The C-terminal domain but not the tyrosine 723 of human DNA topoisomerase I active site contributes to kinase activity

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ABSTRACT

Human DNA topoisomerase I not only has DNA relaxing activity, but also splicing factors phosphorylating activity. Topo I shows strong preference for ATP as the phosphate donor. We used photoaffinity labeling with the ATP analogue \([\alpha\text{-}^{32}\text{P}]8\text{-azidoadenosine-5}\text{-triphosphate}\) combined with limited proteolysis to characterize Topo I domains involved in ATP binding. The majority of incorporated analogue was associated with two fragments derived from N-terminal and C-terminal regions of Topo I, respectively. However, mutational analysis showed that deletion of the first 138 N-terminal residues, known to be dispensable for topoisomerase activity, did not change the binding of ATP or the kinase activity. In contrast, deletion of 162 residues from the C-terminal domain was deleterious for ATP binding, kinase and topoisomerase activities. Furthermore, a C-terminal tyrosine 723 mutant lacking topoisomerase activity is still able to bind ATP and to phosphorylate SF2/ASF, suggesting that the two functions of Topo I can be separated. These findings argue in favor of the fact that Topo I is a complex enzyme with a number of potential intra-cellular functions.

INTRODUCTION

Eukaryotic DNA topoisomerase I changes the linking number of DNA in individual steps, resulting in the release of a positive or negative superhelical tension of a topologically fixed DNA molecule (1). These biochemical properties suggest that this enzyme may participate in all functional aspects of DNA including transcription, replication and recombination (2). However, yeast cells carrying mutations in the gene encoding the major topoisomerase I activity are viable but grow at slower rate than wild type cells (3,4). Because the topoisomerase I gene could complement other mutations in yeast, it was proposed that other proteins provide overlapping functions which prevent the lethality of a top1 null mutant (5,6). In contrast, the DNA topoisomerase I gene is required for complete embryonic development in Drosophila and mouse (7,8).

Lack of complete understanding of the mechanism underlying the lethal phenotype caused by DNA topoisomerase I inactivation in Drosophila and mouse necessitate a better characterisation of its function in multicellular organisms. The discovery that DNA topoisomerase I has an intrinsic protein kinase activity (9,10), that we termed topo I/kinase, led us to propose that this activity may be required to achieve specific phosphorylation of proteins that associate either directly or indirectly with the transcription machinery. We now know that topo I/kinase phosphorylates members of the SR protein family that are associated with actively transcribed regions of Drosophila polytene chromosomes (11). Since SR proteins are involved in the splice site choice of sequentially regulated genes in Drosophila (12–14), variations in the phosphorylation state of these splicing factors may be instrumental in regulating Drosophila development. The finding that reversible phosphorylation can modulate the activity of splicing factors during RNA splicing (15–20) is consistent with this possibility. In addition, SR proteins extracted from HeLa cells treated with DNA topoisomerase I inhibitors have a different pattern of phosphorylation as compared with those extracted from untreated cells (9). However, other protein kinase(s) not affected by the Topo I inhibitors are also active in vivo but have a different specificity from that of topo I/kinase (Labourier et al., accompanying paper). Among kinases phosphorylating SR proteins (21,22), SRPK1 and Clk1 were shown to induce nuclear redistribution of SR proteins (22,23).

Human DNA topoisomerase I is a nuclear phosphoprotein composed of 765 amino acids with a predicted molecular mass of 91 kDa (24). It shares 40–50% identical amino acid sequences with other eukaryotic DNA topoisomerases I and as a result can be divided into four domains based on regions of extensive homology (25,26). The C-terminal domain, which contains the active tyrosine at position 723, is the most conserved domain that spans from residues 697 to 765 (26). This domain is preceded by a linker region of positively-charged residues that are not conserved (26). Residues from positions 198 to 651 form the conserved core domain which is resistant to proteolysis with

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subtilisyn (26). The N-terminal domain is highly charged and contains four putative nuclear localization signals (residues 59–65, 150–156, 174–180 and 192–198) which are conserved in this apparently divergent region of DNA topoisomerase I (27).

We were interested in identifying amino acid regions which are essential for topo I/kinase activity. Here we show that the C-terminal conserved domain contributes to the ATP binding site and is therefore essential for kinase activity. Since substitution of the tyrosine residue at the topoisomerase active site by phenylalanine does not change the kinase activity of topo I kinase, we conclude that the mechanism involved in the DNA-catalyzed reaction is different from the mechanism by which topo I kinase phosphorylates SR proteins.

MATERIALS AND METHODS

Expression and purification of recombinant proteins

The wild type and mutant Y723F recombinant topoisomerase I proteins were produced following infection of monolayer cultures of SF21 cells with the recombinant baculovirus AcMNPV/hTopI (a generous gift from A. M. Zhelkovsky, Tufts University, Boston) or BlueBac (Y/F) virus (a generous gift from J.J. Champoux), respectively, at a multiplicity of infection of 10.

The virus encoding Topo-80 was constructed by inserting the Xho–EcoRI fragment of the human Topo I cDNA into transfer vector (pBlueBacHis2 C) which was co-transfected with linearized Autographa californica multiple polyhedrosis virus (AcMPV) into SF9 cells. This construct encodes additional amino acids (six histidines and the enterokinase cleavage site) before the Pro 139 into SF9 cells. This construct encodes additional amino acids (six histidines and the enterokinase cleavage site) before the Pro 139

To mutate the C-terminal part of human DNA topoisomerase I into Topo-8C, the polymerase chain reaction (PCR) was used to amplify a segment of the human Topo I cDNA from positions 1420 to 1804, taking the first nucleotide of the initiating methionine as position 1. The amplified fragment contained the unique HindIII site of human Topo I cDNA at the 5′ end and a SalI site included in the 3′ end oligonucleotide sequence used for the PCR reaction. The digestion of this fragment with HindIII–SalI was used to replace a HindIII–SalI fragment from the transfer vector (pBlueBacHis2 2A) containing the entire human Topo I cDNA previously cloned between BamHI and EcoRI sites. The entire Topo I sequence inserted in the Topo Transfer vector was sequenced to confirm the proper construction of the initiation end and a SalI transfer vector

To test the DNA relaxation activity of recombinant Topo I mutant proteins, 20 ng of each protein was incubated in buffer B with 500 ng supercoiled DNA plasmid in a final volume of 10 µl at 30°C for 30 min. The DNA was then extracted with phenol–chloroform, precipitated with ethanol and electrophoresed in a 0.8% agarose gel in TBE buffer. The DNA topoisomerases were revealed by ethidium bromide staining.

8N3–ATP cross-linking and chymotrypsin digestion of photolabeled recombinant proteins

The reaction mix for photochemical cross-linking contained, in a total volume of 50 µl, 5 µg of recombinant protein, 50 mM Tris (pH 7.5), 20 mM AMP–α-S and 50 mM 8N3–ATP (unless otherwise indicated). [α–32P]8N3–ATP was used at a specific activity of 0.5–1 Ci/mmol. The photochemical cross-linking of 8N3–ATP to recombinant proteins was performed in 1.5 ml microfuge tubes for 20 min at room temperature in the dark and then irradiated with a transilluminator UVC-254 placed 3 cm under the reaction tubes. Although long exposure times (exceeding 5 min) may result in some proteolysis, it was necessary to expose for 5 min to destroy unreacted 8N3–ATP, which was very important when digesting the photolabeled protein with chymotrypsin. The 8N3–ATP cross-linked topo I/kinase was utilized for kinase assays (1 µl aliquots diluted 10 times in buffer B), for relaxation assays (1 µl aliquots diluted 50 times in buffer B), or for SDS–PAGE (8 µl aliquots), or subjected to limited proteolysis with chymotrypsin as described below.

Digestion of photolabeled proteins was carried out at 30°C by adding 1 µl of chymotrypsin (4 µg/ml) to 40 µl of the cross-linking reaction mixtures immediately after the 5 min exposure to UV light. Aliquots corresponding to 8 µl were withdrawn at intervals and mixed with 5 µl of 4X Laemmli loading buffer containing 1 mM PMSE. The polypeptide fragments were then analyzed by SDS–PAGE on 10–16% gradient gels (34). Protein and proteolytic fragment concentrations were estimated by Coomassie blue staining of SDS gel using Topo I, previously quantitated by protein concentration determinations.
composition analysis, as a standard. Distribution of radioactivity, from radiolabeled samples, was detected by a Molecular Dynamics imaging analyzer and autoradiography of dried gel. The relative amount of [α-32P]8N3-ATP cross-linked to each polypeptide was quantitated by densitometry scanning of the gel using ImageQuant software version 3.22 and counting gel slices using cerenkov counting.

Separation of chymotrypsin peptides and microsequencing

For amino acid microsequencing, the 24K and 80K polypeptides produced at early time of digestion of full length Topo I were separated by reverse phase chromatography using an Applied Biosystem model 130A HPLC and a Spherisorb 300A (100 × 4.6 mm) column. The polypeptides were resolved with a linear gradient of solvent B (95% acetonitrile) in solvent A (0.1% trifluoroacetic acid in H2O) at a flow rate of 45 min (0–95% B). Elution was monitored at 220 nm, and fractions corresponding to the 80K fragment peak were pooled and applied to a C4 Aquapore (100 × 2 mm) column. The 80K fragment was then eluted with a linear acetonitrile gradient 40 min (15–55%) and its N-terminal amino acid sequence was determined by Edman degradation using Applied Biosystems technology protein sequencer (model 470A).

All the other fragments resulting from chymotrypsin digestions were separated by electrophoresis on a 10–16% SDS polyacrylamide gel and electrotransferred to a ProBlott membrane (Applied Biosystems) in 10 mM 3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS), pH 11.0, containing 10% methanol for 1 h. Membranes were stained with Coomassie brilliant blue and the discrete bands (66, 14K for Topo I; 66, 14K for Topo-80; 60, 31K) were excised and subjected to microsequence analysis using an Applied Biosystems Inc. Model 473 protein sequencer. Phenylthiohydantoin (PTH) derivatives of the amino acids were separated and identified by on-line reverse phase HPLC using an RP-18 column (Brownie Labs, Applied Biosystems).

Titration of ATP binding to reconbinant proteins using tryptophan fluorescence

Fluorescence experiments were performed on a Fluorolog-II (Jobin Yvon) spectrophotometer at 25°C. Protein samples (25 nM) were incubated in a buffer containing 50 mM Tris–HCl, 2 mM MgCl2 and 50 mM KCl. The binding of ATP or N3-ATP was monitored by quenching of the intrinsic tryptophan fluorescence of recombinant proteins at 332 nm, upon excitation at 295 nm. The decrease in protein fluorescence was fitted to the appropriate form of the quadratic equation as previously described (30,31). The best fit was obtained, with the Kd corresponding to each protein determined. A maximal fluorescence quenching of 26% for recombinant proteins that bind ATP was observed.

RESULTS

We have shown previously that recombinant human DNA topoisomerase I overexpressed in insect cells using the baculovirus system has the same characteristics as the native HeLa enzyme (9). It binds ATP efficiently and specifically phosphorylates 5′R protein splicing factors at short RS domain regions (Labourier et al., accompanying paper). In order to ascertain whether this enzyme is capable of using other ribonucleotides as phosphate donors, we examined the effect of different nucleotides as competitors in the kinase reaction. The purified recombinant topo I/kinase was used in kinase assays containing increasing concentrations of ATP, GTP, CTP or UTP and kinase activity was detected by its ability to phosphorylate bacterially expressed recombinant SF2/ASF with [γ-32P]ATP. Analysis of the phosphorylated products by SDS–PAGE and densitometric scanning of the autoradiography (Fig. 1) revealed that under conditions where the concentration of [γ-32P]ATP was constant, all four triphosphate nucleotides enhanced 32P incorporation (compare lane 1 and lanes 10, 14, 18 and 22). This indicates that the nucleotides can alter the Kd to an optimal value or may also suggest allosteric interactions or additional binding sites for these nucleotides. Under the conditions of this assay, 100 µM cold ATP completely prevent radiolabeling of SF2/ASF (lane 12), and there was a 50% reduction of maximal activity at 10 µM ATP (compare lanes 10 and 11). In contrast, GTP, UTP and CTP when used at concentrations even as high as 100 µM, did not affect the level of phosphorylation of SF2/ASF (lanes 16, 20 and 24, respectively). These results suggest that at least in vitro, ATP is the preferred phosphate donor used by topo I kinase to phosphorylate SF2/ASF. Consistent with this, in the absence of ATP the [γ-32P]GTP did not act as a phosphate donor (data not shown). However, for reasons that we did not understand, each of the non-radioactive NTPs appeared to behave differently. For example, CTP stimulated kinase activity at all concentrations tested and did not inhibit even at 1 µM (lanes 22–25). GTP and UTP, on the other hand, stimulate differentially at low concentrations (compare lanes 14, 15 with 18, 19). As expected, ADP was also inhibitory but at a 10-fold higher concentration than ATP (lane 9). Because it possesses one less phosphate group, ADP probably fits the ATP binding site more loosely and therefore becomes readily exchangeable with ATP once the phosphate transfer to SF2/ASF has been accomplished. AMP is less effective than ADP as a competitor for the binding of ATP, i.e. a much higher concentration was required to inhibit the phosphorylation of SF2/ASF (compare lanes 5 and 9). By analogy with known NTP binding proteins, DNA topoisomerase I is expected to have an ATP binding motif with a phosphate-loop function.

The photoaffinity probe [α-32P]8-azido-ATP (8N3-ATP) was used to investigate the binding of ATP to highly purified recombinant DNA topoisomerase I. Specific labeling of the binding site requires that the 8N3-ATP binds with a sufficient affinity and selectivity to the same site(s) as the natural ligands do. Therefore, the noncovalent binding of the ATP analogue was first tested. Human DNA topoisomerase I contains a total of 13 tryptophan residues which can be used as selective probes for monitoring the kinetics of enzyme–substrate interactions. Addition
The estimated dissociation constant for ATP and 8N3-ATP binding to recombinant human DNA topoisomerase I (A). Inactivation of kinase activity (B) but not DNA topoisomerase I activity (C) following covalent photocrosslinking of 8N3-ATP to wild type recombinant Topo I. (A) Increasing the concentrations of either ATP (filled symbols) or 8N3-ATP (open symbols) induced marked decrease of 25 and 27% respectively in the relative intrinsic fluorescence of Topo I upon excitation at 295 nm. The relative $K_d$ values derived from the curves fitting are 55 nM for ATP and 65 nM for 8N3-ATP. (B) Kinase assays were carried out with 100 ng of unirradiated (lane 1) or irradiated Topo I in the absence (lane 2) or the presence of either 10 µM (lane 3) or 100 µM (lane 4) of 8N3-ATP. (C, lanes 1–4) 20 ng of the same Topo I samples as those corresponding to lanes 1–4 in (B) were incubated with 500 ng of supercoiled DNA and assayed for DNA relaxation activity (Materials and Methods). For kinase assays the 8N3-ATP cross-linked topo I/kinase was diluted 10 times in buffer B, for relaxation assays it was diluted 50 times in buffer B. (Pi) Supercoiled DNA incubated under the relaxation assay with buffer only. CL stands for UV cross-linking.

Figure 2. Dissociation constants determination for ATP and 8N3-ATP binding to recombinant human DNA topoisomerase I. The concentration of either ATP (filled symbols) or 8N3-ATP (open symbols) induced marked decrease of 25 and 27% respectively in the relative intrinsic fluorescence of Topo I upon excitation at 295 nm. The relative $K_d$ values derived from the curves fitting are 55 nM for ATP and 65 nM for 8N3-ATP. (B) Kinase assays were carried out with 100 ng of unirradiated (lane 1) or irradiated Topo I in the absence (lane 2) or the presence of either 10 µM (lane 3) or 100 µM (lane 4) of 8N3-ATP. (C, lanes 1–4) 20 ng of the same Topo I samples as those corresponding to lanes 1–4 in (B) were incubated with 500 ng of supercoiled DNA and assayed for DNA relaxation activity (Materials and Methods). For kinase assays the 8N3-ATP cross-linked topo I/kinase was diluted 10 times in buffer B, for relaxation assays it was diluted 50 times in buffer B. (Pi) Supercoiled DNA incubated under the relaxation assay with buffer only. CL stands for UV cross-linking.

Figure 3 A and B. For convenience, we will refer to the various proteolytic fragments which were generated following topo I/kinase UV irradiation since they were absent from the unirradiated sample (data not shown). These results indicate that the dose of UV light used to cross-link 8N3-ATP to topo I/kinase does not produce significant amounts of either intra- or interchain cross-links which would alter protein mobility. Further proof of this is that the fragments obtained by chymotrypsin digestion of both unirradiated topo I/kinase and topo I/kinase exposed to UV light, analyzed on SDS–polyacrylamide gels, were identical (Fig. 4, compare A and B left panel).

Previous studies have established that limited proteolysis of DNA topoisomerase I under non-denaturating conditions yields discrete polypeptides which appear to represent independently folded structural domains (26). However, the spatial relationships of the domains have not yet been elucidated. To localize the 8N3-ATP binding, photolabeled topo I/kinase was subjected to proteolytic digestion and analyzed for stoichiometric label incorporation using SDS–PAGE and autoradiography. The time course for chymotrypsin proteolysis of topo I/kinase is shown in Figure 4A and B. For convenience, we will refer to the various proteolytic fragments according to their electrophoretic mobilities relative to protein size markers. Two major fragments of ~24 and 80K are produced at 5 min (lane 2), the earliest time examined. These two fragments were HPLC purified and subjected to N-terminal sequence analysis. The 24K fragment proved to be the N-terminus of DNA topoisomerase I, while the 80K started with Lys 137. Given the unusual gel migration of the first 175 amino acids of DNA topoisomerase I (26), the NH$_2$-terminal sequence information and the relative sizes of the proteolytically released fragments, we propose that chymotrypsin is cleaving predominantly after Leu 136. Given that chymotrypsin has specificity for cleaving on the C-terminal side of aromatic residues and only has weak activity with hydrophobic, aliphatic residues, the cleavage at Leu 136 is likely to reflect accessibility of this region to proteases. Consistent with this interpretation, the finding that NH$_2$-terminal domain (Met 1–Lys 197) is highly accessible.
sensitive to proteolysis (26), in particular, it has been shown that the Arg 138 and Lys 175 were preferentially cleaved by an unidentified insect cell protease.

[α-32P]8N3-ATP was cross-linked to each of the proteolytic fragments, but the efficiency of cross-linking was different (12–20% incorporation) compared with the 80K domain, which showed 70–80% incorporation (lanes 2–6). Continued digestion leads to the release of smaller labeled fragments with molecular weight corresponding to 66, 22, 14 and 12K (derived from the 14K fragment) (Fig. 4 B, lanes 5 and 6 right panel). Those fragments are absent from the proteolysis pattern observed for the core domain of the protein known to be resistant to proteolysis. These findings indicate that there are two polypeptides that contain the bulk of the incorporated label which localize to both the 24K N-terminal (136 amino acids) and 14K C-terminal regions of topo I/kinase; this is consistent with photolabeling at two specific sites in the protein but contrasts with the early finding that topo I/kinase has only a single ATP binding site.

The arrows indicate the cleavage sites at lysine 137 and glutamic acid 641. (B) Coomassie blue stained (left panel) and autoradiography (right panel) of the gel corresponding to the irradiated sample. M, molecular weight. The relative size of proteolytic fragments is indicated. (*) corresponds to Topo I proteolytic fragments resulting from exposure of the protein to UV light. ChTy stands for chymotrypsin.

The Topo I/kinase regions might be cross-linked to SN3-ATP either at the ATP binding domain or at another site in the proximity of this domain. A third possibility could be that the ATP binding domain is formed by amino acids in the N-terminal and C-terminal domains in the tertiary conformation of topo I/kinase. We attempted to distinguish between these possibilities by making deletions in the cDNA encoding human DNA topoisomerase I and expressing the mutated proteins with the baculovirus system. We first constructed a recombinant baculovirus encoding a 82K protein (Topo-80) which lacks the first 138 amino acids of the N-terminal domain of topo I/kinase corresponding to N-terminal 24K domain, but contains 38 amino acids from the transfer vector. These additional N-terminal amino acids include a six histidine tag to allow efficient purification of the recombinant protein by metal affinity chromatography. The recombinant protein purified to apparent homogeneity (Fig. 5A, lane 5) displays kinase and topoisomerase I activities equivalent to that of full-length DNA topoisomerase I protein (Fig. 5C and D, compare lanes 1, 2 and 3, 4, respectively), indicating that the 24K domain is dispensable for these activities.

Figure 3. Time course of cross-linking of [α-32P]8N3-ATP to Topo I. (A) Kinase reactions were performed as described (Materials and Methods) without (lane Ctrl) or with 1 µM (lane 2) 10 µM (lane 3) 100 µM (lane 4) and 1 mM (lane 5) of AMP-α-S, which act as protective compounds in the photolabeling reaction. The photolabeled proteins were analyzed on a 12% SDS–polyacrylamide gel and revealed either by autoradiography (B) or quantitated by phosphor imaging (C). The position of the Topo I on the gel was localized by staining the gel with Coomassie blue. (*) corresponds to Topo I proteolytic fragments resulting from exposure of the protein to UV light.

Figure 4. Limited chymotrypsin digestion of unirradiated (A) and irradiated (B) Topo I. Digestion was performed at 30°C with 0.1% w/w chymotrypsin. Samples (2 µg of protein) were removed at the indicated times and analyzed on a 10–16% SDS–polyacrylamide gel. (A) Coomassie blue stained gel of unirradiated sample (left panel) and schematic representation of Topo I proteolytic fragments obtained by limited chymotrypsin digestion (right panel). The arrows indicates the cleavage sites at lysine 137 and glutamic acid 641. (B) Coomassie blue stained (left panel) and autoradiography (right panel) of the gel corresponding to the irradiated sample. M, molecular weight. The relative size of proteolytic fragments is indicated. (*) corresponds to Topo I proteolytic fragments resulting from exposure of the protein to UV light. ChTy stands for chymotrypsin.
Figure 5. Comparison of chymotrypsin proteolytic patterns of photolabeled recombinant Topo I mutant proteins (A) and $K_d$ determination of ATP binding to each protein (B); protein kinase (C) and topoisomerase I (D) activities. (A) Coomassie blue staining of proteolytic digestion of full length, Topo-80 and Topo-δC (left panel), autoradiography of photolabeled proteins digested with chymotrypsin (right panel). The incubation times are indicated on the top and the relative size of proteolytic fragments are indicated on the left and right of each panel. (*) corresponds to proteolytic fragments resulting from exposure of recombinant proteins to UV light. (B) Curves of intrinsic fluorescence quenching following binding of ATP to full length Topo I (filled circles), Topo-80 (open circles) and Topo-δC (triangle). The $K_d$ values derived from this analysis are indicated on the right of the panel. (C) Protein kinase assays were as described in Materials and Methods with 100 ng (lane 1), or 10 ng (lane 2) of wild type Topo I; 100 ng (lane 3), or 10 ng (lane 4) of Topo-80; 100 ng (lane 5), or 10 ng (lane 6) of Topo-δC. (D) DNA topoisomerase I assays were as described in Materials and Methods with 2 ng (lane 1), or 20 ng (lane 2) of wild type Topo I; 2 ng (lane 3), or 20 ng (lane 4) of Topo-80; 2 ng (lane 5), or 20 ng (lane 6) of Topo-δC. (Pl) Supercoiled DNA incubated under the relaxation assay in the presence of buffer.

For kinase activity. Moreover, this deletion did not greatly interfere with the ability of Topo-80 to bind ATP, since the $K_d$ for ATP was <2-fold of that of the wild type (Fig. 5B). The 8N3-ATP was incorporated in Topo-80 with the same efficiency as the full length protein (Fig. 5A, right panel, compare lanes 1 and 5), demonstrating that efficient crosslinking does not require an intact N-terminal domain. Chymotryptic peptide mapping of Topo-80 photolabeled with $[α-32P]$8N3-ATP revealed that labeling was confined to the same fragments as those derived from the 80K domain of full length topo I/kinase (Fig. 5A, right panel, compare lanes 2–4 and 6–8). Allowing for the absence of the 24K domain, the digestion patterns of this protein and full length topo I/kinase were very similar (Fig. 5A, right panel, compare lanes 2–4 and 6–8), generating two fragments of 66 and 14K with similar kinetics. The slightly slower migration of 66K fragment derived from Topo-80 (right panel, compare lanes 3, 4 and 7, 8) probably reflects the additional 38 amino acids that were inserted during the construction of the expression vector. This was confirmed by amino acid sequencing of the N-terminus (data not shown). It is clear from these results that the N-terminal 24 kDa fragment of topo I/kinase is not necessary for ATP binding and is therefore not important for kinase activity.

We also examined the effect of deleting the C-terminal portion of topo I/kinase on ATP binding and cross-linking to 8N3-ATP. The recombinant fusion protein Topo-δC lacks the C-terminal 162 amino acids of human DNA topoisomerase I, but contains the same N-terminal 38 amino acid tag as Topo-80. Although it has the entire N-terminal amino acid sequence of the wild type topo I/kinase, Topo-δC did not incorporate any label following UV irradiation in the presence of $[α^{-32P}]$8N3-ATP. Background labeling of the mutant protein was negligible compared with wild type or Topo-80 proteins (Fig. 5A, compare lanes 1–8 and lanes 9–12 in the right panel). As shown in Figure 5B, millimolar levels of ATP did not induce quantitative fluorescent quenching of Topo-δC, demonstrating that deletion of the C-terminal domain leads to a mutated protein with strongly impaired capacity to bind ATP. Thus the requirement for ATP binding and 8N3-ATP cross-linking closely paralleled one another. To rule out the trivial explanation that the lack of ATP binding arises from an altered conformation for Topo-δC, we tested its sensitivity to chymotrypsin digestion. Cleavage of Topo-δC with chymotrypsin gave rise to two fragments corresponding to 60 and 31K (Fig. 5A, left panel, lanes 2–4 and 6–8), confirming the 24K and 16K domains, the digestion patterns of this protein and full length topo I/kinase were very similar (Fig. 5A, left panel, compare lanes 2–4 and 6–8), generating two fragments of 66 and 14K with similar kinetics. The slightly slower migration of 66K fragment derived from Topo-80 (right panel, compare lanes 3, 4 and 7, 8) probably reflects the additional 38 amino acids that were inserted during the construction of the expression vector. This was confirmed by amino acid sequencing of the N-terminus (data not shown). It is clear from these results that the N-terminal 24 kDa fragment of topo I/kinase is not necessary for ATP binding and is therefore not important for kinase activity.
domain of DNA topoisomerase I plays an essential role in reactions catalyzed by this enzyme.

The C-terminal domain of DNA topoisomerase I has also been shown to catalyze the cleavage–ligation reactions characteristic of Topo I (32). Since the experiments described above established that the ATP binding site was contained in this domain, we decided to determine whether the mechanism involved in transiently breaking and resealing DNA strand is also involved in transferring the phosphate from ATP to SF2/ASF. The tyrosine at position 723, which serves to form a covalent bond with the 3' phosphate of the cleaved strand during the relaxation reaction, was mutated and its effect on the ability of topo I/kinase to phosphorylate SF2/ASF was examined. Figure 6A shows that substitution of tyrosine 723 by phenylalanine did not change the proteolytic pattern of DNA topoisomerase I when digested with chymotrypsin, confirming that this mutation did not affect the conformation of the protein. Furthermore, the mutated protein binds ATP with a $K_d$ similar to wild type (Fig. 6B) and efficiently incorporates 8N3-ATP at the same region as wild type protein (Fig. 6A, lower panel, compare lanes 1–4 and lanes 5–8). There was no quantitative difference in the phosphorylation of SF2/ASF between wild type Topo I and the mutant protein (Fig. 6C, compare lanes 1, 2, and 3, 4), confirming that the tyrosine at the active site of DNA topoisomerase I is dispensable for the kinase activity. In agreement with previous observations, however, only trace amounts of insect cell-derived topoisomerase I activity could be detected in the purified Y723F Topo I even when assayed at very high protein concentration (Fig. 6D, compare lanes 1, 2 and 3, 4). Therefore we conclude that tyrosine 723 is not involved in the transfer of the $\gamma$-phosphate from ATP to SF2/ASF, but is essential for DNA relaxation activity.

DISCUSSION

Limited proteolysis proved to be a useful tool for defining structural domains whose boundaries closely parallel those predicted from sequence comparisons of cellular Topo I enzymes (26). The present study utilized this approach to define regions of human DNA topoisomerase I contributing to the kinase activity. Photoaffinity labeling with 8N3-ATP localized two regions incorporating this ATP analog within the Topo I molecule. Two observations confirm the significance of this labeling. The reaction is efficient, since $\sim$50% of the input enzyme was modified, and it is specific because ATP competed for the photoinsertion of 8N3-ATP into the enzyme. Assuming that 8N3-ATP binds to topo I/kinase with similar affinity to ATP, and that the covalent photoincorporation of 8N3-ATP into topo I/kinase leads to its inactivation, it is reasonable to conclude that 8N3-ATP binds to the same site as ATP. Surprisingly, the sites labeled with 8N3-ATP are within two distant regions of the primary structure of topo I/kinase. Nevertheless, topo I/kinase is required for efficient photo labeling with 8N3-ATP, since heat denaturation of the protein or deletion of the C-terminal region both abolished cross-linking of 8N3-ATP. The mutant protein lacking the
N-terminal 24K domain behaved as the full length topo I kinase. This deletion did not affect the $K_d$ of the enzyme for ATP, its ability to phosphorylate SF2/ASF, or the cross-linking efficiency to 8N3-ATP. Since we have established that the C-terminal domain contributed to the ATP binding site, the crosslinking result suggest that the N-terminal and C-terminal domains are closely situated in the three dimensional structure of full length DNA topoisomerase I. This conclusion is supported by the finding that SF2/ASF, the kinase substrate, exclusively interacts with amino acids in the N-terminal part of DNA topoisomerase I (Labourier et al., accompanying paper). This interaction is essential for the phosphorylation of SF2/ASF because a deletion mutation that impedes the binding of SF2/ASF (Topo-70) also abolishes the kinase activity.

Further studies will be necessary to define the precise sequences required for the topo I kinase. Nevertheless the results presented here imply that the C-terminal sequences are not obligatorily organized in a structure identical to that required for DNA relaxation activity. Site-directed mutagenesis of highly conserved amino acids at the C-terminal domain of DNA topoisomerase I resulted in relaxation-deficient mutants (32), demonstrating that the latter are involved in cleavage-ligation reactions catalyzed by DNA topoisomerase I. Substitution of tyrosine 723 in the active site (26, 32, 33), arginine 488 or lysine 532, lead to inactive DNA topoisomerase I, and mutation of lysine 720 shows a 50-fold reduction in specific relaxation activity (32). While ATP binds near tyrosine 723 at the active site, mutation of this tyrosine did not affect either the binding of ATP or the kinase activity of TopoI/kinase. This indicates that tyrosine 723 is not involved in the transfer of the $\gamma$-phosphate from ATP to SF2/ASF.

In addition, inclusion of ATP has no inhibitory effect in relaxation reactions (34), however addition of DNA reduced the kinase activity by at least 3-fold (9). This could be brought about by direct competition between the two substrates for binding to the same site and/or by changes in the conformation of the protein concerned to bind ATP. The chymotryptic digestion profile of topo I kinase was the same in the presence or absence of ATP (data not shown), while the binding of DNA to Topo I resulted in differential protease sensitivity (26). Indeed, it was previously shown that noncovalent binding of Topo I to plasmid DNA decreases the proteolysis of a linker region (amino acids 652–696) situated between the core domain (amino acids 175–659) and the C-terminal domain (26, 33).

In contrast, covalent binding of a DNA oligonucleotide to the C-terminal domain did not change the sensitivity of DNA topoisomerase I to proteolysis. Since the conserved core domain alone exhibits preferential binding to superhelical as compared with relaxed DNA (33) and as the C-terminal domain contributes to the binding of ATP, then this may account for changes in the conformation of the C-terminal region during the DNA catalyzed reaction, which would interfere with the binding of ATP thereby impeding the kinase activity.

Understanding how the protein kinase and DNA relaxing activities mediated by the same polypeptide are mechanistically related and determining the way by which the splicing activity of SR proteins is modulated by phosphorylation, will provide us with new insights into functions of this enzyme. Clearly, X-ray crystallography is needed to complete the biochemical characterization of eukaryotic DNA topoisomerase I. Future work must, in addition, concentrate on attempts to integrate our knowledge regarding pathways by which SR proteins regulate splicing with studies of topo I kinase-mediated phosphorylation of SR proteins themselves. Failure to properly phosphorylate SR proteins may perhaps account for the lethality of the DNA topoisomerase I mutations in Drosophila and mouse, and it will be interesting to dissect the DNA relaxing and kinase activities of this molecule in both organisms.

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REFERENCES