An Inhibitor of DNA Topoisomerase I from *Xenopus laevis* Ovaries†

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ABSTRACT: A novel, heat-resistant and Pronase-sensitive, inhibitor of eukaryotic DNA topoisomerase I has been purified from *Xenopus laevis* ovaries. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the most purified fraction revealed three bands with apparent molecular masses of 25, 28.5, and 33.5 kDa. The 25- and 33.5-kDa peptides recovered from an SDS–PAGE gel inhibited *X. laevis* DNA topoisomerase I. The purified inhibitor was specific to DNA topoisomerase I and did not inhibit other DNA enzymes tested. The inhibitor blocked the catalytic activity of DNA topoisomerase I by interacting with the enzyme, rather than by competing for binding sites on substrate DNA. Binding of DNA topoisomerase I to substrate DNA was blocked by the inhibitor, as was the cleavage reaction catalyzed by DNA topoisomerase I. Inhibition of DNA topoisomerase I was relieved by divalent cations Ca^{2+}, Mg^{2+}, or Mn^{2+}.

Two major types of DNA topoisomerase activity that regulate the topological state of DNA molecules in eukaryotes have been characterized (Wang, 1985, 1991). DNA topoisomerase I, as well as DNA topoisomerase II, has been implicated in numerous essential cellular processes including DNA replication, transcription, and recombination (Wang, 1985, 1991; D'Arpa & Liu, 1989; Sternglanz, 1989; Kim & Wang, 1989; Hsieh, 1990; Kroeger & Rowe, 1992).

DNA topoisomerase I activity can be modulated by posttranslational modifications. Phosphorylation of DNA topoisomerase I by casein kinase II or by protein kinase C stimulates the catalytic activity (Durban *et al.*, 1983; Kaiserman *et al.*, 1988; Samuels *et al.*, 1989; Pommier *et al.*, 1990). In contrast, phosphorylation by a tyrosine protein kinase decreases the catalytic activity (Tse-Dinh *et al.*, 1984), as does poly(ADP-ribosylation) (Kasid *et al.*, 1989; Higgins *et al.*, 1990). It has been suggested that these covalent modifications may play an important physiological role since modified forms of DNA topoisomerase I have been detected *in vivo* (Higgins *et al.*, 1990; Samuels & Shimizu, 1992).

The activity of DNA topoisomerase I is also affected by histone and non-histone chromosomal proteins. High mobility group protein HMG17 stimulated DNA topoisomerase I activity by more than 60-fold (Javaherian & Liu, 1983). Rowe *et al.* (1981), Javaherian and Liu (1983), and Kordiyak *et al.* (personal communication) found that histone H1 also stimulated the activity of DNA topoisomerase I. In contrast, however, Bina-Stein and Singer (1977) and Richter and Kapitza (1991) reported that DNA topoisomerase I was inhibited by histone H1.

Certain organic molecules and the polyanion heparin also inhibit the activity of DNA topoisomerase I (Ishii *et al.*, 1982, 1987; Douc-Rasy *et al.*, 1983; Liu, 1989; Mortensen *et al.*, 1990; Champoux, 1990; Riou et al., 1991). Camptothecin, the most extensively studied inhibitor of eukaryotic DNA topoisomerase I, inhibits the closure step in the cleavage–resealing cycle by trapping DNA topoisomerase I covalent intermediates (D'Arpa & Liu, 1989; Liu, 1989, 1990). Camptothecin converts DNA topoisomerase I into a DNA damaging-agent (Giovanella *et al.*, 1989; D'Arpa & Liu, 1989; Liu, 1989, 1990).

In this study, we describe a novel protein from *Xenopus laevis* ovaries that efficiently and specifically inhibits the catalytic activity of purified DNA topoisomerase I from *X. laevis*, calf thymus, or wheat germ. Although no physiological role has been established for the inhibitor, the abundance of the inhibitor and its properties are consistent with this possibility. Moreover, the inhibitor potentially will interfere with the reconstitution of *X. laevis* cell-free systems that are increasingly being used to study molecular events in nucleic acid metabolism. For these reasons, therefore, it is important to describe its properties.

EXPERIMENTAL PROCEDURES

Materials

Frog. Adult *X. laevis* females were purchased from Xenopus I (Ann Arbor, MI).

Enzymes. X. laevis DNA topoisomerase I was purified from *X. laevis* ovaries as described previously (Kaiserman *et al.*, 1988). Calf thymus DNA topoisomerase I was from GIBCO BRL. Wheat germ DNA topoisomerase I and *Eco*RI methylase were from Promega. Calf thymus DNA topoisomerase II was from TopoGEN, Inc. Pronase was obtained from Boehringer Mannheim Biochemicals.

Chromatography Media. DEAE[1]-cellulose (DE52), CM-cellulose (CM52), and cellulose phosphate (P11) were from Whatman. Affi-Gel Blue gel was from Bio-Rad. Hydroxyapatite–agarose (HA-Ultrogel) was from IBF Biotechnics Inc.

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[1] Abbreviations: DEAE, diethylaminoethyl; CM, carboxymethyl; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; DS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Polylysine–agarose was from Sigma. Sephadex G100 was from Phamacia LKB.

DNA. Plasmids pUC19 (Yanisch-Perron *et al.*, 1985) and pXY65 (Hiraga *et al.*, 1982) were isolated from *Escherichia coli* HB101 by alkaline lysis and purified by CsCl gradient centrifugation (Sambrook *et al.*, 1989). [^{3}H]-labeled pXY65 was prepared as described by Hines and Benbow (1982). ΦX174 (+) strand DNA and λ DNA were purchased from GIBCO BRL. Kinetoplast DNA (KDNA) was from Topo-GEN,Inc.

Methods

DNA Topoisomerase I Assay. DNA topoisomerase I activity was determined by monitoring the relaxation of supercoiled (form I) pUC19 plasmid DNA (Kaiserman *et al.*, 1988). Unless otherwise stated, the reaction mixture (20 μL) contained 50 mM Tris-HCl, pH 7.5, 60 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 30 μg/mL nuclease-free BSA, and the indicated amounts of pUC19 DNA and DNA topoisomerase I. Reactions were carried out for 15 min at 23 °C (or 30 °C to determine units for *X. laevis* DNA topoisomerase I) and were terminated by the addition of 2 μL of 10% SDS. Reaction products were analyzed by electrophoresis on 1% agarose gels. Supercoiled and relaxed pUC19 DNA was quantitated by densitometry using a GS300 transmittance/reflectance scanning densitometer (Hoefer Scientific Instruments). One unit of *X. laevis* DNA topoisomerase I was defined as the amount required to relax 250 ng of supercoiled (form I) pUC19 DNA in 30 min. One unit of *Xenopus* DNA topoisomerase I is equivalent to 23 ng of purified protein on the basis of the specific activity determined by Kaiserman *et al.* (1988). Units of calf thymus and wheat germ DNA topoisomerase I were as defined by the manufacturers.

Assays for Inhibition of DNA Topoisomerase I. Inhibitors were mixed with pUC19 DNA and reaction buffer before addition of DNA topoisomerase I. One unit of the inhibitor was defined as the amount that inhibits 50% of the relaxation of supercoiled (form I) pUC19 DNA by 1 unit of *X. laevis* DNA topoisomerase I under the above assay conditions.

Protein concentration was measured by the method of Bradford (1976) using bovine plasma γ-globulin (Bio-Rad) as standard.

Purification of DNA Topoisomerase I Inhibitor from X. laevis Ovaries. Ovaries were removed from decapitated frogs and washed with modified Barth solution (Ford & Gurdon, 1977). All subsequent manipulations were carried out at 4 °C. Ovaries were washed with buffer A [30 mM Tris-HCl, pH 8.5, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 25% (v/v) glycerol, 0.5 mM PMSF, 1 mM benzamidine hydrochloride, and 0.5 mg/L leupeptin]. Homogenization and low-speed and high-speed centrifugation (fractions I–III) were performed as described by Kaiserman and Benbow (1987). The high-speed supernatant (fraction III) was loaded onto a DEAE-cellulose column (11 × 12.1 cm) equilibrated with buffer B [25 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 25% (v/v) glycerol, 0.5 mM PMSF, 1 mM benzamidine hydrochloride, and 0.5 mg/L leupeptin]. The column was washed with 4 volumes of buffer B at a flow rate of 154 mL/h and eluted with 3 volumes of buffer B containing 250 mM KCl (fraction IV) at a flow rate of 180 mL/h. Fraction IV was precipitated at 55% saturation (25 °C) ammonium sulfate and collected by centrifugation at 11 000*g* for 90 min at 4 °C. The pellet was dissolved with buffer C [25 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 25% (v/v) glycerol, 0.5 mM PMSF, 0.2 mM benzamidine hydrochloride, 0.5 mg/L leupeptin] and dialyzed in the same buffer (fraction V). Fraction V was applied to a cellulose phosphate column (2.5 × 47 cm) equilibrated with buffer C and washed with 3 volumes of buffer C at a flow rate of 50 mL/h. The flow-through and washes, which inhibited *X. laevis* DNA topoisomerase I activity, were concentrated using an Amicon ultrafiltration stirred cell fitted with a PM-10 membrane (fraction VI). Fraction VI was loaded onto an Affi-Gel Blue gel column (2.2 × 25 cm) equilibrated with buffer C and washed with 3 volumes of buffer C at a flow rate of 60 mL/h. The inhibitor was in the flow-through and washes, which were combined (fraction VII). Fraction VII was adjusted to pH 8.0 with 2.5 M Tris base and applied to a DEAE-cellulose column (2.5 × 20.4 cm) equilibrated with buffer B. The column was washed with 3 volumes of buffer B and eluted with a gradient of 60–600 mM KCl in buffer B at a flow rate of 60 mL/h. The inhibitor eluted at 180 mM KCl. Pooled DEAE-cellulose fractions (fraction VIII) were dialyzed against buffer D [10 mM K_xPO_4, pH 7.0, 10 mM KCl, 1 mM DTT, 25% (v/v) glycerol, 0.5 mM PMSF, and 0.2 mM benzamidine hydrochloride], loaded onto a CM-cellulose column (2.2 × 10.5 cm) equilibrated with buffer D, and washed with buffer D at a flow rate of 30 mL/h. The flow-through and washes, which contained the inhibitor, were combined (fraction IX), applied to an HA-Ultrogel column (2.5 × 22.4 cm) equilibrated with buffer D, and washed with buffer D. No inhibitor was detected in the flow-through fraction. Initially, a gradient of 20–700 mM K_xPO_4 in buffer D was applied to elute the column as suggested by the manufacturer. No inhibitor was detected, however, and a wash of 1 M K_xPO_4, pH 7.0, 0.5 M NaCl, 1 mM DTT, 0.5 mM PMSF, 0.2 mM benzamidine hydrochloride, and 20% (v/v) glycerol was applied to the column at a flow rate of 80 mL/h. The eluate contained the inhibitor (fraction X). Fraction X was concentrated, dialyzed against buffer E [10 mM Na_xPO_4, pH 7.5, 0.2 mM EDTA, 1 mM DTT, 25% (v/v) glycerol, 0.2 mM PMSF, and 0.2 mM benzamidine hydrochloride], and loaded onto a 5-mL polylysine–agarose column equilibrated with buffer E. After washing with 4 volumes of buffer E at a flow rate of 15 mL/h, the column was eluted with 5 volumes of 0.2, 0.5, 1 M, and 2 M NaCl in buffer E, respectively. The inhibitor eluted in both the 1 M (>80%) and 2 M (<20%) NaCl fractions. The fractions containing the inhibitor eluted with 1 M NaCl were pooled, concentrated (fraction XI), and dialyzed against buffer F [25 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 15% (v/v) glycerol, 0.2 mM PMSF, and 0.2 mM benzamidine hydrochloride]. Fraction XI was applied to a Sephadex G100 column (2.2 × 62.2 cm) equilibrated with buffer F and eluted at a flow rate of 9.1 mL/h. The inhibitor eluted anomalously (see Discussion). Pooled fractions were dialyzed against buffer G containing 10 mM K_xPO_4, pH 7.0, 10 mM KCl. 1 mM DTT, 25% (v/v) glycerol, 0.2 mM PMSF, and 0.2 mM benzamidine hydrochloride (fraction XII) and loaded onto a 5-mL HA-Ultrogel column equilibrated with the same buffer. The column was washed with 3 volumes each of buffer G, buffer G containing 0.2 M K_xPO_4, and buffer G containing 0.5 M K_xPO_4. The column was eluted with a gradient of 0.5–1.5 M K_xPO_4 in buffer G at a flow rate of 8 mL/h. The inhibitor eluted as two peaks, at 0.85 M K_xPO_4 and at 1.4 M K_xPO_4. We were unable to detect any difference between the two fractions except in the relative abundance of peptide subunits on SDS–PAGE. The fraction eluting at 1.4 M K_xPO_4 was not further characterized. The fraction eluting at 0.85 M K_xPO_4 was dialyzed against buffer H [25 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 15%

Table I: Purification of an Inhibitor of DNA Topoisomerase I from *X. laevis* Ovaries[a]

fraction	vol (mL)	total protein (mg)	total units[b] ($\times 10^{-3}$)	sp act. (units/mg)	yield
(I) crude extract	4240	nd	nd		
(II) low-speed supernatant	3830	54700	nd		
(III) high-speed supernatant	3740	46500	nd		
(IV) DEAE-cellulose	3350	8210	nd		
(V) $(NH_4)_2SO_4$ precipitation	455	3460	nd		
(VI) cellulose phosphate	826	2480	826	333	100
(VII) Affi-Gel Blue gel	1120	2130	747	351	90.4
(VIII) DEAE-cellulose	300	360	600	1667	72.6
(IX) CM-cellulose	396	333	566	1700	68.5
(X) hydroxyapatite	14	4.34	350	80600	42.4
(XI) polylysine–agarose[c]	7.53	1.13	251	222000	30.4
(XII) Sephadex G100	59	0.84	197	235000	23.8
(XIII) hydroxyapatite[d]	0.78	0.20	78	390000	9.4

[a] Based on 603 g of starting material. [b] nd: not determined. DNA topoisomerase I activity could not be measured reproducibly in the presence of fractions I–V because of nucleases and endogenous DNA topoisomerase I activity. [c] Inhibitor in the 2 M NaCl fraction was not included. [d] Inhibitor in the 1.4 M K_xPO_4 fraction was not included.

(v/v) glycerol, 0.2 mM PMSF, and 0.2 mM benzamidine hydrochloride], concentrated (fraction XIII), and stored at −20 °C.

SDS-PAGE was carried out according to Laemmli (1970). Silver staining was performed with Bio-Rad silver stain kit as described by the manufacturer.

Elution and Renaturation of DNA Topoisomerase I Inhibitor from SDS–Polyacrylamide Gels. Aliquots of fraction XIII were electrophoresed through a 15% SDS–polyacrylamide gel. The location of peptides was determined using prestained molecular weight markers (Bio-Rad and Sigma). Peptides of interest were cut out, and the remaining gel was stained to verify the location of the peptides. The peptides were eluted with a Bio-Rad electro-eluter (Model 422) in 25 mM Tris and 192 mM glycine. Nuclease-free BSA (50 μg) was added to the recovered samples, which were renatured as described by Hager and Burgess (1980) and concentrated with Amicon Centricon 10. Control gel slices from a lane with no sample were treated as described above.

Pronase Treatment of the Inhibitor. The purified inhibitor was dialyzed against 20 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM DTT, 0.2 mM EDTA, and 5% (v/v) glycerol and incubated with 0.5 mg/mL Pronase for 2 h at 37 °C. Pronase was then inactivated at 80 °C for 10 min.

DNA Competition Assay. Assays were carried out in a total volume of 20 μL. Four units of purified *X. laevis* DNA topoisomerase I was incubated with increasing amounts of pUC19 DNA in the presence of 6 units of fraction XIII.

DNA Binding Assay. DNA binding was performed in a total reaction volume of 30 μL in a binding buffer of 50 mM Tris-HCl, pH 7.5, 60 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, and 30 μg/mL nuclease-free BSA. Purified *X. laevis* DNA topoisomerase I (7.5 units) was incubated with 150 ng [^{3}H]-labeled pXY65 DNA in the presence of the indicated amounts of fraction XIII for 10 min at 23 °C. The reaction was stopped by adding 500 μL of ice-cold binding buffer. Reaction mixtures were filtered at a flow rate of 400 μL/min through a nitrocellulose membrane (Schleicher and Schuell BA-85) in a microfiltration apparatus (Bio-Rad), and the membrane was washed twice with 500 μL of cold binding buffer without BSA. The membrane was dried under an infrared lamp, and radioactivity retained on the membrane was determined in Scintiverse (Fisher) in an LKB 1218 Rackbeta scintillation counter. Percentage DNA binding was calculated using the binding of purified *X. laevis* DNA topoisomerase I to [^{3}H]-pXY65 in the absence of fraction XIII as 100% binding.

DNA Cleavage Assay. Assays were performed as described by Been and Champoux (1981). Wheat germ DNA topoisomerase I (5 units) was incubated with 125 ng of ΦX174 DNA in a reaction volume of 20 μL for 30 min at 37 °C.

Reversibility of Inhibition of DNA Topoisomerase I by Purified Inhibitor. Purified *X. laevis* DNA topoisomerase I (2.5 units) was incubated with 250 ng of pXY65 plus 50 units of fraction XIII in the standard DNA topoisomerase I assay buffer for 10 min (reaction A). pUC19 (300 ng) and 5 mM $MgCl_2$ were then added to the mixture, and the reaction was carried out for another 20 min (reaction B) before termination by SDS.

Assays for DNA Topoisomerase II and EcoRI Methylase. The activity of calf thymus DNA topoisomerase II was measured as described by the manufacturer. The activity of *Eco*RI methylase was assayed by its protection of λ DNA against cleavage by *Eco*RI, using procedures suggested by the manufacturer: 1 μg of λ DNA was incubated with 1 unit of *Eco*RI methylase with or without the presence of fraction XIII for 60 min, and then the DNA was digested with 20 units of *Eco*RI for 30 min. The reaction products were analyzed by electrophoresis on 0.8% agarose gels.

RESULTS

Identification and Purification of an Inhibitor of DNA Topoisomerase I. During characterization of circular plasmid DNA replication in cell-free extracts of *X. laevis* eggs (Benbow et al., 1990), we noted that extracts of *X. laevis* oocytes inhibited both replication and DNA topoisomerase I activity. In this study, an inhibitor of DNA topoisomerase I has been extensively purified from extracts of *X. laevis* ovaries (Table I) using inhibition of relaxation of supercoiled pUC19 DNA by *X. laevis* DNA topisomerase I as an assay. An aliquot of fraction VI (the first fraction we were able to quantitate accurately) inhibited the catalytic activity of *X. laevis* DNA topoisomerase I (Figure 1A). The most purified fraction (fraction XIII) was heat-resistant and Pronase-sensitive (Figure 1B). Three bands of apparent molecular mass 25, 28.5, and 33.5 kDa, respectively, were detected on SDS–PAGE gels of the most purified fraction (Figure 2). The 25-kDa peptide, which was predominant in the most purified fraction (Figure 2), and the 33.5-kDa peptide, which predominated (data not shown) in the 2 M polylysine–agarose fraction (see Experimental Procedures), were eluted from SDS–PAGE gel, renatured, and tested for their ability to inhibit DNA topoisomerase I. Both peptides inhibited *X. laevis* DNA topoisomerase I (Figure 1C).

Mechanism of Inhibition of DNA Topoisomerase I by the Purified Inhibitor. The reaction catalyzed by DNA topoisomerase I proceeds in several steps: binding of the enzyme

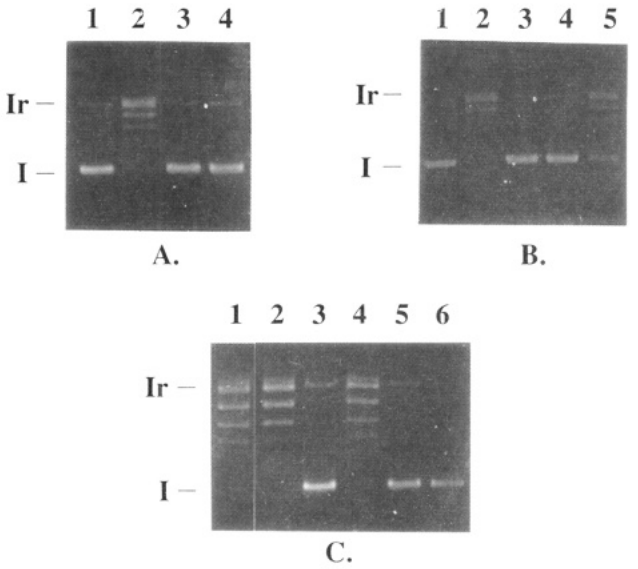

FIGURE 1: Inhibition of *X. laevis* DNA topoisomerase I by a protein from *X. laevis* ovaries. (A) Inhibition of *X. laevis* DNA topoisomerase I by fraction VI. DNA topoisomerase I assay was carried out in the absence (lane 2) or presence of 4 μL of fraction VI (lane 3) or 4 μL of heat-treated (80 °C, 10 min) fraction VI (lane 4). Lane 1 contained 500 ng of pUC 19 DNA. The positions of supercoiled (form I) and relaxed (form Ir) DNA are indicated. Purified *X. laevis* DNA topoisomerase I (2 units) was used in each reaction. (B) Heat resistance and Pronase sensitivity of the purified inhibitor. The purified inhibitor was heated (80 °C, 10 min) or treated with Pronase (see Experimental Procedures) before addition to a DNA topoisomerase I assay. Lane 1: 500 ng of pUC19 DNA; lane 2: 2 units of *X. laevis* DNA topoisomerase I; lane 3: 2 units of *X. laevis* DNA topoisomerase I plus 50 units of untreated fraction XIII; lane 4: 2 units of *X. laevis* DNA topoisomerase I plus 50 units of heat-treated (80 °C, 10 min) fraction XIII; lane 5: 2 units of *X. laevis* DNA topoisomerase I plus 50 units of Pronase-treated fraction XIII. (C) Inhibition of *X. laevis* DNA topoisomerase I by peptides from the most purified fraction (see Experimental Procedures). The recovered 25-kDa peptide (lane 3) and 33.5-kDa (lane 5) peptide were added to DNA topoisomerase I assay. Lane 1: Control (see Experimental Procedures); lanes 2 and 4: filtrates obtained during the concentration of recovered 25- and 33.5-kDa peptides, respectively, which are also used as controls; lane 6: pUC19 DNA. pUC19 DNA (250 ng) and 1.2 units of *X. laevis* DNA topoisomerase I were used in the reactions.

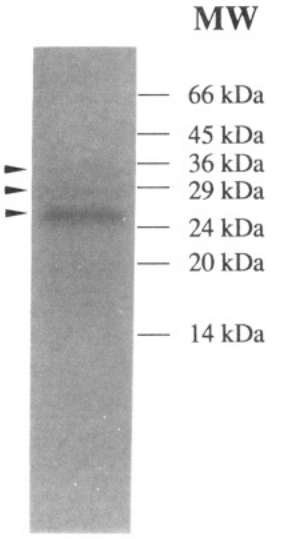

FIGURE 2: SDS–PAGE analysis of the most purified fraction. Fraction XIII (0.20 μg) was electrophoresed through a 15% polyacryamide gel. The gel was stained as described in Experimental Procedures. The arrows indicate the locations of stained peptides. The positions of molecular mass markers are also shown.

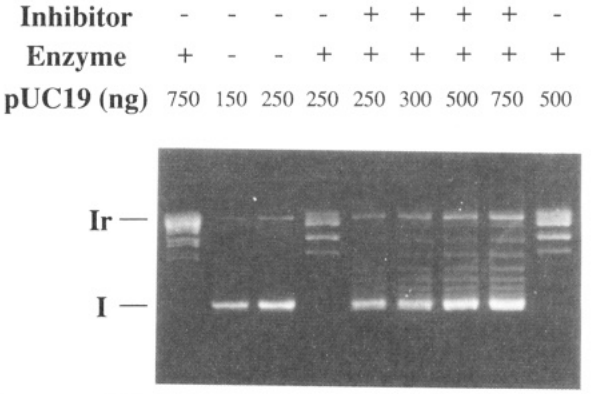

FIGURE 3: Effect of DNA concentration on inhibition of DNA topoisomerase I. Four units of *X. laevis* DNA topoisomerase I was incubated with the indicated concentration of pUC19 DNA in the absence or presence of 6 units of the most purified fraction.

to substrate DNA (noncleavable complex); strand cleavage during which the enzyme is covalently linked to the 3′ end of the broken strand (cleavable complex); and passage of the unbroken strand through the break and religation (Wang, 1985; D'Arpa & Liu, 1989; Champoux, 1990).

To test the possibility that the inhibitor could inhibit the catalytic activity of DNA topoisomerase I by competing with the enzyme for binding sites on substrate DNA, increasing amounts of pUC19 DNA were incubated with *X. laevis* DNA topoisomerase I under conditions where the enzyme activity was partially inhibited. As shown in Figure 3, increasing concentrations of DNA did not result in a significant increase in the amount of relaxed DNA in the presence of the inhibitor, even though enough DNA topoisomerase I to completely relax the supercoiled DNA at a DNA concentration of 750 ng was present. This suggests there is little competition between the inhibitor and DNA topoisomerase I for binding sites on DNA.

To test whether the inhibitor interfered with binding of the enzyme to DNA, binding of *X. laevis* DNA topoisomerase I to DNA was examined using [^{3}H]-labeled pXY65 DNA in a filter binding assay. Binding of the enzyme to DNA was inhibited by the most purified fraction (Figure 4), and inhibition of DNA binding exhibited similar concentration dependence as inhibition of relaxation of supercoiled DNA (Figure 4 insert).

The effect of the most purified fraction on the cleavage reaction catalyzed by DNA topoisomerase I was characterized using single-stranded circular ΦX174 DNA (Been & Champoux, 1981). The cleavage reaction was blocked by the inhibitor (Figure 5).

Effects of Divalent Cations on Inhibition of DNA Topoisomerase I. It has been reported that Mg^{2+}, Ca^{2+}, and Mn^{2+} stimulate, whereas Co^{2+}, Zn^{2+}, and Cu^{2+} inhibit, the activity of DNA topoisomerase I (Liu & Miller, 1981; Goto *et al.*, 1984; Svejstrup *et al.*, 1991). The effect of Mg^{2+}, Ca^{2+}, and Mn^{2+} on the inhibition of DNA topoisomerase I by the purified inhibitor was examined. Inhibition of DNA topoisomerase I by the most purified fraction was sensitive to Mg^{2+}, Ca^{2+}, and Mn^{2+} (Figure 6). This sensitivity was not simply the result of elevated activity of DNA topoisomerase I in the presence of Mg^{2+}, Ca^{2+}, or Mn^{2+}, since the catalytic activity of a high concentration of DNA topoisomerase I was completely inhibited by the inhibitor at a concentration which had no effect on a much lower concentration of DNA topoisomerase I in the presence of Mg^{2+}, Ca^{2+}, or Mn^{2+} (data not shown).

Reversibility of Inhibition of DNA Topoisomerase I by the Purified Inhibitor. To test whether inhibition of DNA topoisomerase I by the most purified fraction was reversible, we carried out a two-step reaction. DNA topoisomerase I was first incubated with pXY65 DNA plus the inhibitor in

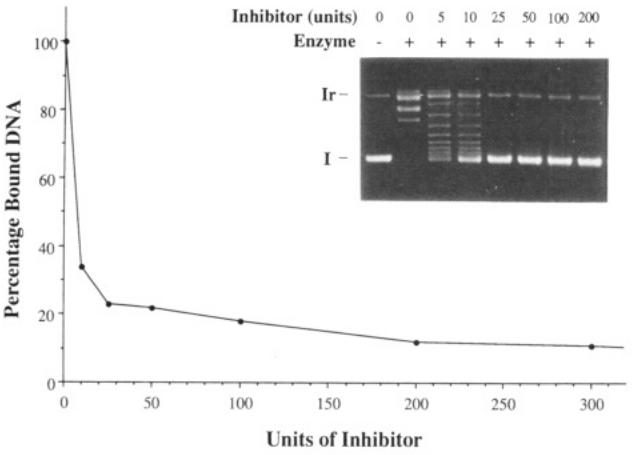

FIGURE 4: Inhibition of DNA binding to DNA topoisomerase I by the most purified fraction. DNA binding assays were carried out as described in Experimental Procedures. Under the experimental conditions used, protein-free DNA retained on the membrane was less than 2.5% of the bound DNA resulting from DNA topoisomerase I–DNA complex, and most of the DNA binding (>95%) in the presence of DNA topoisomerase I resulted from formation of noncleavable complex between DNA topoisomerase I and DNA (data not shown). The inhibitor did not bind to DNA in this and other DNA binding assays (data not shown). Inset: Inhibition of catalytic activity of DNA topoisomerase I by the most purified fraction. *X. laevis* DNA topoisomerase I (7.5 units) was incubated with 500 ng of pUC19 in the presence of the indicated amounts of purified inhibitor. Reactions were carried out as for the DNA topoisomerase I assay.

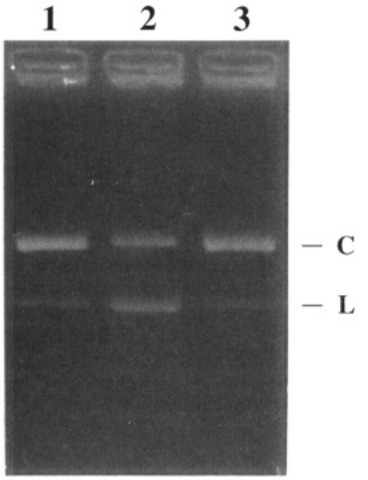

FIGURE 5: Effect of the most purified fraction on cleavage by DNA topoisomerase I. Single stranded ΦX174 DNA (lane 1) was incubated with DNA topoisomerase I (lane 2) or incubated with DNA topoisomerase I in the presence of fraction XIII (lane 3). The positions of circular (C) and linear (L) ΦX174 DNA are indicated. The assays were carried out as described in Experimental Procedures.

the standard assay buffer (reaction A), and then Mg^{2+} and pUC19 DNA were added to the reaction mixture (reaction B). Inhibition of DNA topoisomerase I by the inhibitor was reversible (Figure 7).

Species Specificity of the Inhibitor. The catalytic activity of DNA topoisomerase I from calf thymus and wheat germ was inhibited by the most purified fraction, as was the *Xenopus* ovarian enzyme (Figure 8). Interestingly, DNA topoisomerases I from calf thymus and wheat germ were not inhibited by a partially purified fraction (fraction VI), although *Xenopus* enzyme was strongly inhibited by this fraction (Figure 8). In addition, inhibition of the calf thymus and wheat germ enzymes by the most purified fraction was relieved by fraction VI.

Effect of the Inhibitor on Other DNA Enzymes. To test whether the inhibitor is specific to DNA topoisomerase I, the

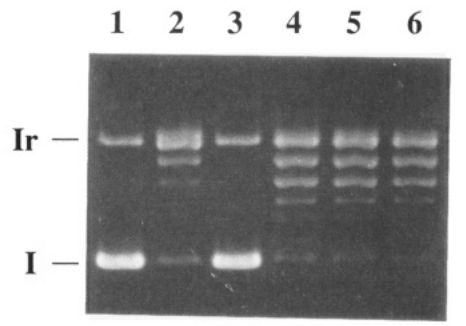

FIGURE 6: Effects of divalent cations on the inhibition of DNA topoisomerase I. DNA topoisomerase I activity was assayed with or without the most purified fraction and individual divalent cation. pUC19 DNA (500 ng), 2 units of *X. laevis* DNA topoisomerase I, and 100 units of fraction XIII were used in the reactions. Lane 1: 500 ng of pUC19; lane 2: enzyme alone, no inhibitor; lane 3: enzyme plus inhibitor; lane 4: enzyme plus inhibitor and 4 mM Mg^{2+}; lane 5: enzyme plus inhibitor and 4 mM Ca^{2+}; lane 6: enzyme plus inhibitor and 4 mM Mn^{2+}.

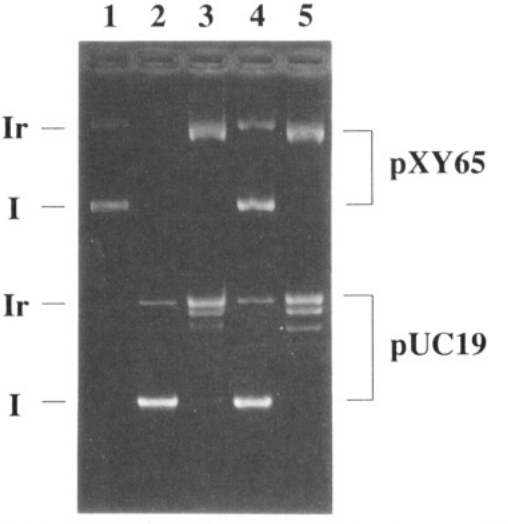

FIGURE 7: Reversibility of inhibition of DNA topoisomerase I by the most purified fraction. Reactions were carried out as described in Experimental Procedures. Lane 1: pXY65 DNA; lane 2: pUC19 DNA; lane 3: DNA topoisomerase I, no inhibitor or Mg^{2+} was added; lane 4: same as lane 3 except that fraction XIII was added in reaction A; lane 5: same as lane 4 except that Mg^{2+} was added in reaction B.

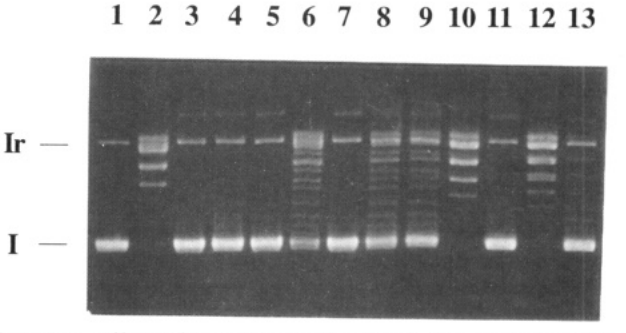

FIGURE 8: Effects of *X. laevis* inhibitor on catalytic activity of DNA topoisomerases I from three different species. DNA topoisomerase I from *X. laevis* ovaries (2.5 units, lanes 2–5), calf thymus (2.5 units, lanes 6–9), and wheat germ (0.1 unit, lanes 10–13) was incubated with 500 ng of pUC19 DNA (lane 1) in standard DNA topoisomerase I assay buffer (lanes 2, 6, and 10) or incubated with pUC19 DNA in the presence of 100 units of fraction XIII (lanes 3, 7, and 11), 5 μL of fraction VI (lanes 4, 8, and 12), or 100 units of fraction XIII plus 5 μL of fraction VI (lanes 5, 9, and 13).

effect of the most purified fraction on other DNA enzymes was examined. As shown in Figure 9, the inhibitor did not inhibit reactions catalyzed by calf thymus DNA topisomerase II or *Eco*RI methylase.

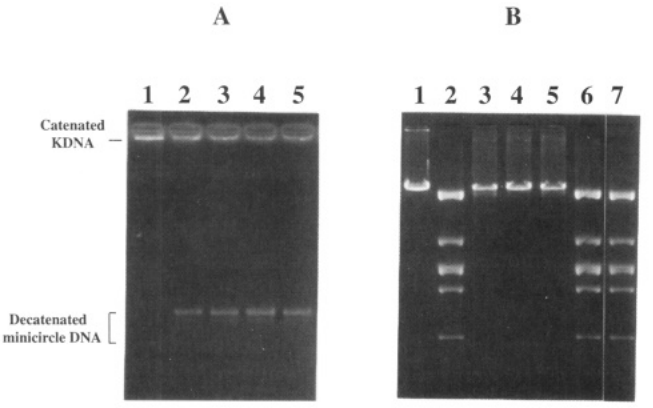

FIGURE 9: Effect of the purified inhibitor on other DNA enzymes. (A) Effect of the inhibitor on DNA topoisomerase II. Lane 1: 200 ng of KDNA; lane 2: 0.2 unit of DNA topoisomerase II; lane 3: 0.2 unit of DNA topoisomerase II plus 500 units of fraction XIII: lane 4: 0.6 unit of DNA topoisomerase II; lane 5: 0.6 unit of DNA topoisomerase II plus 500 units of fraction XIII. (B) Effect of the inhibitor on *Eco*RI methylase. Lane 1: λ DNA; lane 2: λ DNA digested with *Eco*RI; lane 3: λ DNA treated with *Eco*RI methylase before digestion with *Eco*RI; lane 4: λDNA treated with *Eco*RI methylase in the presence of 200 units of fraction XIII before digestion with *Eco*RI; lane 5: λ DNA treated with *Eco*RI methylase in the presence of 400 units of fraction XIII before digestion with *Eco*RI; lane 6: λ DNA digested with *Eco*RI in the presence of 400 units of fraction XIII; lane 7: λ DNA treated with *Eco*RI methylase without *S*-adenosylmethionine before digestion with *Eco*RI.

DISCUSSION

An inhibitor of DNA topoisomerase I from *X. laevis* ovaries has been purified and characterized. The most purified fraction (fraction XIII) contained peptides with apparent molecular masses of 25, 28.5, and 33.5 kDa. The 25- and 33.5-kDa peptides inhibited DNA topoisomerase I (Figure 1C). We speculate that the much less abundant, copurifying 28.5-kDa peptide would also have inhibitory activity. It is not known whether the smaller peptides are degradation products of the 33.5-kDa peptide, whether the peptides represent posttranslational modifications, or whether the inhibitor is heterogeneous. The inhibitor eluted anomalously from Sephadex G100, with apparent molecular mass ranging from 13 to 54 kDa. This might have resulted from the interaction of the inhibitor with the Sephadex matrix.

The most purified fraction was a specific inhibitor for DNA topoisomerase I. It did not inhibit the activities of calf thymus DNA topoisomerase II, *X. laevis* DNA polymerase α, and T_4 DNA ligase (Figure 9A and data not shown), which require a divalent cation for their activities, nor did it inhibit the activity of *Eco*RI methylase (Figure 9B), which has no requirement for a divalent cation.

The mechanism of inhibition of DNA topoisomerase I by the purified inhibitor was investigated. Increasing the amount of DNA did not result in increased relaxation of supercoiled DNA (Figure 3). Increasing amounts of DNA topoisomerase I, however, overcame inhibition of relaxation of supercoiled DNA (data not shown). These observations suggest that inhibition of DNA topoisomerase I was not due to the competition of inhibitor with enzyme for binding sites on substrate DNA. In addition, DNA binding experiments showed that binding of enzyme to substrate DNA decreased as the concentration of inhibitor increased (Figure 4). It seems likely, therefore, that the inhibitor interferes with binding of DNA topoisomerase I to DNA by interacting with the enzyme. The observation that the cleavage reaction catalyzed by DNA topoisomerase I was inhibited (Figure 5) is consistent with the hypothesis that the inhibitor interferes with binding of DNA topoisomerase I to DNA.

The catalytic activities of calf thymus and wheat germ DNA topoisomerase I were not inhibited by fraction VI although these enzymes were inhibited by purified inhibitor (Figure 8). A probable explanation is that the inhibitor binds to the *X. laevis* enzyme more tightly than it binds to the calf thymus and wheat germ enzymes. In the presence of other factors, which may bind either to DNA topoisomerase I or to the inhibitor, only the *X. laevis* enzyme would remain bound to the inhibitor.

Several forms of DNA topoisomerase I have been purified from a number of organisms (Liu & Miller, 1981; Attardi *et al.*, 1981; Javaherian *et al.*, 1982; Martin *et al.*, 1983; Kaiserman *et al.*, 1988; Richard & Bogenhagen, 1989). It is believed that the low molecular mass forms of DNA topoisomerase I, which exhibit catalytic activity, are proteolytic products of higher molecular mass forms. Consistent with this hypothesis, a fusion protein containing only a 67.7-kDa fragment of the carboxy terminus of human DNA topoisomerase I was reported to have catalytic activity (D'Arpa *et al.*, 1988). The function of the domains missing in the lower molecular mass forms is not clear. One possibility is that the missing domains may play roles in regulation of the enzyme by interacting with other proteins to modulate the activity of DNA topoisomerase I. In support of this hypothesis, we found that a low molecular mass (<60-kDa) DNA topoisomerase I from *X. laevis* ovaries (gift from Drs. S. Jakes and G. Kordiyak) was insensitive to a partially purified fraction (fraction VI), while higher molecular weight forms of the enzyme (Richard & Bogenhagen, 1989)were strongly inhibited by the same fraction (data not shown). Recently, Richard and Bogenhagen (1991) showed that DNA topoisomerase I in *X. laevis* is tissue specific. The primary enzyme from oocytes has a molecular mass of 165 kDa, while the enzyme from liver is 110 kDa. Antiserum raised against liver 110-kDa DNA topoisomerase I did not cross-react with the 165-kDa enzyme from ovaries and vice versa. Richard and Bogenhagen (1991) speculate that the additional domains present in the 165-kDa topoisomerase I may serve as the target for the regulated destruction of the enzyme during the development. More recently, Alsner *et al.* (1992) found that the *in vitro* catalytic activity of human DNA topoisomerase I is not affected by deletion of a 70-amino acid domain, spanning positions 141–210 in the N-terminal part of the enzyme. They demonstrated, however, that the domain is required for nuclear localization of the enzyme *in vivo*.

DNA topoisomerase I activity at different stages of the cell cycle has been measured in several different systems. Some investigators found no change of DNA topoisomerase I activity (Champoux *et al.*, 1979), while others reported significant increases in activity during or just prior to S-phase (Rosenberg *et al.*, 1976; Tricoli *et al.*, 1985). The identification of an inhibitor of DNA topoisomerase I in extracts of *X. laevis* ovaries suggests that similar inhibitors should be considered during quantitation of DNA topoisomerase I activity in cell extracts. On the basis of the levels of inhibitor in Table I, we estimate that the abundance of the inhibitor in *X. laevis* ovaries is comparable to the abundance of DNA topoisomerase I.

At present, it is not known whether the inhibitor affects the catalytic activity of DNA topoisomerase I *in vivo*. If the physiological activity of DNA topoisomerase I is modulated by the inhibitor, this modulation might in turn be regulated by protein phosphorylation/dephosphorylation, since we have observed that treatment of the most purified fraction with alkaline phosphatase, either soluble or immobilized, dramatically decreased its inhibitory activity (data not shown). Although the inhibitory activity of the inhibitor is modulated

by calcium *in vitro* (Figure 6), it seems unlikely that the inhibitor is regulated by Ca^{2+} *in vivo*, since more than 100 μM Ca^{2+} was required for the reversal of the inhibition in our assays.

The fact that cell-free *X. laevis* extracts are increasingly being used to study replication, transcription, chromatin assembly, DNA repair, and recombination *in vitro* makes it important to describe and thoroughly characterize the inhibitor proteins irrespective of whether they have a physiological role. Since DNA topoisomerase I is likely to be involved in each of these processes, attempts to reconstitute these *in vitro* could be confounded by the unsuspected presence of the inhibitor.

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