Purification and Characterization of Type II DNA Topoisomerase from Mouse FM3A Cells: Phosphorylation of Topoisomerase II and Modification of Its Activity†

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ABSTRACT: Type II topoisomerase has been purified from mouse FM3A cells by using P4 phage knotted DNA as a substrate. Analysis of the purified enzyme by sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed two bands of apparent molecular masses of 167 and 151 kDa. Partial digestion of the two bands with *Staphylococcus aureus* V8 protease indicated that the two polypeptides were structurally related. The enzyme required ATP and Mg^{2+} for activity. dATP could substitute for ATP, and ITP was slightly effective at 5–10 mM. The activity was sensitive to 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA), coumermycin, and ethidium bromide. A protein kinase activity was detected in the partially purified topoisomerase II fraction, and this protein kinase was further purified. The protein kinase phosphorylated the purified topoisomerase II, and the phosphorylation of topoisomerase II by the kinase increased the activity by 8.6-fold over that of the unmodified enzyme. The treatment of the purified topoisomerase II with alkaline phosphatase abolished the enzyme activity almost completely, and the treatment of the dephosphorylated topoisomerase II with the protein kinase restored the enzyme activity. The protein kinase activity was not stimulated by Ca^{2+} or cyclic nucleotides, and the aminoacyl residue phosphorylated by the kinase was serine. Enzymatic properties of the kinase were very similar to those of the kinase reported to be tightly associated with the *Drosophila* topoisomerase II [Sander, M., Nolan, J. M., & Hsieh, T.-S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6938–6942]. The immunoprecipitation of nuclear extracts prepared from ^{32}P-labeled cells with anti-mouse topoisomerase II antiserum indicated that DNA topoisomerase II existed in mouse cells as a phosphoprotein.

DNA topoisomerases are a class of enzymes which catalyze the interconversion of the topological states of DNA. Two classes of enzymes have been found in prokaryotes and eukaryotes (Gellert, 1981; Wang, 1985; Vosberg, 1985). Type I topoisomerases change the linking number in steps of one by making a transient single-stranded nick, while type II topoisomerases introduce a transient double-stranded break through which another DNA helix passes, resulting in a change of linking number in steps of two.

Biological roles of topoisomerase II in eukaryotes are not well understood, and it has been suggested that it may be implicated in several genetic processes, including replication, transcription, recombination, sister chromatid exchange, and chromosome disjunction at mitosis. Recently, genetic studies of yeast revealed that topoisomerase II is encoded by a single-copy, essential gene and is necessary for the segregation of daughter chromosomes at termination of replication (Yanagida & Wang, 1987). Using specific antibodies, it was shown that topoisomerase II is a component of the nuclear matrix (Berrios et al., 1985) and mitotic chromosome scaffold fractions (Earnshaw et al., 1985) and that topoisomerase II is localized at the bases of the radial loop domains of mitotic chromosomes (Earnshaw & Heck, 1985; Gasser et al., 1986).

Several studies have shown that the level of topoisomerase II activity and the amount of the enzyme in proliferating cells were higher than those in quiescent cells (Miskimins et al., 1983; Duguet et al., 1983; Taudou et al., 1984; Sullivan et al., 1986; Heck & Earnshaw, 1986; Zwelling et al., 1987; Markovits et al., 19877; Nelson et al., 1987; Chow & Ross, 1987; Hsiang et al., 1988). During the development of *Drosophila*, the expression of topoisomerase II is high at the stages with increased mitotic activity (Fairman & Brutlag, 1988). Furthermore, it was observed that both the amount and the stability of topoisomerase II alter during the cell cycle (Heck et al., 1988).

At present, little is known about the mechanism to modulate the intracellular activity of topoisomerae II. Phosphorylation is one of the possible modifications to regulate the enzyme activity. *Drosophila* topoisomerase II was shown to copurify with a tightly associated protein kinase activity (Sander et al., 1984). Ackerman et al. (1985) showed that casein kinase II is capable of increasing the activity of *Drosophila* topoisomerase II and provided several lines of evidence indicating that the phosphorylation of topoisomerase II in *Drosophila* Kc cells is catalyzed by casein kinase II (Ackerman et al., 1988).

In this study, we have purified topoisomerase II from mouse FM3A cells and characterized its physical and enzymatic properties. In addition, we have examined whether its activity can be modulated by phosphorylation using a protein kinase which was detected in the partially purified topoisomerase II fraction from FM3A cells.

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Materials and Methods

Materials. [γ-^{32}P]ATP and [^{32}P]orthophosphate were purchased from NEN; nucleoside triphosphates, ADP, and AMP from Yamasa Biochemicals; 5'-adenylyl methylenediphosphate (AMP-PCP),[1] AMP-PNP, and ATPγS from Boehringer Mannheim; novobiocin, coumermycin A1, nalidixic acid, oxolinic acid, actinomycin D, ethidium bromide, PMSF, *Staphylococcus aureus* V8 protease, and insoluble alkaline phosphatase from Sigma; heparin from Fluka; DEAE-cellulose from Brown; phosphocellulose (P11) from Whatman; hydroxyapatite (Bio-Gel HTP) from Bio-Rad; Mono Q, Mono S, and protein A–Sepharose CL-4B from Pharmacia. Antipain and *m*-AMSA were generous gifts from Dr. T. Aoyagi (Institute of Microbiological Chemistry, Tokyo) and Dr. Y. Hashimoto (University of Tokyo), respectively. VP-16 and camptothecin were kindly provided by Nihon Kayaku Co., Ltd., and Yakult Co., Ltd., respectively. P4 phage knotted DNA and pBR322 supercoiled DNA were prepared according to Liu et al. (1981) and Maniatis et al. (1982), respectively.

Buffers. Buffer 1 contained 20 mM potassium phosphate buffer, pH 7.5, 0.1 mM Na_3EDTA, 1 mM 2-mercaptoethanol, 0.25 mM PMSF, and 1% ethanol. Buffer 2 consisted of all components of buffer 1, 20% ethylene glycol, and 0.01% Triton X-100. Buffer 3 consisted of all components of buffer 1, 50% glycerol, and 0.01% Triton X-100. Buffer A contained 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM EDTA, 2 mM 2-mercaptoethanol, and 1 mM PMSF. Buffer B contained 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 2 mM 2-mercaptoethanol, and 1 mM PMSF.

Cells. FM3A cells were originally established from a spontaneous mammary carcinoma in a C3H/He mouse. Cells were grown intraperitoneally in ddY mice and harvested as described previously (Hanaoka et al., 1981). Cells were stored at −80 °C until use.

DNA Topoisomerase II Assay. The standard reaction mixture (20 μL) for DNA unknotting assay consisted of 50 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT, 0.5 mM Na_3EDTA, 30 μg/mL BSA, and 0.3 μg of P4 phage knotted DNA. Incubation was carried out for 30 min at 30 °C, and the reaction was stopped by the addition of 5 μL of stop solution containing 5% SDS, 25% Ficoll, and 0.05% bromophenol blue. The sample was incubated for 15 min at 50 °C and then loaded on a 0.7% agarose gel in TBE buffer (89 mM Tris–borate, pH 8.2, and 2 mM EDTA). After electrophoresis, the gel was stained with ethidium bromide and photographed under UV illumination. One unit of the activity is defined as the amount of enzyme that unknots 0.3 μg of knotted DNA under standard conditions. DNA relaxation activity was assayed as described above for the unknotting assay except that supercoiled pBR322 DNA was used as the substrate DNA.

DNA Topoisomerase I Assay. DNA relaxation activity of topoisomerase I was assayed as described above using supercoiled pBR322 DNA except that ATP was omitted. One unit of the activity is defined as the amount of enzyme that relaxes 0.3 μg of supercoiled DNA under standard conditions.

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out essentially according to the method of Laemmli (1970).

Peptide Mapping Analysis of Topoisomerase II. Partial proteolytic digestion with *S. aureus* V8 protease was done according to Cleveland et al. (1977).

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

Purification of DNA Topoisomerase II. All operations were carried out at 0–4 °C. A total of 1×10^{11} frozen FM3A cells were thawed, suspended in buffer 1 containing 2 μg/mL antipain at a concentration of 1.25×10^8 cells/mL, and sonicated 5 times for 10 s each with 20-s intervals with a Branson Model 185 sonifier. The sonicate was made 0.3 M in KCl by the addition of 0.1 volume of buffer 1 containing 3.3 M KCl. After being stirred for 30 min, the extract was centrifuged for 30 min at 10000*g*, and the supernatant was recentrifuged for 1 h at 125000*g*. To remove nucleic acids, the supernatant (fraction I) was loaded onto a DEAE-cellulose column (800 mL) equilibrated with 0.3 M KCl in buffer 1. The flow-through fractions were pooled (fraction II) and dialyzed against 50 mM KCl in buffer 2 after the addition of Triton X-100 at a final concentration of 0.01% (v/v). The dialysate was centrifuged for 30 min at 10000*g* to remove the precipitates formed during dialysis. The supernatant was loaded onto a second DEAE-cellulose column (500 mL) equilibrated with 50 mM KCl in buffer 2. The column was washed with 3 bed volumes of the same buffer and eluted with 10 bed volumes of a linear gradient of KCl from 50 to 400 mM in buffer 2. Topoisomerase I and II activities eluted between 55 and 190 mM KCl. The active fractions were pooled (fraction III) and loaded onto a phosphocellulose column (160 mL) equilibrated with 150 mM KCl in buffer 2. The column was washed with 3 bed volumes of the same buffer and eluted with 12 bed volumes of a linear gradient of KCl from 150 mM to 1.1 M in buffer 2. Topoisomerase II activity eluted between 550 and 700 mM KCl, and topoisomerase I activity eluted between 740 and 920 mM KCl. Each active fraction was pooled separately. The pooled fraction of topoisomerase II (fraction IV) was dialyzed against 400 mM KCl in buffer 2 and loaded onto a hydroxyapatite column (15 mL) equilibrated with 400 mM KCl in buffer 2. The column was washed with 3 bed volumes of the same buffer and eluted with 10 bed volumes of a linear gradient of potassium phosphate buffer, pH 7.5, from 20 to 500 mM in buffer 2 containing 400 mM KCl. Topoisomerase II activity eluted from the column at 270 mM potassium phosphate. The active fractions were pooled (fraction V). Two hundred microliter aliquots of the hydroxyapatite fraction were layered onto 4.8-mL linear gradients of glycerol from 15 to 35% (v/v) in buffer 1 containing 400 mM KCl and 0.01% Triton X-100. Centrifugation was performed for 14 h at 216000*g* in a Hitachi RPS65T roter at 4 °C. Fractions were collected from the bottom of the tube. The active fractions were pooled (fraction VI) and dialyzed against 100 mM KCl in buffer 3. The dialysate was stored at −20 or −80 °C until use.

Protein Kinase Assay. The assay mixture (50 μL) consisted of 20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 1 μM [γ-^{32}P]ATP (1–2 Ci/mmol), and 1 mg/mL substrate proteins. Incubation was carried out for 30–60 min at 30 °C, and the reaction was stopped by the addition of 1 mL of 20% trichloroacetic acid–0.1 M sodium pyrophosphate. Acid-insoluble materials were collected on a Whatman GF/C glass-fiber filter. The filter was washed 5 times with 3 mL of 20% tri-

[1] Abbreviations: AMP-PCP, 5'-adenylyl methylenediphosphate; AMP-PMP, 5'-adenylyl imidodiphosphate; ATPγS, adenosine 5'-*O*-(3-thiotriphosphate); *m*-AMSA, 4'-(9-acridinylamino)methanesulfon-*m*-anisidide; BSA, bovine serum albumin; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; kDa, kilodalton(s); PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; VP-16, 4'-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside).

chloroacetic acid–0.1 M sodium pyrophosphate and twice with ethanol, and dried. The radioactivity was measured with a liquid scintillation counter.

Purification of Protein Kinase. The glycerol density gradient fractions containing protein kinase activity were pooled and dialyzed against 50 mM KCl in buffer 2. The dialysate was loaded onto a Mono Q column (0.5 × 5 cm) equilibrated with 50 mM KCl in buffer 2 at 0.5 mL/min. The column was washed with 5 bed volumes of the same buffer and eluted with a 20-min linear gradient of KCl from 50 to 500 mM in buffer 2 at 0.5 mL/min. The kinase activity eluted between 350 and 400 mM KCl. The active fractions were pooled and dialyzed against 50 mM KCl in buffer 2. The dialysate was loaded onto a Mono S column (0.5 × 5 cm) equilibrated with 50 mM KCl in buffer 2 at 0.3 mL/min. The column was washed with 5 bed volumes of the same buffer and eluted with a 40-min linear gradient of KCl from 50 to 600 mM in buffer 2 at 0.3 mL/min. The kinase activity eluted between 410 and 460 mM KCl. The active fractions were pooled and dialyzed against 100 mM KCl in buffer 3. The dialysate was stored at −20 °C.

Phosphoamino Acid Analysis. Phosphorylated proteins were separated by electrophoresis in 8% acrylamide gels containing 0.1% SDS. The band corresponding to phosphorylated topoisomerase II was located by autoradiography and excised. The gel slice was soaked in 45% ethanol for 30 min, 10% ethanol for 30 min, and 10% 2-propanol for 30 min and washed with water several times. The gel slice was crushed, and the polypeptides in the gel were hydrolyzed in 6 N HCl for 2 h at 110 °C. The sample was mixed with phosphorylated amino acid standards and analyzed by thin-layer electrophoresis at 1000 V, at pH 3.5 for 45 min (one dimensional), or first at pH 1.9 for 90 min and then at pH 3.5 for 45 min (two dimensional) as described (Cooper et al., 1983). Standards were located by ninhydrin staining, and ^{32}P was visualized by autoradiography.

Treatment of Topoisomerase II with Alkaline Phosphatase. One unit of topoisomerase II (fraction VI) was incubated with 1 unit of immobilized calf intestinal alkaline phosphatase for 30 min at 30 °C in the kinase buffer as described above.

Preparation of Antisera. Purified topoisomerase II (100 μg) was electrophoresed on SDS–polyacrylamide gels. The 167- and 151-kDa bands were excised from the gel. The gel slices were crushed with a Dounce homogenizer and mixed with an equal volume of Freund's complete adjuvant. The emulsified sample was injected subcutaneously into a New Zealand White rabbit. The rabbit was booster-injected at intervals of 1–2 months, and blood was collected from the ear vein 1 week after boosting. Sera were prepared and fractionated by ammonium sulfate precipitation.

Labeling Cells with ^{32}P. Exponentially growing cells were washed once with phosphate-free RPMI 1640 and inoculated into 60-mm plastic dishes at a concentration of 1.5×10^6 cells/mL with 5 mL of the above medium supplemented with 10% dialyzed fetal bovine serum. The cells were incubated for 1 h at 37 °C and then labeled with [^{32}P]orthophosphate (60 μCi/mL) for 2 h at 37 °C.

Immunoprecipitation of ^{32}P-Labeled Topoisomerase II. ^{32}P-Labeled cells (3×10^6) were washed with buffer A, suspended in buffer A containing 0.05% Triton X-100 at a concentration of 2×10^6 cells/mL, and stood at 0 °C for 20 min to be lysed. Nuclei were separated from cytosol by centrifugation for 5 min at 1000*g* and washed with buffer A. Nuclear extracts were prepared by adding buffer B containing 0.4% SDS (1.5×10^6 nuclei/mL) and heating in boiling water for 3 min. The extracts were centrifuged for 10 min at 13000*g*, and the supernatant was recovered. An equal volume of buffer B, $^1/_4$th volume of 10% Triton X-100, and $^1/_{40}$th volume of 0.25 M PMSF were added to the supernatant. Then, 200 μL of nuclear extracts or cytosol was incubated with 10–15 μL of anti-mouse topoisomerase II antiserum for 2–4 h at 4 °C, and subsequently mixed with 5 mg of protein A–Sepharose. The mixture stood at 4 °C for 1–4 h, and the beads were washed several times with buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and 1 mM PMSF and once with water. The proteins adsorbed to the beads were extracted with 40 μL of buffer containing 125 mM Tris-HCl, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 20% glycerol, and 0.02% bromophenol blue by heating at 90 °C for 3 min, and the beads were removed by centrifugation. The supernatant was subjected to electrophoresis on 8% acrylamide gel containing 0.1% SDS. The gel was dried and autoradiographed at −80 °C.

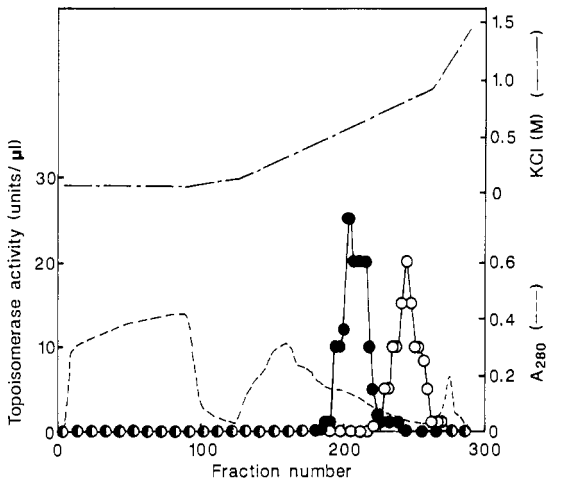

FIGURE 1: Phosphocellulose column chromatography of topoisomerases I and II. Topoisomerase II (●) and topoisomerase I (○) activities were determined by serial dilution. Absorbance at 280 nm (---); concentration of KCl (–·–).

RESULTS

Purification of Mouse FM3A DNA Topoisomerase II. To assay type II topoisomerase activity, we used phage P4 knotted DNA as a substrate because only type II topoisomerase can unknot the DNA (Liu et al., 1981). The extraction of DNA topoisomerase II was performed with buffer containing 0.3 M KCl. Increasing the concentration of KCl up to 1 M resulted in essentially no increase in the yield of topoisomerase II activity.

Topoisomerase II was purified by sequential column chromatography on the first DEAE-cellulose, the second DEAE-cellulose, phosphocellulose, and hydroxyapatite and by following glycerol density gradient centrifugation as described under Materials and Methods. Almost all of the topoisomerase II activity and half of the topoisomerase I activity bound to the second DEAE-cellulose column. As shown in Figure 1, topoisomerase was completely separated from topoisomerase I on the phosphocellulose column. In a glycerol gradient containing 0.4 M KCl, topoisomerase II sedimented at 9.6 S as a single peak (Figure 2A). A similar result was obtained in the presence of 1 M KCl. The specific activity of purified topoisomerase II was 560000 units/mg of protein. A summary of the purification of FM3A topoisomerase II is presented in Table I.

Analysis of Constituting Polypeptides. SDS–polyacrylamide gel electrophoresis of the peak fraction of the glycerol

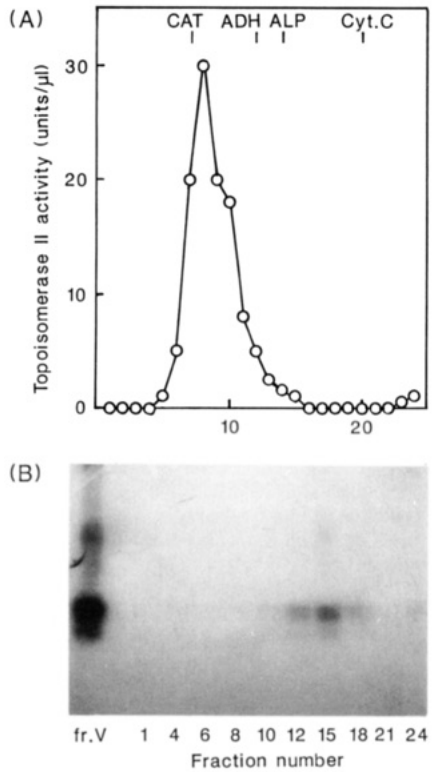

FIGURE 2: Glycerol gradient centrifugation of topoisomerase II. Two hundred microliters of fraction V was layered onto a 15–35% glycerol gradient as described under Materials and Methods. Centrifugation was performed for 14 h at 216000*g* in a Hitachi RPS65T roter at 4 °C. Fractions were collected from the bottom of the tube. Marker proteins used were catalase (CAT, 11.3 S), alcohol dehydrogenase (ADH, 7.4 S), alkaline phosphatase (ALP, 6.1 S), and cytochrome *c* (Cyt. C, 1.8 S). (A) DNA topoisomerase II activity. (B) Protein kinase activity. A portion of each glycerol gradient fraction was assayed for protein kinase activity using core histones as a substrate. After incubation, the reaction mixture was subjected to 15% SDS–polyacrylamide gel electrophoresis followed by autoradiography.

Table I: Purification of DNA Topoisomerase II from FM3A Cells

step	protein (mg)	total units ($\times 10^{-3}$)	sp act. (units/mg)
crude extract	11386	30960	2720
first DEAE-cellulose	9495	36000	3790
second DEAE-cellulose	2010	24000	11940
phosphocellulose	221.5	2840	12820
hydroxyapatite	30.4	2125	70000
glycerol density gradient[a]	2.7	1519	560000

[a] A portion of the hydroxyapatite fraction was subjected to glycerol density gradient centrifugation.

gradient revealed the doublet of 167- and 151-kDa bands (Figure 3A). Topoisomerase II activity correlated very closely with the appearance of the both bands (data not shown).

To ascertain the relationship between the 167- and 151-kDa polypeptides, the cleavage patterns of these polypeptides by *Staphylococcus aureus* V8 protease were compared. The cleavage patterns of the two polypeptides were similar at each concentration of the protease used (Figure 3B).

The treatment of the purified enzyme with alkaline phosphatase before electrophoresis caused no change in the migration of the two bands (data not shown).

Characterization of Enzymatic Properties. The properties of topoisomerase II activity were examined with the peak fraction of the glycerol density gradient. The optimal concentration of KCl for activity was 100 mM. In the presence of 1 mM ATP, the optimal concentrations of Mg^{2+} and Mn^{2+} were 10 and 1 mM, respectively. The maximal activity ob-

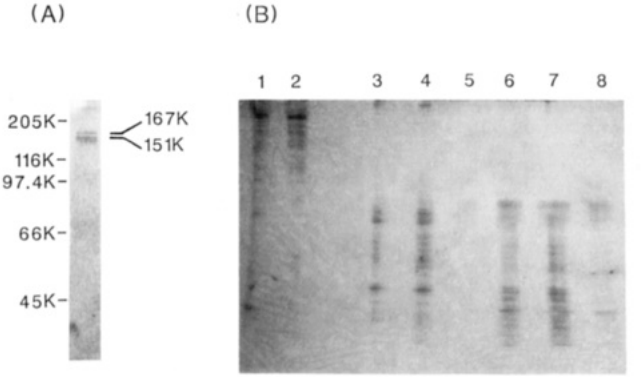

FIGURE 3: Partial proteolytic analysis of 167- and 151-kDa polypeptides with *S. aureus* V8 protease. (A) Four micrograms of the peak fraction of the glycerol density gradient was electrophoresed on an 8% polyacrylamide gel containing 0.1% SDS. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. (B) 167- and 151-kDa bands were excised from the gel, and each gel slice was loaded onto a 3% polyacrylamide stacking gel underlaid by a 15% polyacrylamide gel containing 0.1% SDS. Polypeptides in the slices were proteolyzed in the wells of the second SDS–polyacrylamide gel with various amounts of V8 protease as described under Materials and Methods, electrophoresed, and silver stained. The concentrations of the protease were 0 (lanes 1 and 2), 10 ng (lanes 3–5), and 100 ng (lanes 6–8) per slot. Lanes 1, 3, and 6 and lanes 2, 4, and 7 show cleavage patterns of 167- and 151-kDa polypeptides, respectively. Lanes 5 and 8 were protease only.

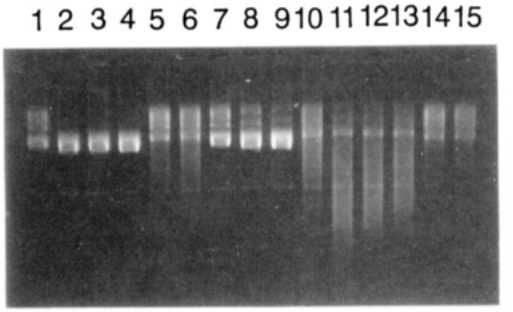

FIGURE 4: Effect of concentration of ATP, dATP, and ITP on topoisomerase II activity. DNA unknotting assay was performed with 1 unit of topoisomerase II under the conditions described under Materials and Methods in the presence of ATP (lanes 1–5), dATP (lanes 6–10), and ITP (lanes 11–15). Concentration of nucleotides were 0.1 (lanes 1, 6, and 11), 0.5 (lanes 2, 7, and 12), 1 (lanes 3, 8, and 13), 5 (lanes 4, 9, and 14), and 10 (lanes 5, 10, 15) mM.

Table II: Drug Sensitivity of Topoisomerase II Actvity[a]

inhibitor	concn of 50% inhibn (μM)	inhibitor	concn of 50% inhibn (μM)
m-AMSA	13	oxolinic acid	1600
VP-16	99	camptothecin	520
novobiocin	100	actinomycin D	130
coumermycin	4.2	ethidium bromide	1.3
nalidixic acid	5200		

[a] DNA relaxation assays were performed with 1 unit of topoisomerase II under the conditions described under Materials and Methods in the presence of the indicated reagents.

tained with Mn^{2+} was about half of that with Mg^{2+}, and the range of effective concentrations of Mn^{2+} was narrow; 0.2 and 2 mM were not effective. At concentrations between 1 and 20 mM, Ca^{2+} was not effective. In the presence of 10 mM Mg^{2+}, the optimal concentration of ATP was 0.5–5 mM (Figure 4). dATP could be substituted for ATP, although the maximal activity obtained with dATP was slightly lower than that with ATP. Low but appreciable activity was detected with ITP at 5 and 10 mM. No topoisomerase II activity

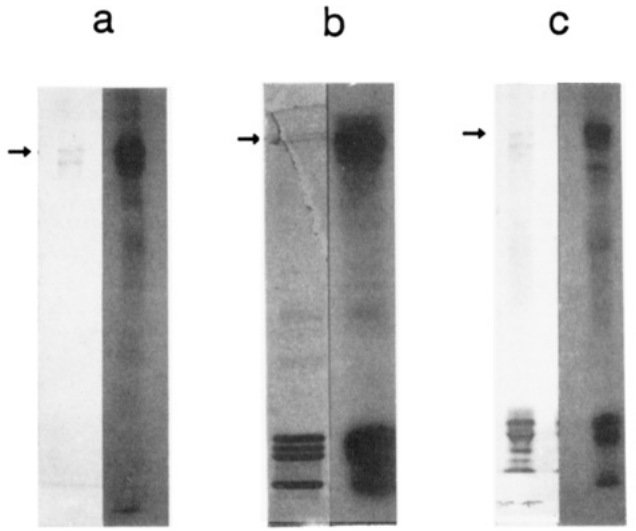

FIGURE 5: Detection of protein kinase activity in fraction V. 2.5 μg of the hydroxyapatite fraction (fraction V) was assayed for protein kinase activity in the presence or absence of exogenously added substrates. Reaction products were electrophoresed on a 10% (a and c) or 15% (b) SDS–polyacrylamide gel. Gels were stained with Coomassie Brilliant Blue (each left lane), and phosphorylated proteins were visualized by autoradiography (each right lane). Arrows indicate the location of topoisomerase II. (a) Fraction V alone; (b) fraction V plus 20 μg of core histones; (c) fraction V plus 10 μg of casein

was observed in the presence of GTP, CTP, UTP, or dTTP. The ATP analogues (AMP-PCP, AMP-PNP, and ATPγS), ADP, and AMP were also not effective.

Table II presents the sensitivity of mouse topoisomerase II to various inhibitors. The assay to measure the relaxation of supercoiled pBR322 DNA was performed in the presence of various concentrations of the inhibitors. FM3A topoisomerase II was highly sensitive to *m*-AMSA, coumermycin, and ethidium bromide. The concentrations required for 50% inhibition were 13, 4.2, and 1.3 μM for *m*-AMSA, coumermycin, and ethidium bromide, respectively. The enzyme was also sensitive to VP-16. Nalidixic acid and oxolinic acid, which are specific inhibitors for *Escherichia coli* DNA gyrase, were inhibitory only when they werre added at high concentrations. Camptothecin inhibited topoisomerase II at a much higher concentration than that required to inhibit topoisomerase I. FM3A topoisomerase I was inhibited by the drug by 60% at 29 μM and almost completely at 290 μM, but only 50% of inhibition was observed at 520 μM with topoisomerase II.

Detection of Protein Kinase Activity in the Partially Purified Topoisomerase II Fraction. It has been reported that a protein kinase activity is tightly associated with the purified *Drosophila* topoisomerase II (Sander et al., 1984). Then, the association of protein kinase activity with mouse topoisomerase II was examined with the hydroxyapatite column fraction. A protein kinase activity was detected in the hydroxyapatite fraction, and topoisomerase II was phosphorylated by the kinase (Figure 5a). This protein kinase also phosphorylated exogenously added core histones and casein (Figure 5b,c). However, the protein kinase phosphorylated topoisomerase II more efficiently than these exogenously added substrates (compare Coomassie Brilliant Blue staining with autoradiography).

Although a protein kinase activity was detected in the hydroxyapatite column fraction, the protein kinase sedimented at 4–5 S, separating from topoisomerase II activity in the glycerol gradient (Figure 2B).

Characterization of Protein Kinase Activity. The protein kinase separated from topoisomerase II by glycerol gradient

Table III: Effect of Various Treatments on Protein Kinase Activity[a]

addition or omission	% of control	addition or omission	% of control
none	100	+heparin, 10 ng/mL	106.9
$-Mg^{2+}$	3.2	+heparin, 100 ng/mL	71.0
$-Mg^{2+} + Ca^{2+}$ [b]	50.0	+heparin, 1 μg/mL	51.7
$-Mg^{2+} + Mn^{2+}$	27.4	+GTP, 20 μM	93.3
$-Mg^{2+} + Zn^{2+}$	1.9	+GTP, 100 μM	88.7
+10 μM cAMP	104.1	+GTP, 500 μM	83.7
+10 μM cGMP	112.2		

[a] Protein kinase activity (10 ng of Mono S fraction) was measured with phosvitin as a substrate. [b] The concentration of divalent cations was 10 mM.

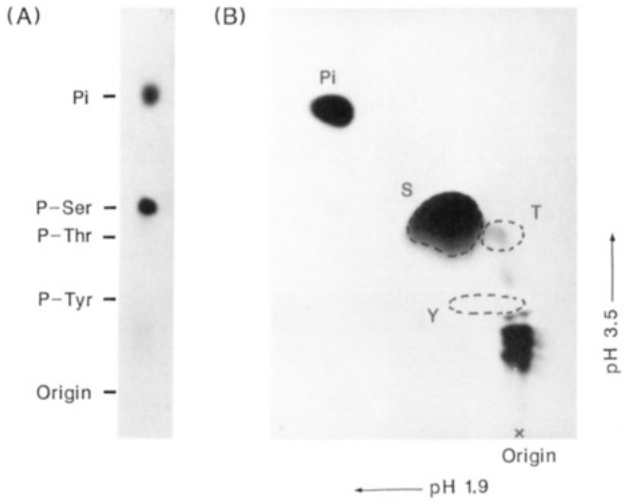

FIGURE 6: Phosphoamino acid analysis of ^{32}P-labeled topoisomerase II. Phosphorylated topoisomerase II was hydrolyzed in 6 N HCl at 110 °C for 2 h. The hydrolysate was mixed with phosphorylated amino acid standards (P-Ser, P-Thr, P-Tyr) and analyzed by thin-layer electrophoresis as described under Materials and Methods. Autoradiograms of the thin-layer plates are shown. (A) One-dimensional electrophoresis. (B) Two-dimensional electrophoresis. Dashed circles indicate the positions of the standards: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine; Pi, inorganic phosphate.

centrifugation was further purified by chromatography on Mono Q and Mono S columns. The protein kinase activity was characterized with this Mono S fraction (Table III). The kinase required a divalent cation for activity. Mg^{2+} was the preferred cation. Ca^{2+} and Mn^{2+} could substituted for Mg^{2+}, but they supported the activity less efficiently, resulting in 50% and 27% of maximal activity, respectively. Essentially no activity was observed with Zn^{2+}. In the presence of 10 mM $MgCl_2$, Ca^{2+} did not stimulated the kinase activity at concentrations of 10 μM–1 mM. cAMP or cGMP was not stimulatory. Inhibition by heparin was observed with the protein kinase, but the concentration required for 50% inhibition of the kinase activity was much higher than that for previously reported casein kinase II (Hathaway et al., 1980; Glover et al., 1983; Padmanabha et al., 1987). Little inhibition was observed with nonradioactive GTP. Phosphoamino acid analysis revealed that the aminoacyl residue phosphorylated by the kinase was serine (Figure 6).

Modulation of Topoisomerase II Activity by Phosphorylation. The effect of phosphorylation on the activity of topoisomerase II was examined. The kinase used in these experiments was the Mono S fraction, which contained no topoisomerase I and II activities.

The purified topoisomerase II was first incubated with agarose bead conjugated alkaline phosphatase or agarose beads (mock incubation), and after removal of the agarose beads, the enzyme was further incubated in the presence or absence

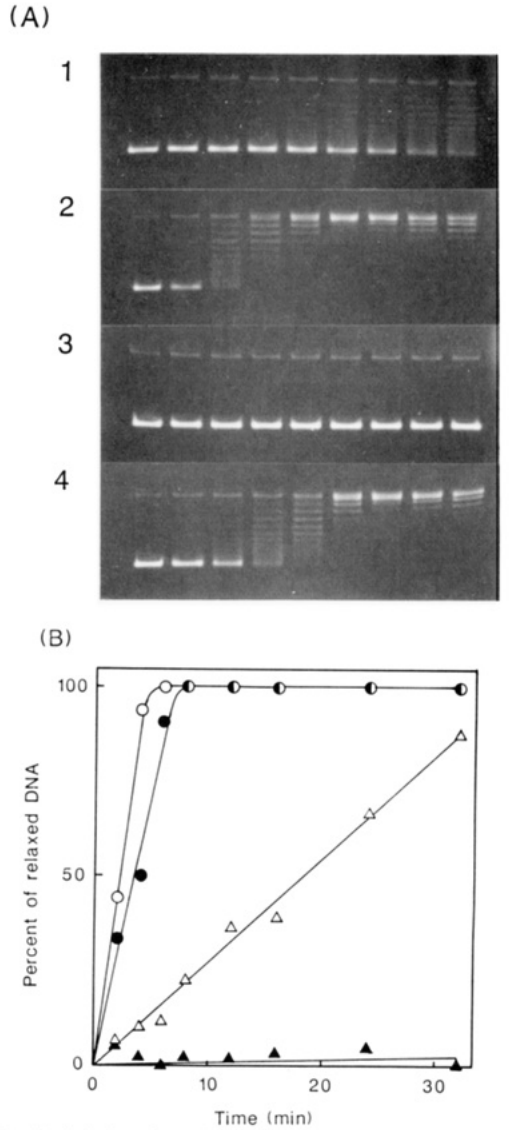

FIGURE 7: Modulation of topoisomerase II activity by phosphorylation. Topoisomerase II (1 unit) was incubated with agarose–alkaline phosphatase (1 unit) or agarose (mock incubation) for 30 min at 30 °C and then centrifuged at 10000 rpm for 1 min. The supernatant was incubated with or without the protein kinase (1.6 ng) for 30 min at 30 °C. After treatment, topoisomerase activity was assayed by using pBR322 DNA as a substrate, and the reaction was stopped at the indicated time. (A) Agarose gel electrophoresis of reaction products. Reactions were performed for 0, 2, 4, 6, 8, 12, 16, 24, and 32 min (left to right lanes). (B) Topoisomerase activity determined by densitometric tracing. Panel 1 or (△), topoisomerase II which was mock-treated and then incubated in the absence of the kinase; panel 2 or (○), mock-treated and then incubated in the presence of the kinase; panel 3 or (▲), treated with alkaline phosphatase and then incubated in the absence of the kinase; panel 4 or (●), treated with alkaline phosphatase and then incubated in the presence of the kinase.

of the protein kinase. The initial velocity of the mock-treated topoisomerase II activity was increased 8.6-fold by the treatment of the protein kinase (Figure 7A, panels 1 and 2, and Figure 7B). The treatment of the enzyme with alkaline phosphatase abolished the enzyme activity almost completely (panels 3 and B). The treatment of the dephosphorylated topoisomerase II with the protein kinase restored topoisomerase activity (panels 4 and B). On the contrary, successive treatment of the protein kinase and alkaline phosphatase abolished topoisomerase II activity (data not shown).

Phosphorylation of Topoisomerase II in FM3A Cells. As described above, topoisomerase II activity is modulated by phosphorylation. To ascertain whether topoisomerase II is

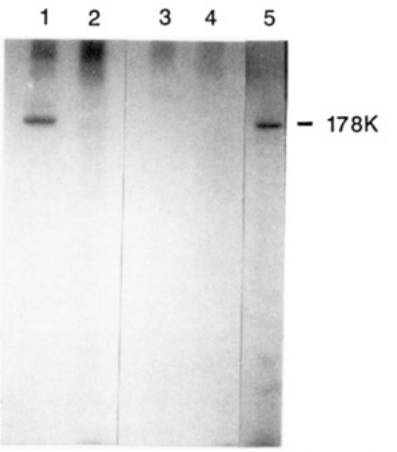

FIGURE 8: Immunoprecipitation of ^{32}P-labeled proteins with anti-topoisomerase II antiserum. Immunoprecipitation was performed as described under Materials and Methods. Lane 1, the sample precipitated from nuclear extracts with antiserum; lane 2, from cytosol with antiserum; lane 3, from nuclear extracts with preimmune serum; lane 4, from cytosol with preimmune serum; lane 5, same as lane 1 except that the sample was treated with 50 μg/mL DNase I in buffer containing 10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, and 50 mM NaCl for 20 min at 30 °C.

phosphorylated in the cells, FM3A cells were labeled with [^{32}P]orthophosphate, and topoisomerase II was immunoprecipitated with anti-topoisomerase II antiserum, which recognized only one polypeptide with a molecular mass of 178 kDa in crude nuclear extracts of FM3A cells. As shown in Figure 8, radioactivity was detected at 178 kDa with the nuclear extract but not with the cytosol (lanes 1 and 2). No radioactive band was observed at 178 kDa with the nuclear extract and cytosol by the immunoprecipitation with preimmune serum (lanes 3 and 4). The smear near the top of the gel disappeared by treatment of DNase I (lane 5).

Discussion

We have purified a type II topoisomerase from mouse FM3A cells using an assay to unknot phage P4 DNA in order to avoid the influence of topoisomerase I activity (Liu et al., 1981). The enzymatic properties of the purified mouse topoisomerase II are similar to those of other eukaryotic topoisomerases II (Miller et al., 1981; Osheroff et al., 1983; Benedetti et al., 1983; Goto et al., 1984; Schomburg & Grosse, 1986).

SDS–PAGE of the purified mouse topoisomerase II revealed two polypeptide bands with apparent molecular masses of 167 and 151 kDa. Previous studies have also reported the existence of multiple bands in the purified topoisomerase II fractions. Halligan et al. (1985) reported two bands of molecular masses of 140 and 125 kDa, and Schomburg and Grosse (1986) showed 175- and 150-kDa bands with calf thymus topoisomerase II. Several bands were observed in the purified *Drosophila* topoisomerase II fractions (Sander & Hsieh, 1983; Shelton et al., 1983). All these authors indicated that the existence of multiple bands was caused by proteolysis.

In contrast, Drake et al. (1987) isolated from mouse leukemia cells two distinct forms of topoisomerase II with molecular masses of 180 and 170 kDa, whose V8 protease cleavage patterns differed from each other. In addition, specific polyclonal antibodies to either polypeptide did not cross-react. The V8 protease cleavage patterns shown in Figure 3, however, indicate that two polypeptides of mouse FM3A topoisomerase II are closely related. A polyclonal antibody against the mixture of the two polypeptides recognized a 178-kDa polypeptide in crude extracts of FM3A cells.

Thus, the 167- and 151-kDa polypeptides of FM3A topoisomerase II are most likely to be proteolytic products of the 178-kDa form found by the immunoblotting of cell lysates. It is not clear whether this 178-kDa form corresponds to the 180-kDa or 170kDa form of leukemia cells. In addition, the reason why only Drake et al. have detected two distinct forms of topoisomerase II is not obvious at present.

The activity of topoisomerase II has been reported to fluctuate markedly during the transition of the cell cycle (Miskimins et al., 1983; Duguet et al., 1983; Taudou et al., 1984; Sullivan et al., 1986; Zwelling et al., 1987; Markovits et al., 1987; Nelson et al., 1987; Chow & Ross, 1987). Although nothing is known about the mechanism to modulate intracellular topoisomerase II activity, the following observations seem to indicate that phosphorylation plays a role in regulating topoisomerase II activity.

First, topoisomerase II was phosphorylated by casein kinase II and protein kinase C (Ackerman et al., 1985; Sahyoun et al., 1986), and FM3A topoisomerase II was phosphorylated by an unidentified protein kinase (Figure 5). The phosphorylation of topoisomerase II by these protein kinases resulted in the increase in enzyme activity.

Second, the treatment of purified topoisomerase II with alkaline phosphatase abolished enzyme activity as indicated in Figure 7.

Third, *Drosophila* topoisomerase II exists in the cells as a phosphoprotein (Ackerman et al., 1988). We have also observed that mouse topoisomerase II is phosphorylated in the cells (Figure 8). In addition, preliminary experiments with Swiss 3T3 cells have indicated that the phosphorylation of topoisomerase II increases during the transition from G1 to S phase.

The protein kinase that is responsible for the phosphorylation of topoisomerase II in the cells has not been definitely confirmed yet. Rottmann et al. (1987) proposed that topoisomerase II is phosphorylated primarily by protein kinase C in sponge cells, because levels of phosphorylation increased following the treatment of cells with 12-*O*-tetradecanoylphorbol-13-acetate. Recently, Ackerman et al. (1988) have provided several lines of evidence indicating that the phosphorylation of topoisomerase II in *Drosophila* Kc cells is catalyzed by casein kinase II.

The protein kinase activity detected in this study is cyclic nucleotide and Ca^{2+} independent. The degree of sensitivity to heparin and the insensitivity to GTP differentiate this activity from the previously reported casein kinase II. The enzymatic properties (Table III) of this kinase are very similar to those of the kinase which is reported to be tightly associated with *Drosophila* topoisomerase II. Thus, further studies are required to confirm the protein kinase that is responsible for the phosphorylation of topoisomerase II in mouse cells.

Acknowledgments

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Registry No. *m*-AMSA, 51264-14-3; VP-16, 33419-42-0; Ca, 7440-70-2; Mg, 7439-95-4; Mn, 7439-96-5; DNA topoisomerase, 80449-01-0; protein kinase, 9026-43-1; novobiocin, 303-81-1; coumermycin, 4434-05-3; nalidixic acid, 389-08-2; oxolinic acid, 14698-29-4; camptothecin, 7689-03-4; actinomycin D, 50-76-0; ethidium bromide, 1239-45-8.

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Thermotropic Properties of Saturated Mixed Acyl Phosphatidylethanolamines†

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ABSTRACT: The mixed acyl phosphatidylethanolamine (PE) series C(18)C(18)PE, C(18)C(16)PE, C(18)C(14)PE, C(18)C(12)PE, and C(18)C(10)PE has been prepared from the corresponding phosphatidylcholines by phospholipase D mediated transphosphatidylation. The thermotropic behavior of unhydrated and hydrated preparations of these PEs has been investigated by differential scanning calorimetry and ^{31}P NMR spectroscopy. Unhydrated preparations of the PEs undergo crystalline to liquid-crystalline transitions (T_{m+h}), which correspond to the simultaneous hydration and acyl chain melting of poorly hydrated crystalline samples. Hydrated preparations of the PEs undergo gel to liquid-crystalline transitions (T_m) when scanned immediately subsequent to cooling from temperatures above their respective T_{m+h}s. Multilamellar bilayers of C(18)C(18)PE, C(18)C(16)PE, and C(18)C(14)PE pack without significant interdigitation of the phospholipid acyl chains across the bilayer center in the gel phase. C(18)C(10)PE multilamellar preparations exhibit a mixed-interdigitated gel phase packing of the phospholipid acyl chains. Hydrated bilayers of C(18)C(12)PE adopt a mixed-interdigitated gel phase packing at temperatures below 13.9 °C. Between 13.9 °C and the gel to liquid-crystalline transition temperature of 36.9 °C, the C(18)C(12)PE bilayer adopts a noninterdigitated gel phase packing. The metastable behavior of fully hydrated and partially hydrated preparations of the mixed acyl PEs has been investigated. Bilayers of C(18)C(18)PE, C(18)C(16)PE, and C(18)C(14)PE exhibited little or no tendency toward regeneration of the crystalline phase. In contrast, bilayers of C(18)C(12)PE and C(18)C(10)PE exhibited a metastability of the liquid-crystalline phase in the temperature interval between T_m and T_{m+h}, which can allow for the regeneration of the crystalline phase under certain conditions. Bilayers of C(18)C(12)PE exhibited an additional metastability of the noninterdigitated gel phase.

Phosphatidylethanolamines (PEs)[1] represent the second largest phospholipid component of mammalian plasma membranes after the PCs (Rouser et al., 1986). In addition, PE has been shown to be the major membrane phospholipid in many prokaryotes (Overath & Thilo, 1978). For these reasons, the membrane properties of naturally occurring and synthetic PEs are a topic of considerable interest in membrane research.

Until a few years ago, it was thought that the thermotropic properties of saturated chain PEs were simpler than those of their PC counterparts However, recent studies have revealed a complex polymorphism for these PEs. When saturated symmetric chain PEs with chain lengths from C(11) to C(20) are suspended in buffer at room temperature, a crystalline subphase (L_c) results. When these phases are heated, crystalline to liquid-crystalline ($L_c \rightarrow L_\alpha$) phase transitions are observed. These transitions are assigned to the simultaneous

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[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; C(x)C(y)PC(PE), a saturated acyl phosphatidylcholine (phosphatidylethanolamine) with x carbons in the *sn*-1 acyl chain and y carbons in the *sn*-2 acyl chain; DSC, differential scanning calorimetry; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; CSA, chemical shift anisotropy; T_m, gel to liquid-crystalline transition temperature; T_{m+h}, crystalline to liquid-crystalline transition temperature; L_β, gel bilayer phase (untilted chains); L_c, crystalline bilayer phase; L_α, liquid-crystalline bilayer phase.

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