Regulation of *Xenopus laevis* DNA Topoisomerase I Activity by Phosphorylation in Vitro†

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ABSTRACT: DNA topoisomerase I has been purified to electrophoretic homogeneity from ovaries of the frog *Xenopus laevis*. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the most purified fraction revealed a single major band at 110 kDa and less abundant minor bands centered at 62 kDa. Incubation of the most purified fraction with immobilized calf intestinal alkaline phosphatase abolished all DNA topoisomerase enzymatic activity in a time-dependent reaction. Treatment of the dephosphorylated *X. laevis* DNA topoisomerase I with a *X. laevis* casein kinase type II activity and ATP restored DNA topoisomerase activity to a level higher than that observed in the most purified fraction. In vitro labeling experiments which employed the most purified DNA topoisomerase I fraction, [γ-^{32}P]ATP, and the casein kinase type II enzyme showed that both the 110- and 62-kDa bands became phosphorylated in approximately molar proportions. Phosphoamino acid analysis showed that only serine residues became phosphorylated. Phosphorylation was accompanied by an increase in DNA topoisomerase activity in vitro. Dephosphorylation of DNA topoisomerase I appears to block formation of the initial enzyme–substrate complex on the basis of the failure of the dephosphorylated enzyme to nick DNA in the presence of camptothecin. We conclude that *X. laevis* DNA topoisomerase I is partially phosphorylated as isolated and that this phosphorylation is essential for expression of enzymatic activity in vitro. On the basis of the ability of the casein kinase type II activity to reactivate dephosphorylated DNA topoisomerase I, we speculate that this kinase may contribute to the physiological regulation of DNA topoisomerase I activity.

DNA topoisomerases are enzymes that alter and control the topological states of DNA, thereby influencing the many processes in which DNA serves as a template in vivo (Wang, 1985; Liu, 1983; Cozzarelli, 1980). In eukaryotic organisms, DNA topoisomerases fall into two distinct classes. Type II DNA topoisomerases require ATP and catalyze double-stranded DNA breakage, passage, and rejoining. In contrast, type I DNA topoisomerases do not require ATP and catalyze transient breakage of one DNA strand, permitting passage of another single strand through the break.

RNA transcription and DNA replication are processes in which strand passage must take place in order to maintain or relieve a torsionally stressed state, thus facilitating the translocation of RNA or DNA polymerases (and associated proteins) along the template DNA strand. DNA topoisomerases presumably play a key role in maintaining eukaryotic chromatin in the proper topological states. Moreover, the level of DNA topoisomerase activity must somehow be coupled to the need to change the topological states of the DNA. In fact, Poccia et al. (1978) have found that DNA topoisomerase activity levels were linked to the cell cycle, although it was not determined if the increase in activity was due to an increase in the actual number of DNA topoisomerase molecules.

As possible regulatory mechanisms, one might expect the in vivo levels of DNA topoisomerase activities to vary during the cell cycle or development, perhaps being regulated in vivo by covalent modification. In particular, phosphorylation is a covalent modification that has been intimately linked to mitotic events. Maturation promoting factor (MPF) can induce breakdown of the nuclear envelope during mitosis (Miake-Lye & Kirschner, 1985), a process that has been correlated with hyperphosphorylation of nuclear lamina (Gerace & Blobel, 1980). In *Xenopus*, Lohka et al. (1987) have shown that at least six proteins are found as phosphoproteins during M phase, but not in G_1/S phase, and apparently become phosphorylated in response to MPF. An attractive hypothesis is that MPF induces a phosphorylation cascade leading to mitotic events (Miake-Lye & Kirschner, 1985). Moreover, monoclonal antibodies exist that recognize mitosis-specific phosphoproteins (Davis et al., 1983), further implying that the role of phosphorylation in mitosis should not be underestimated.

A similar cascade of phosphorylation events may occur prior to S phase and may ultimately lead to activation of the enzymes responsible for DNA replication. A putative DNA replication enzyme, DNA polymerase α, has been shown to be phosphorylated in vivo (Donaldson & Gerner, 1987; Wong et al., 1986) and to change its catalytic properties upon exposure to protein kinase C (Krauss et al., 1987). DNA topoisomerase II, another putative DNA replication enzyme, was shown by Sander et al. (1984) to copurify with a tightly associated protein kinase activity. Ackerman et al. (1985) subsequently showed that casein kinase II was capable of modulating DNA topoisomerase II activity in vitro. Phosphorylation by casein kinase II of yet another putative DNA replication enzyme, DNA topoisomerase I from Novikoff hepatoma cells, has been reported in vivo and in vitro by Durban et al. (1983), Mills et al. (1982), and Durban et al. (1985). Phosphorylation was also shown to increase DNA

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topoisomerase I activity in vitro. It has not yet been established, however, whether this control mechanism is operative in other eukaryotic cells. Consistent with this posibility, one other laboratory has reported ATP to be an effector molecule of DNA topoisomerase I isolated from human leukemia cells (Castora & Kelly, 1986).

In the present study we isolated DNA topoisomerase I from ovaries of *Xenopus laevis* and asked whether its activity could be modulated in vitro by phosphorylation/dephosphorylation. We then addressed the question how dephosphorylation might interfere with the ability of DNA topoisomerase I to relax supercoiled DNA.

Experimental Procedures

Adult *Xenopus laevis* females were obtained from the South African Snake Farm (Fish Hoek, Cape Providence, South Africa).

Chemicals. Tris(hydroxymethyl)aminomethane (Tris), glycerol, and other buffer components were of enzyme-grade quality from various sources. Cellulose phosphate (P11) and DEAE-cellulose (DE-52) ion-exchange resins were from Whatman. Hydroxylapatite (HA-Ultrogel) was from LKB. Carboxymethyl-Sephadex (CM-Sephadex) was from Pharmacia. Electrophoresis reagents were purchased from BRL. Silver Stain Kit was from Bio-Rad. Electrophoresis molecular weight markers were obtained from Sigma and Bio-Rad Laboratories. Agarose-immobilized calf intestinal alkaline phosphatase, heparin, hydrolyzed casein, phosvitin, and unlabeled phosphoamino acid standards were obtained from Sigma. Nuclease-free bovine serum albumin was obtained from Boehringer Mannheim. Camptothecin was obtained through the courtesy of Dr. Ven Narayanan of the National Cancer Institute and was stored at −20 °C as a 25 mM stock solution in dimethyl sulfoxide. Cellulose thin-layer chromatography (TLC) plates were obtained from Brinkmann. Nonradioactive nucleotides were obtained from Pharmacia P-L Biochemicals. [γ-^{32}P]ATP (3000 Ci/mmol) was from New England Nuclear. [γ-^{32}P]GTP (1361 Ci/mmol) was purchased from ICN Radiochemicals.

Protein was measured by the method of Bradford (1976) with bovine serum albumin as standard.

Agarose gel electrophoresis was performed with Ficoll-based loading buffer and Tris–borate (TBE) electrophoresis buffer according to Maniatis et al. (1982).

Phosphoamino acid analysis was performed according to Hunter and Sefton (1980). Samples for analysis were dissolved in 6 N HCl and incubated at 100 °C for 2 h under nitrogen after which time the solvent was removed under vacuum. The dried samples were then taken up in electrophoresis buffer (glacial acetic acid/pyridine/H_2O, 50:5:945) containing phosphoamino acid standards (1 mg/mL each). A total of 1 μL was applied to a cellulose TLC plate. Electrophoresis was performed at 1 kV for 3 h. Phosphoamino acid standards were visualized by ninhydrin staining after which the plate was exposed to Kodak XAR-5 X-ray film at −70 °C to determine the positions of the labeled amino acids.

DNA topoisomerase I activity was assayed by agarose gel electrophoresis to monitor the relaxation of supercoiled (form I) pUC19 plasmid DNA. The procedure was based on that used by Liu and Miller (1981). The assay mixture contained 50 mM Tris-HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM ethylenediaminetetraacetic acid (EDTA), nuclease-free bovine serum albumin (30 μg/mL), pUC19 DNA (0.5 μg), and enzyme in a total volume of 30 μL. Reactions were incubated for 30 min at 30 °C, terminated by the addition of 1.5 μL of 20% sodium dodecyl sulfate, and electrophoresed on a 1.1% agarose gel at 30 V. One unit of DNA topoisomerase I activity is the amount of enzyme that relaxes 0.25 μg of DNA in 30 min.

Protein kinase assays were performed according to the procedure used by Glover et al. (1983). Assay mixtures contained 50 mM Tris-HCl (pH 8.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 μM [γ-^{32}P]ATP (35 Ci/mmol, 1 μCi/reaction), and 1 mg/mL casein in a total volume of 30 μL. In some experiments phosvitin at 1 mg/mL was substituted for casein. Samples to be assayed were diluted in 20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 200 mM NaCl, 0.5 mM dithiothreitol, 10% glycerol, and 0.05% Triton X-100. Reactions were incubated at 30 °C for 15 min after which 20% trichloroacetic acid containing 0.1 M sodium pyrophoshate was added. Precipitates were collected onto Whatman GF/C filters, washed with 20% trichloroacetic acid, dried under infrared lamps, and counted in fluor (Liquifluor, New England Nuclear). One unit of protein kinase activity is defined as the incorporation of 1 pmol of ATP with casein as substrate under the conditions of the assay above in 15 min at 30 °C.

Purification of X. laevis Ovarian DNA Topoisomerase I. All manipulations were carried out at 4 °C. Unless otherwise stated, all buffers contained 2 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 mM benzamidine, 10 mM sodium bisulfite, and 25% glycerol (v/v). Ovaries were removed from decapitated frogs, washed several times with 25 mM Tris-HCl (pH 9.3) containing 5 mM KCl, and homogenized in the same buffer at a concentration of 1 g wet weight per 8 mL of buffer. Homogenization and low-speed and high-speed centrifugation (fractions I–III) were performed according to Kaiserman and Benbow (1987). Portions of 1350 mL of fraction III were mixed with 1000-mL portions of DEAE-cellulose equilibrated with 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM $MgCl_2$, and 5 mM KCl and stirred occasionally for 90 min. Nonadsorbed material was removed by filtration, followed by four 1-L washes. Adsorbed material was eluted by resuspending the cellulose in 1 L of the same buffer containing 200 mM KCl and stirring occasionally for 1 h, followed by filtration. The cellulose was washed 3 times with 500 mL of elution buffer, with 15-min equilibration periods between washes. The filtrate and three elution washes were combined to give 2.5 L (fraction IV). Fraction IV was added to 1500-mL settled volume of phosphocellulose equilibrated with 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 50 mM KCl and stirred for 2 h. Nonadsorbed material was removed by washing 3 times with 500 mL of 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 150 mM KCl. Two 5 × 30 cm columns were prepared and eluted with a 2-L linear gradient of 0.15–1.2 M KCl in the same buffer, followed by a 500-mL wash with 1.2 M KCl. DNA topoisomerase activity eluted at approximately 0.75 M KCl (fraction V). Fraction V was dialyzed into 50 mM K_xPO_4 (pH 7.5) containing 700 mM NaCl and applied to an 80-mL hydroxylapatite column equilibrated with the same buffer. After no more protein was evident in the flow-through as monitored by A_{280}, a 300-mL 0.2–0.7 M K_xPO_4 linear gradient was applied to the column. DNA topoisomerase activity eluted as a broad peak at 0.45 M K_xPO_4. Active fractions were pooled, concentrated to 25 mL to give fraction VI, and equilibrated in 25 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 0.1 M NaCl with an Amicon pressure dialysis cell fitted with a PM-10 membrane (fraction VI). Fraction VI was applied to a DNA–agarose column (4 × 50 cm) that had been previously equilibrated with the same buffer and eluted with a 1-L linear 0.1–1.0 M NaCl gradient. DNA topoisomerase

Table I: Purification of DNA Topoisomerase I from *Xenopus laevis* Ovaries[a]

fraction	vol (mL)	total protein (mg)	total units	sp act. (units/mg)	yield (%)
(I) crude extract	10500	230000			
(II) low-speed supernatant	6000	132000			
(III) high-speed supernatant	5400	52000			
(IV) DEAE-cellulose	2500	2400			
(V) phosphocellulose	380	144	152000	1060	100
(VI) hydroxylapatite	25	16	81000	5063	53
(VII) DNA–agarose	23	2.7	56000	20740	37
(VIII) CM-Sephadex	1.2	0.8	35000	43750	23

[a] Based on 990 g of starting material.

activity eluted at 0.33 M NaCl. No DNA topoisomerase activity was detected above 0.6 M NaCl. Active fractions were pooled and concentrated to give fraction VII (23 mL). Fraction VII was dialyzed against 50 mM potassium phosphate (pH 7.0) containing 1 mM EDTA and applied to a 2.5 × 28 cm column of carboxymethyl-Sephadex equilibrated with the same buffer. The column was washed until the A_{280} had reached base line and subsequently eluted with a 500-mL linear 0.05–1.0 M potassium phosphate gradient. DNA topoisomerase activity eluted at approximately 0.18 M potassium phosphate coincident with a peak of A_{280}-absorbing material. Active fractions were combined and concentrated to 1.2 mL (fraction VIII) for storage at −70 °C.

Dephosphorylated DNA topoisomerase I was prepared by incubating 100 ng of fraction VIII enzyme with 5 units (defined by the supplier) of agarose-immobilized calf intestinal alkaline phosphatase in DNA topoisomerase assay buffer (minus BSA and DNA) at 4 °C. The ability of DNA topoisomerase I to relax supercoiled DNA decreased in a time-dependent manner. Approximately 20% DNA topoisomerase activity remained after 5 h. No DNA topoisomerase activity was detectable after 12 h. The mixture was centrifuged at 15000*g* for 10 min. The supernatant was then centrifuged an additional 10 min and stored for further use. Dephosphorylated DNA topoisomerase I for reactivation experiments was prepared as above except that 30 units of alkaline phosphatase was used. Under these conditions no DNA topoisomerase activity was detectable after 5 h.

Xenopus laevis casein kinase type II activity copurified with DNA topoisomerase I through step VI as measured by the protein kinase assay. Single-stranded DNA–agarose chromatography (step VII) separated DNA topoisomerase I from casein kinase type II activities found in the flow through. The casein kinase type II activity was sensitive to heparin (15% activity remained at 10 μg/mL heparin) and used phosvitin as substrate at 60% efficiency compared to casein. The kinase activity was able to utilize GTP as donor molecule at 80% of the efficiency of ATP using casein as acceptor. Upon incubation with [γ-^{32}P]ATP, a band at 31 kDa became labeled, presumably corresponding to the casein kinase II β subunit. Because of the properties described above as well as its chromatographic behavior, we tentatively identified this activity as a casein kinase type II activity as reviewed by Hathaway and Traugh (1982).

Subcellular fractionation of X. laevis oocytes was performed exactly as described by Fox et al. (1980) and Breaux (1981).

Isoelectric focusing of X. laevis DNA topoisomerase I was carried out with a 100-mL LKB type 8101 isoelectric focusing column (LKB-Produkter AB, Bromma, Sweden) as described by Vesterberg (1971) and Breaux (1981).

Results

Purification of X. laevis DNA Topoisomerase I. The purification scheme we employed for *X. laevis* DNA topoisomerase I is similar to that used for other eukaryotic DNA topoisomerase I activities [reviewed by Wang (1985)] and is outlined in Table I. Gel electrophoretic analysis of the most highly purified enzyme (fraction VIII) revealed a single major band at 110 kDa and minor less abundant bands at 62 kDa (Figure 1). Smearing of the gel in the high molecular weight region was an apparent artifact of silver staining. Liu and Miller (1981) have shown similar minor bands at 62 kDa in HeLa cell DNA topoisomerase I preparations to be catalytically active proteolytic products of the 110-kDa protein. Fraction VIII *X. laevis* DNA topoisomerase I did not require ATP. Morever, catalytic activity was inhibited by camptothecin, a selective inhibitor of DNA topoisomerase I. All of these properties are therefore consistent with the fraction VIII enzyme being a near homogeneous DNA topoisomerase I with no apparent contamination by DNA topoisomerase II.

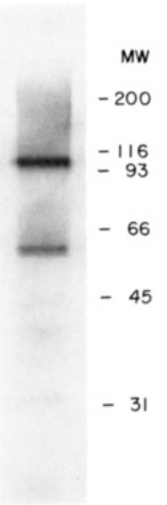

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoretic analysis of fraction VIII DNA topoisomerase I. A total of 100 ng of fraction VIII DNA topoisomerase I was applied to a 3.75% stacking gel and electrophoresed through a 10% resolving gel according to Laemmli (1970). Proteins were visualized by silver staining according to the method of Merrill et al. (1981).

Intracellular Localization of DNA Topoisomerase I. Extracts of subcellular components of *X. laevis* oocytes were prepared as described under Experimental Procedures and assayed for DNA topoisomerase activity. The results in Figure 2 show that DNA topoisomerase activity was found not only in germinal vesicles but also in enucleated oocytes and mitochondria.

Isoelectric Focusing of DNA Topoisomerase I. Fraction VIII DNA topoisomerase I was applied to an isoelectric focusing column and analyzed as described in the legend to Figure 3. We obtained a p*I* of 9.1 ± 0.2 using this method. DNA topoisomerase activity from fractions III and IV exhibited significantly lower p*I* values (p*I*'s of 4.0–5.0) presumably due to association of DNA topoisomerase I with nucleic acids.

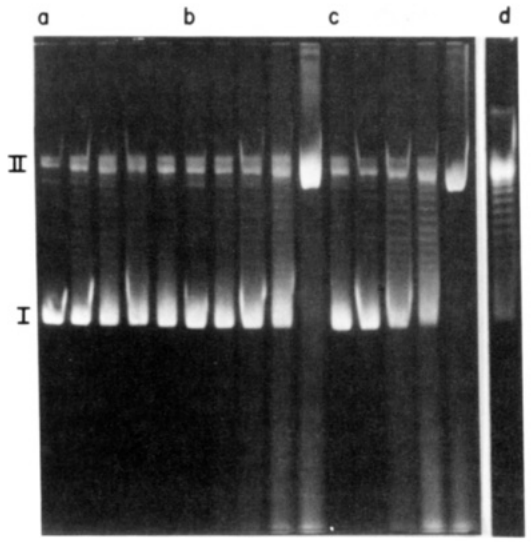

FIGURE 2: Intracellular localization of *X. laevis* DNA topoisomerase I. Manually isolated germinal vesicles, enucleated oocytes, whole oocytes, and mitochondrial extracts were prepared as described by Fox et al. (1980). Each preparation was adjusted to a concentration of 60 oocytes/mL, and aliquots of 2.5, 5.0, 10, 20, and 40 μL were assayed for DNA topoisomerase activity. A single aliquot of 10 μL was assayed from the mitochondrial fraction. I denotes closed circular DNA. II denotes open circular DNA. (a) Germinal vesicle extract; (b) enucleated oocyte extract; (c) whole oocyte extract; (d) mitochondrial extract.

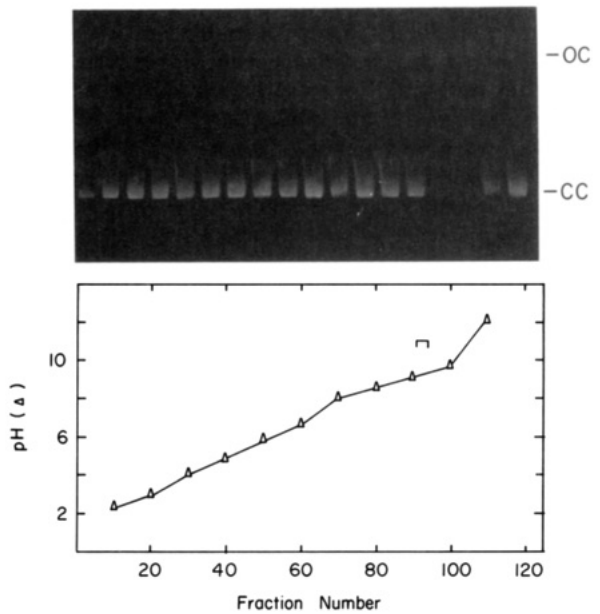

FIGURE 3: Isoelectric focusing. The isoelectric focusing column was filled with a linear 5–50% (v/v) sucrose density gradient. Fraction VIII DNA topoisomerase I [previously equilibrated into 25 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, and 25% glycerol] was added to the dense sucrose layer before forming the gradient. Electrophoresis was performed at a constant voltage of 400 V (5.0–0.5 mA) for 40 h at 0 °C, and 1-mL fractions were collected. pH values were determined immediately, and aliquots were assayed for DNA topoisomerase activity.

Phosphorylation of DNA Topoisomerase I in Vitro. Fraction VIII DNA topoisomerase I was incubated with [γ-^{32}P]ATP and a highly purified *X. laevis* casein kinase type II activity. As shown in Figure 4a, the 110- and 62-kDa forms of DNA topoisomerase I became phosphorylated in approximate proportion (3.5:1) to their molar representation (3.6:1) as seen in Figure 1. In addition, a band at 31 kDa was also phosphorylated, most likely corresponding to autophosphorylation of a casein kinase type II β subunit. The

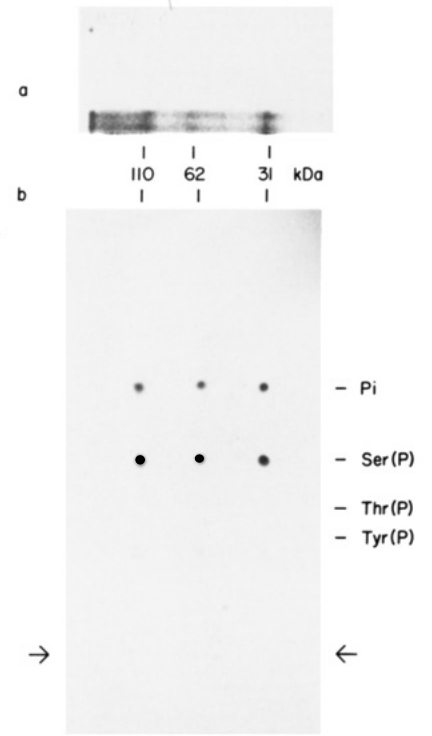

FIGURE 4: (a) Phosphorylation of DNA topoisomerase I by a *X. laevis* casein kinase type II enzyme activity. A total of 100 ng of fraction VIII DNA topoisomerase I was incubated with 50 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and 2 units of *X. laevis* casein kinase type II activity under DNA topoisomerase assay conditions (minus BSA and DNA). The reaction was terminated by the addition of SDS loading buffer and electrophoresed as in Figure 1. After electrophoresis the gel was exposed to Kodak XAR-5 film to localize the labeled proteins. The clear area above the visible lane shows control lanes containing reactions from which either the casein kinase or the ATP had been omitted. These lanes are not visible on the autoradiogram. (b) Phosphoamino acid analysis of the bands from (a). Labeled proteins were excised, and ^{32}P-labeled phosphoamino acids were identified as described under Experimental Procedures. The migration positions of phosphoamino acid standards and inorganic phosphate are indicated. The origin of electrophoresis is denoted by the arrows.

labeled bands in Figure 4a were excised from the gel and subjected to phosphoamino acid analysis. As shown in Figure 4b, the bands at 110 and 62 kDa, representing DNA topoisomerase I, and the 32-kDa band all contained phosphoserine as the only labeled amino acid liberated upon protein hydrolysis.

Phosphate Incorporation by DNA Topoisomerase I. In a separate experiment, DNA topoisomerase I was incubated with casein kinase type II activity and [γ-^{32}P]ATP (4.5 Ci/mmol) under the conditions of the DNA topoisomerase assay. Following incubation, the reaction products were subjected to SDS–polyacrylamide gel electrophoresis, and the bands corresponding to DNA topoisomerase I (110 and 62 kDa) were excised from the gel, macerated, dissolved in NCS tissue solubilizer, and counted in 3A70B aqueous fluor. One microgram of the 110-kDa DNA topoisomerase I (as compared to protein standards) incorporated 10.9 pmol of PO_4, giving a ratio of 1.2 mol of phosphate/protein (±10%). A similar number was obtained for the 62-kDa DNA topoisomerase I band.

Dephosphorylation Eliminates DNA Topoisomerase I Catalytic Activity. As shown in Figure 5, incubation of the most purified DNA topoisomerase I with immobilized calf intestinal alkaline phosphatase completely abolished the ability of the enzyme to relax supercoiled DNA. Incubation of the supercoiled DNA template with alkaline phosphatase (lane

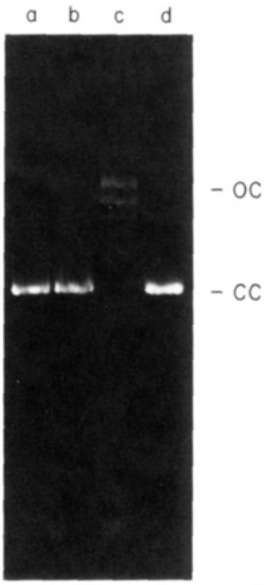

FIGURE 5: Inactivation of DNA topoisomerase I activity by alkaline phosphatase. A total of 1 ng of fraction VIII DNA topoisomerase I inactivated by preincubation with immobilized calf intestinal alkaline phosphatase (see Experimental Procedures) was incubated with 0.5 μg of supercoiled pUC19 DNA for 30 min at 37 °C. The reaction products were assayed by electrophoresis on a 1.1% agarose gel. (a) No enzyme. (b) No enzyme, preincubation of DNA with immobilized alkaline phosphatase. (c) Mock preincubation of DNA topoisomerase I. (d) DNA topoisomerase I preincubated with immobilized alkaline phosphatase. CC denotes closed circular form I DNA. OC denotes open circular (presumably forms II and IV) DNA.

b) or mock incubation of DNA topoisomerase I (lane c) had no effect on DNA topoisomerase activity.

Stimulation of Native DNA Topoisomerase I by Phosphorylation. As shown in Figure 6a, in the presence of ATP and casein kinase type II activity, DNA topoisomerase I activity was stimulated severalfold (lanes 5–7). Moreover, this effect was dependent on the amount of casein kinase type II activity added (lanes 5–7). Casein kinase II itself showed no ability to relax supercoiled DNA (lane 2), and ATP itself did not stimulate DNA topoisomerase I (lanes 3 and 4). This indicates that stimulation was due to phosphorylation of DNA topoisomerase I by the casein kinase type II activity.

Incubation with Casein Kinase II and ATP Restores Catalytic Activity to Dephosphorylated DNA Topoisomerase I. Casein kinase type II activity was able to reactivate dephosphorylated DNA topoisomerase I in the presence of ATP as shown in Figure 6b. The restoration of catalytic activity was dependent on the amount of casein kinase type II activity added (lanes 2–7). With the highest casein kinase II concentrations, the level of DNA topoisomerase I activity was greater than that observed prior to dephosphorylation (lanes 5, 6, and 7 versus lane 1). This result is consistent with the stimulation of DNA topoisomerase I activity by casein kinase II prior to dephosphorylation (Figure 6a).

Dephosphorylation Blocks Camptothecin-Induced DNA Topoisomerase I Nicking of DNA. As shown by Hsiang et al. (1985), in the presence of camptothecin, catalytically active DNA topoisomerase I from calf thymus introduces nicks into DNA molecules. Figure 7 (lanes c and d) shows that DNA topoisomerase I from *X. laevis* will convert supercoiled (CC) DNA to nicked or relaxed (OC) DNA in the presence of camptothecin. We attribute the formation of relaxed DNA in the presence of camptothecin (lane d) to camptothecin-induced nicking (rather than relaxation) of the DNA template [see Hsiang et al. (1985)] since the number of molecules with intermediate linking numbers was greatly reduced when

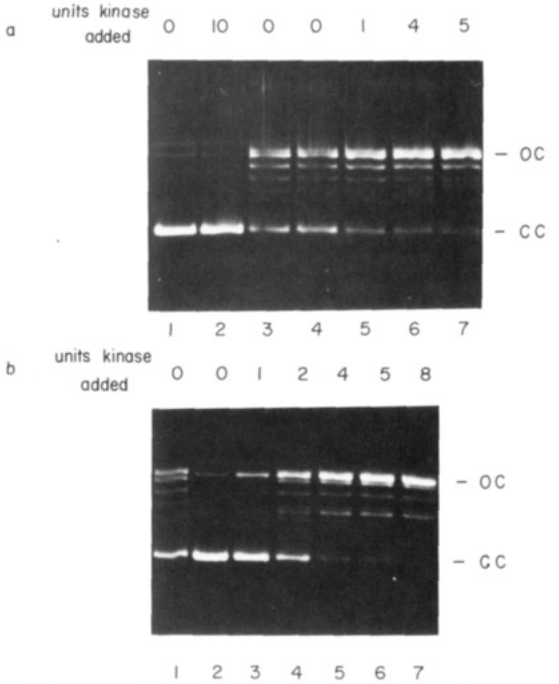

FIGURE 6: (a) Stimulation of fraction VIII *X. laevis* DNA topoisomerase I by *X. laevis* casein kinase type II activity. (a) A total of 1 ng of fraction VIII DNA topoisomerase I was incubated with 30 μM ATP and the indicated number of units of casein kinase type II activity: (1) no enzymes, ATP; (2) no DNA topoisomerase I, 10 units of casein kinase type II activity, ATP; (3) DNA topoisomerase I; (4) DNA topoisomerase I, ATP; (5–7) DNA topoisomerase I, ATP; 1, 4, or 5 units of casein kinase type II activity. (b) Restoration of catalytic activity to dephosphorylated DNA topoisomerase I. Dephosphorylated DNA topoisomerase I (1 ng) was incubated with the indicated number of units of casein kinase type II activity in the presence of 30 μM ATP (lanes 2–7) under the conditions for the DNA topoisomerase assay. Fraction VIII DNA topoisomerase I (1 ng) activity prior to dephosphorylation is shown in lane 1.

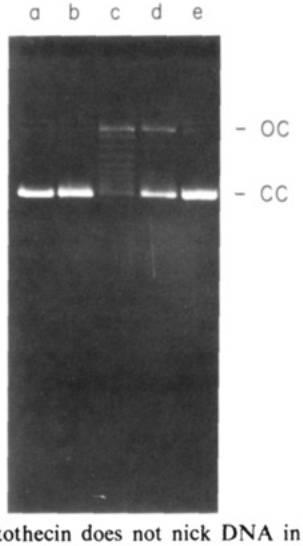

FIGURE 7: Camptothecin does not nick DNA in the presence of alkaline phosphatase treated DNA topoisomerase I. A total of 1 ng of fraction VIII DNA topoisomerase I preincubated with immobilized alkaline phosphatase was incubated with 0.5 μg of pUC19 supercoiled DNA as under Experimental Procedures in the presence of 25 μM camptothecin. The reaction products were analyzed on a 1.1% agarose gel. (a) No enzyme, no drug; (b) no enzyme, 25 μM camptothecin; (c) DNA topoisomerase I, no drug; (d) DNA topoisomerase I, 25 μM camptothecin; (e) alkaline phosphatase inactivated DNA topoisomerase I, 25 μM camptothecin. All reactions contained an equal amount of dimethyl sulfoxide, which was necessary to dissolve the camptothecin.

compared with the number of intermediates observed in the absence of camptothecin (lane c). Figure 7 (lane e) shows that

dephosphorylated DNA topoisomerase I fails to introduce similar nicks into supercoiled DNA molecules.

Discussion

We have purified to near electrophoretic homogeneity DNA topoisomerase I from ovaries of the frog *X. laevis* (Table I and Figure 1). The molecular weight of the single major 110-kDa band observed in Figure 1 differs from the single major 67-kDa band reported for *X. laevis* ovarian DNA topoisomerase I by Attardi et al. (1981). We think it is likely, however, that their 67-kDa band is analogous to the minor 62-kDa bands we observed [and therefore is also related to the 110-kDa band described here; see Liu and Miller (1981)]. The properties of our fraction VIII *X. laevis* DNA topoisomerase I also differ from the properties of the *Xenopus* enzyme described by Attardi et al. (1981) in p*I* (Figure 3, 9.4 vs 4.2–4.4 for the Attardi et al. enzyme) and localization (Figure 2, we found high levels of activity in enucleated oocytes as well as in nuclei; the Attardi et al. enzyme was exclusively nuclear). We speculate that the p*I* of 4.2–4.4 observed by Attardi et al. corresponded to the p*I* of a DNA topoisomerase I–nucleic acid complex, and that their cytoplasmic enzyme became inactivated during preparation of enucleated oocytes. If these speculations are correct, the properties of the *X. laevis* DNA topoisomerase I then become consistent with the properties of other eukaryotic DNA topoisomerase I activities [see review by Wang (1985)]. One additional property of the *X. laevis* DNA topoisomerase I, not noted for other eukaryotic DNA topoisomerases, was potent inhibition of catalytic activity by the *X. laevis* linear concatenation protein described by Bayne et al. (1984).

We have shown that the *X. laevis* DNA topoisomerase I enzyme is phosphorylated in vitro by an endogenous casein kinase type II activity, which is found at high levels in *X. laevis* ovaries. The molar ratios of phosphorylation were ca. 1.2 mol of PO_4/mol of protein for both the 110- and 62-kDa topoisomerase I bands (compare Figures 1 and 4a). Phosphorylation by incubation of fraction VIII enzyme with casein kinase type II activity and ATP stimulated DNA topoisomerase I catalytic activity severalfold (Figure 6a). Conversely, dephosphorylation by immobilized calf intestinal alkaline phosphatase totally abolished DNA topoisomerase catalytic activity (Figure 5) in a time-dependent manner (see Experimental Procedures). Phosphorylation of the dephosphorylated DNA topoisomerase I with *X. laevis* casein kinase type II activity and ATP restored catalytic activity to a higher level than in the fraction VIII enzyme prior to dephosphorylation (Figure 6b), suggesting that ovarian DNA topoisomerase I may exist in a hypophosphorylated state.

We have referred to the *X. laevis* ovarian enzyme catalyzing the phosphorylation of DNA topoisomerase I as a casein kinase type II activity. This attribution is tentative and is based solely on sensitivity to heparin, ability to phosphorylate phosvitin and to utilize both ATP and GTP as donor molecules, and autophosphorylation of a presumptive 31-kDa β subunit [see Hathaway and Traugh (1982)]. A more detailed characterization, however, is necessary before the kinase can be rigorously designated as the *X. laevis* casein kinase II enzyme.

Our observations are qualitatively consistent with similar reports from the laboratory of Busch (Mills et al., 1982; Durban et al., 1983, 1985) for mammalian DNA topoisomerase I from Novikoff hepatoma cells. A potentially significant difference, however, is that the *X. laevis* DNA topoisomerase I is totally dependent on phosphorylation for catalytic activity. This implies that the *X. laevis* DNA topoisomerase must be phosphorylated in vivo in order to be active. Moreover, the *X. laevis* DNA topoisomerase I was shown to be partially phosphorylated as isolated (Table I and Figure 6a), and it is likely that this phosphorylation was also catalyzed by the casein kinase type II activity. Durban et al. (1985) have shown that a casein kinase type II enzyme phosphorylates Novikoff hepatoma cell DNA topoisomerase I in vivo.

In the presence of camptothecin, covalent intermediates between eukaryotic DNA topoisomerase I and DNA are trapped, forming nicks in supercoiled DNA molecules (Hsiang et al., 1985). Dephosphorylation totally abolishes this camptothecin-induced nicking by the *X. laevis* DNA topoisomerase I (Figure 7). This suggests that phosphorylation may regulate formation of the initial covalent enzyme–substrate complex, rather than functioning during subsequent strand passage.

The modulation of DNA topoisomerase I activity by phosphorylation/dephosphorylation in vitro suggests an obvious means of physiologically regulating DNA topoisomerase I activity. Microinjection experiments, which can readily be carried out in *X. laevis* embryos, should therefore have the potential to determine whether this mechanism actually functions in vivo.

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Registry No. Ser, 56-45-1; DNA topoisomerase, 80449-01-0; casein kinase, 52660-18-1.

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Phosphorylation of Insulin-like Growth Factor I Receptor by Insulin Receptor Tyrosine Kinase in Intact Cultured Skeletal Muscle Cells†

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ABSTRACT: The interaction between insulin and insulin-like growth factor I (IGF I) receptors was examined by determining the ability of each receptor type to phosphorylate tyrosine residues on the other receptor in intact L6 skeletal muscle cells. This was made possible through a sequential immunoprecipitation method with two different antibodies that effectively separated the phosphorylated insulin and IGF I receptors. After incubation of intact L6 cells with various concentrations of insulin or IGF I in the presence of [^{32}P]-orthophosphate, insulin receptors were precipitated with one of two human polyclonal anti-insulin receptor antibodies (B2 or B9). Phosphorylated IGF I receptors remained in solution and were subsequently precipitated by anti-phosphotyrosine antibodies. The identities of the insulin and IGF I receptor β-subunits in the two immunoprecipitates were confirmed by binding affinity, by phosphopeptide mapping after trypsin digestion, and by the distinct patterns of expression of the two receptors during differentiation. Stimulated phosphorylation of the β-subunit of the insulin receptor correlated with occupancy of the β-subunit of the insulin receptor by either insulin or IGF I as determined by affinity cross-linking. Similarly, stimulation of phosphorylation of the β-subunit of the IGF I receptor by IGF I correlated with IGF I receptor occupancy. In contrast, insulin stimulated phosphorylation of the β-subunit of the IGF I receptor at hormone concentrations that were associated with significant occupancy of the insulin receptor but negligible IGF I receptor occupancy. These findings indicate that the IGF I receptor can be a substrate for the hormone-activated insulin receptor tyrosine kinase activity in intact L6 skeletal muscle cells.

The insulin receptor contains an intrinsic protein kinase activity that phosphorylates tyrosine residues in the β-subunit of the receptor after hormone binding (Kasuga et al., 1982a,b; Roth & Cassell, 1983; Ullrich et al., 1985). Similarly, the structurally homologous receptor for insulin-like growth factor I (IGF I)[1] (Kasuga et al., 1981; Kull et al., 1983; Massague & Czech, 1982; Rechler et al., 1980; Rechler & Nissley, 1985) becomes phosphorylated on tyrosine residues of its β-subunit in response to IGF I binding (Jacobs et al., 1983; Rubin et al., 1983; Zick et al., 1984). It has been hypothesized that tyrosine phosphorylation plays a role in mediating the biological actions of these two hormones, either via the phosphorylation of other proteins by the activated receptor kinases or as a result of the modified properties of the receptor that

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[1] Abbreviations: IGF I, insulin-like growth factor I; IGF II, insulin-like growth factor II; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; MSA, multiplication stimulating activity; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; α-ptyr, anti-phosphotyrosine antibody; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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