acid sequence requirements.

The observation that the linker domain of permanently trapped covalent complexes is sensitive to proteolysis both in the presence and absence of added duplex DNA is not necessarily inconsistent with the direct binding model if one assumes that the short segment of single-stranded DNA is insufficient to protect the linker from proteolysis. In this case, one
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would have to further assume that the suicided oligo would sterically prevent the covalent complex from binding to additional duplex DNA molecules. In an alternative model, it could be envisioned that upon cleavage of DNA the COOH-terminal and/or core domains undergo a conformational shift that renders the linker susceptible to proteolysis in the absence and presence of added DNA.

Though the exact nature of DNA-induced changes that result in differential protease sensitivity remains unclear, our results indicate that the linker domain of Topo I can exist in one of two different states. The linker domain of the free enzyme or of the suicided covalent complex is in an "open" state and is sensitive to proteolysis. However, when Topo I is noncovalently bound to duplex DNA, the linker is in a "closed" state and is less sensitive to proteolysis. These results suggest the intriguing possibility that the linker domain may switch from the "closed" to the "open" state upon formation of the covalent enzyme-DNA complex. We are currently investigating this possibility by examining the proteolytic sensitivity of the linker domain when the enzyme is noncovalently and covalently complexed to a suicide substrate comprised of duplex DNA flanking both sides of the cleavage site. The results from this experiment will also help to resolve which of the two above models better explains the proteolytic sensitivity of the linker domain in the suicided covalent complex.

Comparison with the Vaccinia Viral Topo I—Limited proteolysis studies of the vaccinia virus Topo I have revealed that the enzyme has a tripartite domain organization, being comprised of a 9-kDa NH2-terminal domain, an 8-kDa central domain, and a 20-kDa COOH-terminal domain, which contains the active site tyrosine (4, 28). The three domains are connected via protease-sensitive linker regions. Hence, the limited trypptic digestion pattern of the vaccinia enzyme generates fragments of 9, 8, and 20 kDa. This pattern differs substantially from the trypptic digestion pattern of the human enzyme. For example, the COOH-terminal domain of the human enzyme is only ~10 kDa in size and is connected to a core domain of ~54 kDa via a short proteolytically sensitive linker domain. Furthermore, proteolytic cleavage of the vaccinia enzyme within either of the two linker regions results in loss of activity (4). In contrast, cleavage within the linker domain of the human enzyme has little effect on Topo I activity. Relative to this observation, we have recently found that Topo I activity can be reconstituted by mixing individually expressed core and COOH-terminal domains, the latter of which is inactive by itself.

The structural differences between the human and vaccinia enzymes can be further contrasted when considering the effects of DNA on limited proteolysis. Covalent and noncovalent binding of vaccinia Topo I to duplex oligonucleotide results in 10-fold inhibition of proteolysis at both linker domains (4). In the case of the human enzyme, noncovalent binding to duplex DNA results in protection of the linker domain but has a negligible effect on proteolysis of the NH2-terminal domain. Furthermore, when the human enzyme is covalently bound to a single-stranded substrate, the linker region is rendered sensitive to proteolysis regardless of the presence or absence of duplex DNA. The vaccinia enzyme has only 43% sequence similarity and 20% sequence identity with the human enzyme (28).

Thus, even though the viral and cellular enzymes display very similar catalytic and biochemical properties, the weak sequence similarity between the two and the differences in domain organization as assayed by limited proteolysis strongly suggest that the cellular and viral topoisomerases comprise separate families of type I enzymes. More detailed structural and/or crystallographic analyses of the two proteins will be required to confirm or disprove this prediction.

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