Protein Kinase C Modulates the Catalytic Activity of Topoisomerase II by Enhancing the Rate of ATP Hydrolysis: Evidence for a Common Mechanism of Regulation by Phosphorylation†

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ABSTRACT: The catalytic activity of topoisomerase II is stimulated ~2–3-fold following phosphorylation by either casein kinase II or protein kinase C. A previous study [Corbett, A. H., DeVore, R. F., & Osheroff, N. (1992) *J. Biol. Chem. 267*, 20513–20518] demonstrated that casein kinase II regulates the activity of topoisomerase II by specifically enhancing the ability of the enzyme to hydrolyze its ATP cofactor. To determine whether other protein kinases use a similar mechanism to activate the enzyme, the effects of protein kinase C mediated phosphorylation on the individual steps of the topoisomerase II catalytic cycle were assessed. Modification stimulated rates of enzyme-mediated ATP hydrolysis ~2.7-fold, but had no effect on any reaction that preceded this step, including enzyme·DNA binding, pre- or poststrand passage DNA cleavage/religation, or the double-stranded DNA strand passage event. Furthermore, the activation of ATP hydrolysis was reversed following treatment of phosphorylated topoisomerase II with alkaline phosphatase. As determined by partial proteolytic mapping, the site(s) of protein kinase C modification was (were) localized to the 350 amino acid C-terminal regulatory domain of topoisomerase II within ~50 amino acids of the site(s) phosphorylated by casein kinase II. Finally, while protein kinase C and casein kinase II were able to modify the enzyme simultaneously, rates of ATP hydrolysis for doubly-modified topoisomerase II were comparable to those observed for the enzyme following phosphorylation by either individual kinase. On the basis of these findings, it is concluded that protein kinase C and casein kinase II regulate the catalytic function of topoisomerase II by a common mechanism and that the ATP hydrolysis step of the enzyme's catalytic cycle is the control point for activation of topoisomerase II by these two disparate protein kinases.

The topological state of DNA is regulated in vivo by enzymes known as topoisomerases (Wang, 1985). Type II topoisomerases alter DNA topology by the double-stranded DNA passage reaction (Wang, 1985; Osheroff, 1989; Sutcliffe et al., 1989). A number of recent mechanistic studies on the enzyme [reviewed in Osheroff et al. (1991)] have broken this reaction into six discrete steps. These are shown in Figure 1 which depicts one round of enzyme catalysis. Briefly, the steps that comprise the catalytic cycle of topoisomerase II are (1) enzyme·DNA binding, (2) prestrand passage DNA cleavage/religation, (3) DNA strand passage (which requires ATP binding), (4) poststrand passage DNA cleavage/religation, (5) ATP hydrolysis, and (6) enzyme turnover.

Despite the fact that topoisomerase II is required for a number of fundamental DNA processes (DiNardo et al., 1984; Goto & Wang, 1984; Uemura & Yanagida, 1984; Holm et al., 1985; Wang, 1985; Osheroff, 1989; Sutcliffe et al., 1989), little is understood concerning the mechanism by which its catalytic cycle is regulated in the eukaryotic cell. The only known physiological effector of the type II enzyme is serine/threonine phosphorylation (Rottman et al., 1987; Ackerman et al., 1988; Heck et al., 1989; Kroll & Rowe, 1991; Takano et al., 1991; Cardenas et al., 1992; Saijo et al., 1992).

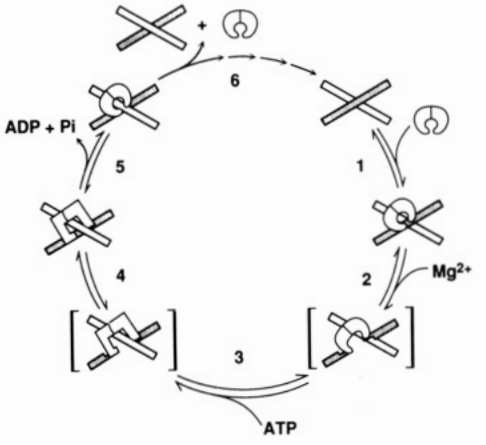

FIGURE 1: Catalytic cycle of topoisomerase II (Osheroff et al., 1991). The homodimeric enzyme is represented by the croissant-shaped structure, and the two DNA helices are represented by the open and stippled bars. The change in enzyme structure that takes place following step 3 represents the structural transition that occurs upon ATP binding (Lindsley & Wang, 1991). The double-stranded DNA passage reaction of topoisomerase II is made up of at least six steps: (1) enzyme·DNA binding; (2) prestrand passage DNA cleavage/religation; (3) double-stranded DNA passage; (4) poststrand passage DNA cleavage/religation; (5) ATP hydrolysis; (6) enzyme turnover. Transient enzyme–DNA cleavage complexes are shown in brackets.

Topoisomerase II appears to be phosphorylated throughout the cell cycle; however, levels of modification are highest at G_2/M (Heck et al., 1989; Cardenas et al., 1992; Saijo et al., 1992). The increase in phosphorylation that occurs over the cell cycle coincides with a 2–3-fold increase in enzyme activity (Chow & Ross, 1987; Heck et al., 1988).

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Although topoisomerase II can be phosphorylated by a number of serine/threonine kinases in vitro (Sander et al., 1984; Ackerman et al., 1985; Sahyoun et al., 1986; Rottman et al., 1987; Saijo et al., 1990; Cardenas et al., 1992), only casein kinase II and protein kinase C have been implicated in the cellular modification of the enzyme. Genetic, metabolic labeling, and peptide mapping studies in *Drosophila* embryonic cells (Ackerman et al., 1988) and in budding yeast (Cardenas et al., 1992) indicate that casein kinase II phosphorylates topoisomerase II in vivo. Metabolic labeling studies in sponge (Rottman et al., 1987; Müller et al., 1990) and human systems (Constantinou et al., 1989; Matthes et al., 1990; Song et al., 1992) treated with a variety of protein kinase C activators or inhibitors suggest that this latter kinase also modifies the enzyme in vivo.

In vitro, topoisomerase II is a high-affinity substrate for both casein kinase II and protein kinase C (Ackerman et al., 1985; Sahyoun et al., 1986). Moreover, maximal modification by either kinase (approximately one to two serines per homodimer of topoisomerase II) stimulates the overall catalytic activity of the enzyme ~2–3-fold (Ackerman et al., 1985; Sahyoun et al., 1986; Rottman et al., 1987; Corbett et al., 1992; DeVore et al., 1992). Since casein kinase II modifies serine/threonine residues flanked by acidic residues and protein kinase C requires basic amino acids adjacent to its phosphorylation site (Kemp & Pearson, 1990; Kennelly & Krebs, 1991), it is likely that these two kinases phosphorylate different aminoacyl residues in topoisomerase II. This prediction was confirmed by peptide mapping studies (Cardenas et al., 1992) and by experiments demonstrating that casein kinase II and protein kinase C could modify the enzyme simultaneously (DeVore et al., 1992). Although the phosphorylation of topoisomerase II by these two kinases was additive, the effect on overall rates of catalysis was not (DeVore et al., 1992). The activity of doubly-modified topoisomerase II was comparable to that observed following phosphorylation by either individual kinase. This latter finding suggests that casein kinase II and protein kinase C employ the same mechanism to regulate the activity of topoisomerase II. In this regard, a previous study demonstrated that casein kinase II enhances enzyme activity by specifically stimulating the ability of topoisomerase II to hydrolyze its ATP cofactor (Corbett et al., 1992).

In order to compare the mechanisms by which casein kinase II and protein kinase C modulate enzyme function, the effect of protein kinase C mediated phosphorylation on each step of the topoisomerase II catalytic cycle was analyzed. As found for casein kinase II (Corbett et al., 1992), stimulation of enzyme activity by protein kinase C correlated with an increased rate of topoisomerase II catalyzed ATP hydrolysis. Moreover, modification did not affect any step of the enzyme's catalytic cycle that preceded the ATPase step. Therefore, it appears that these two disparate protein kinases modulate the overall catalytic activity of topoisomerase II by a common mechanism and that the ATP hydrolysis step is the control point for regulation by phosphorylation.

EXPERIMENTAL PROCEDURES

Topoisomerase II was purified from the nuclei of *Drosophila melanogaster* Kc tissue culture cells or 6–12-h-old embryos by the procedure of Shelton et al. (1983). Negatively supercoiled pBR322 plasmid DNA was isolated from *Escherichia coli* DH1 by a Triton X-100 lysis procedure followed by double banding in cesium chloride (Sambrook et al., 1989). Protein kinase C was purchased from Lipidex as a mixture of isozymes and was stored at –80 °C in 20 mM Tris-HCl, pH 7.5, 400 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.05% Triton X-100, and 10% glycerol. Casein kinase II was purified from the cytosol of *Drosophila* Kc tissue culture cells by the protocol of Glover et al. (1983) and was stored at 20 °C in 30 mM Tris-HCl, pH 8.0, 1 M NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 20% glycerol. Tris, ethidium bromide, agarose-linked alkaline phosphatase, adenyl-5′-yl β,γ-imidodiphosphate [APP(NH)P],[1] *Staphylococcus aureus* V8 protease, and porcine pancreatic elastase were obtained from Sigma, α-chymotrypsin and trypsin (TPCK-treated) were from Worthington, ATP was from LKB Pharmacia Biotechnology, SDS and proteinase K were from Merck, Ecolume aqueous counting scintillant was from ICN, [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham, and histone H1 was from Boehringer Mannheim. All other chemicals were analytical reagent grade.

Phosphorylation of Topoisomerase II. Phosphorylation reactions were carried out as previously described (Corbett et al., 1992; DeVore et al., 1992). Reactions contained 0.8 ng/μL protein kinase C or 1.4–2.8 nM casein kinase II or a combination of both kinases at these concentrations. Protein kinase C phosphorylation reactions contained 400 nM topoisomerase II, 100 μg/mL phosphatidylserine, and 30 μM ATP in 50 mM Tris-HCl, pH 8.0, 25 mM NaCl, 7 mM $MgCl_2$, and 100 μM $CaCl_2$. Casein kinase II phosphorylation reactions contained 400 nM topoisomerase II and 30 μM ATP in 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 2.5% glycerol. Phosphorylation reactions were incubated at 25 °C for 12 min before topoisomerase II assays were carried out. Mock phosphorylation reactions were carried out in the presence of the appropriate kinase storage buffer. Unless stated otherwise, all activity assays described below compared topoisomerase II that had been mock-phosphorylated to enzyme that had been phosphorylated by protein kinase C. Levels of phosphorylation were confirmed periodically by including [γ-^{32}P]-ATP (2 μCi/reaction) in reaction mixtures followed by precipitation with trichloroacetic acid and scintillation counting (Ackerman et al., 1985). For all experiments, topoisomerase II was modified to its maximal extent.

Dephosphorylation of Topoisomerase II. Protein kinase C modified topoisomerase II was dephosphorylated utilizing insoluble agarose-linked alkaline phosphatase as previously described (Ackerman et al., 1989).

Catalytic Catenation of pBR322 Plasmid DNA by Topoisomerase II. Catenation assays were carried out as previously described (Shelton et al., 1983). Reactions contained 2.7 nM topoisomerase II and 5 nM pBR322 plasmid DNA in a total of 20 μL of assay buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, and 2.5% glycerol) that contained 5 mM $MgCl_2$, 15 μg/mL histone H1, and 1 mM ATP. Reaction mixtures were incubated for various times up to 7.5 min and were stopped by the addition of 3 μL of 0.77% SDS/77 mM EDTA. Two microliters of loading buffer (60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol FF, and 10 mM Tris-HCl, pH 7.9) was added, and products were resolved by electrophoresis in 1% agarose (MCB) gels in 100 mM Tris–borate, pH 8.3, and 2 mM EDTA at 5 V/cm for ~3 h. Following electrophoresis, gels were stained in a 1 μg/mL solution of ethidium bromide. DNA bands were visualized by transillumination with ultraviolet light (300 nm) and photographed through Kodak no. 23A

[1] Abbreviation: APP(NH)P, adenyl-5′-yl β,γ-imidodiphosphate.

and no. 12 filters with Polaroid type 665 positive/negative film. Levels of DNA catenation were monitored by quantitating the accumulation of high molecular mass catenenes (which remained at the gel origin) or the loss of supercoiled plasmid DNA. This was accomplished by scanning negatives with an E-C Apparatus Model EC910 scanning densitometer in conjunction with Hoefer GS-370 data system software. Under the conditions employed, the intensity of the bands in the negative was proportional to the amount of DNA present.

Topoisomerase II·DNA Binding. Topoisomerase II·DNA binding was analyzed by an electrophoretic mobility shift assay (Osheroff & Brutlag, 1983; Osheroff, 1986). Reactions contained 0–30 nM topoisomerase II and 5 nM pBR322 DNA in 20 μL of assay buffer that contained 5 mM $MgCl_2$. Binding was for 6 min at 30 °C and was stopped by the addition of 2.5 μL of loading buffer. Reaction products were separated by agarose gel electrophoresis and quantitated as described above.

Topoisomerase II Mediated Pre- and Poststrand Passage DNA Cleavage. DNA cleavage assays were performed as previously described (Osheroff & Zechiedrich, 1987; Robinson & Osheroff, 1991). All assays contained 100 nM topoisomerase II and 5 nM pBR322 in 20 μL of assay buffer. Reactions that monitored the DNA cleavage/religation equilibrium established prior to strand passage included either 5 mM $MgCl_2$ or 5 mM $CaCl_2$. Assays that monitored the DNA cleavage/religation equilibrium established after strand passage contained 5 mM $MgCl_2$ and 1 mM APP(NH)P. Reaction mixtures were incubated at 30 °C for 6 min. Cleavage products were trapped (Gale & Osheroff, 1990; Anderson et al., 1991) by the addition of 2 μL of 10% SDS following by 1.5 μL of 250 mM EDTA. Topoisomerase II was digested by incubation with 2 μL of 0.8 mg/mL proteinase K for 45 min at 45 °C. Final products were mixed with 2.5 μL of loading buffer and subjected to electrophoresis in 1% agarose gels in 40 mM Tris–acetate, pH 8.3, and 2 mM EDTA. Gels were stained and visualized as described above. Double-stranded DNA cleavage was quantitated by monitoring the accumulation of the linear DNA band as described above.

Topoisomerase II Mediated Pre- and Poststrand Passage DNA Religation. DNA religation reactions were carried out as previously described (Robinson & Osheroff, 1991). All reactions contained 100 nM topoisomerase II and 5 nM pBR322 in 20 μL of assay buffer. Initial DNA cleavage/religation equilibria were established at 30 °C for 6 min. As described in the preceding section, prestrand passage DNA cleavage/religation equilibria were established in the presence of 5 mM $CaCl_2$, and poststrand passage DNA cleavage/religation equilibria were established in the presence of 5 mM $MgCl_2$ and 1 mM APP(NH)P. DNA religation was initiated by rapidly shifting samples from 30 to 55 °C (Robinson & Osheroff, 1991). Reactions were terminated by the addition of SDS (1% final concentration) at various time points up to 30 s. Samples were treated with EDTA and proteinase K followed by agarose gel electrophoresis in 40 mM Tris–acetate as described above. The apparent first-order rate of DNA religation was determined by quantitating the loss of the linear DNA band.

Nonturnover DNA Catenation Mediated by Topoisomerase II. Nonturnover DNA catenation assays were carried out as previously described (Corbett et al., 1991). Assays included 100 nM topoisomerase II and 5 nM pBR322 in 20 μL of assay buffer that contained 5 mM $MgCl_2$, 6 μg/mL histone H1, and 1 mM APP(NH)P. Reaction mixtures were incubated at room temperature for various times up to 20 s and stopped by the addition of EDTA (25 mM final concentration). Topoisomerase II was incubated with SDS and proteinase K as described above, and final products were resolved by agarose gel electrophoresis in 100 mM Tris–borate, pH 8.3, and 2 mM EDTA. DNA strand passage was quantitated as described for catalytic catenation.

Hydrolysis of ATP by Topoisomerase II. ATPase assays were carried out as described (Osheroff et al., 1983). Reactions contained 10 nM topoisomerase II that had been phosphorylated by protein kinase C, casein kinase II, or both kinases as appropriate and 250 nM negatively supercoiled pBR322 plasmid DNA. Reactions were carried out in 20 μL of assay buffer containing 5 mM $MgCl_2$ and 1 mM [γ-^{32}P]ATP (3 μCi/reaction). Mixtures were incubated at 30 °C. Two-microliter samples were removed at various intervals up to 20 min, spotted onto thin-layer cellulose plates impregnated with poly(ethylenimine) (Polygram CEL 300 PEI, Brinkmann), and chromatographed in freshly made 400 mM NH_4HCO_3. Reaction products were visualized by autoradiography with Kodak XAR film. Radioactive areas corresponding to inorganic phosphate released by ATP hydrolysis were cut out of the chromatograms and quantitated by liquid scintillation counting. Ten milliliters of Ecolume aqueous counting scintillant was added, and radioactivity was determined with a Beckman LS-7500 liquid scintillation counter.

Partial Proteolytic Mapping of Topoisomerase II. Topoisomerase II was phosphorylated with casein kinase II, protein kinase C, or a combination of the two kinases. Samples were subjected to electrophoresis in 7% polyacrylamide gels as described by Laemmli (1970). Bands corresponding to phosphorylated topoisomerase II were located by autoradiography and excised. Labeled enzyme was extracted by electroelution in 25 mM Tris base, 192 mM glycine, and 0.1% SDS for 8 h at 23 °C using a Bio-Rad Model 422 electroeluter. Eluates were pooled, dialyzed overnight against 100 mM ammonium bicarbonate, and concentrated by centrifugation under reduced pressure. Samples were dissolved in proteolysis buffer (125 mM Tris-HCl, pH 6.8, 0.5% SDS, 10% glycerol, and 0.001% bromphenol blue) containing 0.5 mg/mL bovine serum albumin and digested by the method of Cleveland et al. (1977) as modified by Ackerman et al. (1988). Proteolytic enzymes were freshly dissolved in proteolysis buffer and added immediately to phosphorylated topoisomerase II samples. Final enzyme concentrations were α-chymotrypsin, 120 μg/mL; elastase, 60 μg/mL; V8 protease, 20 μg/mL; and trypsin, 120 μg/mL. Mixtures were incubated at 37 °C for 30 min. Following the addition of β-mercaptoethanol (10% final concentration) and SDS (2% final concentration), samples were heated to 95 °C for 5 min and subjected to electrophoresis in 12.5% polyacrylamide gels by the procedure of Laemmli (1970). Phosphorylated peptides were visualized by autoradiography as described above.

RESULTS

Effects of Protein Kinase C Mediated Phosphorylation on the Overall Catalytic Activity of Topoisomerase II. Previous studies indicate that phosphorylation of topoisomerase II by protein kinase C enhances the ability of the type II enzyme to unknot or relax DNA by ~2–3-fold (Sahyoun et al., 1986; Rottman et al., 1987; DeVore et al., 1992). In order to confirm these findings and as a prelude to more detailed mechanistic studies, the effects of protein kinase C mediated phosphorylation on topoisomerase II catalyzed DNA catenation were determined. Results are shown in Figure 2. Maximal phosphorylation (approximately two phosphates per enzyme

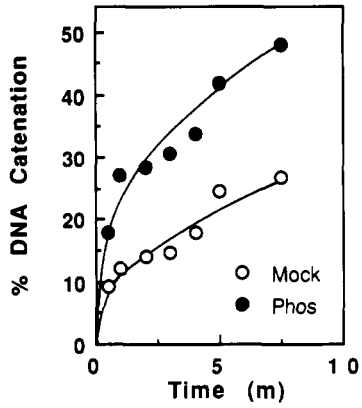

FIGURE 2: Effect of protein kinase C mediated phosphorylation on the catalytic activity of topoisomerase II. A catalytic DNA catenation assay was employed. Results compare the activities of mock-phosphorylated (○) and phosphorylated (●) topoisomerase II and are typical of three independent experiments.

homodimer) stimulated the catalytic activity of topoisomerase II by ~2.5-fold.

When *Drosophila* topoisomerase II prepared by the method of Shelton et al. (1983) is treated extensively with alkaline phosphatase, catalytic activity drops by only 30% (Ackerman et al., 1985). Since this loss of activity is minimal, untreated enzyme (i.e., topoisomerase II that was treated with neither alkaline phosphatase nor protein kinase C) was used for most of the experiments described in this paper. As a control, however, topoisomerase II was always mock-phosphorylated in reactions that contained protein kinase storage buffer prior to its inclusion in activity assays.

Finally, while some preparations of topoisomerase II apparently contain an associated low-level protein kinase activity (Sander et al., 1984; Saijo et al., 1990), the enzyme preparation (Shelton et al., 1983) used in this study contains no such activity (Ackerman et al., 1985; Ackerman and Osheroff, unpublished results). Therefore, the phosphorylation observed in the present study was due solely to the actions of protein kinase C.

Stimulation of Topoisomerase II Catalyzed ATP Hydrolysis by Protein Kinase C Mediated Phosphorylation. While the binding of ATP to topoisomerase II induces the DNA strand passage event (Osheroff et al., 1983; Osheroff, 1986) (step 3 in Figure 1), hydrolysis of this high-energy cofactor is a prerequisite for dissociation of the enzyme from its DNA substrate and the initiation of a new round of catalysis (i.e., enzyme turnover, step 6) (Osheroff, 1986). A previous study demonstrated that casein kinase II regulates the catalytic activity of topoisomerase II by specifically enhancing the rate of enzyme-mediated ATP hydrolysis (Corbett et al., 1992). To determine whether protein kinase C also stimulates this critical reaction step, the ATPase activity of phosphorylated topoisomerase II was compared to that of the mock-phosphorylated enzyme. As shown in Figure 3, protein kinase C mediated modification increased the rate of ATP hydrolysis ~2.7-fold. This increase correlates with the stimulation of overall catalytic activity observed following phosphorylation by protein kinase C.

To demonstrate that the stimulation of ATPase activity resulted from phosphorylation, modified topoisomerase II was incubated with alkaline phosphatase. This treatment removed ≥90% of the radioactive phosphate that had been incorporated by protein kinase C. As expected, dephosphorylation diminished the rate of topoisomerase II mediated ATP hydrolysis nearly to that of the mock-phosphorylated enzyme (Figure 3).

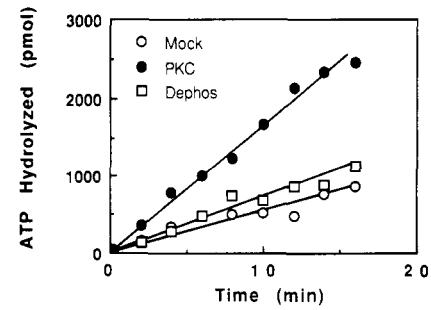

FIGURE 3: Stimulation of topoisomerase II catalyzed ATP hydrolysis. The data are plotted as pmol of ATP hydrolyzed/20-μL reaction versus time. Results compare the activities of mock-phosphorylated (○) and phosphorylated (●) topoisomerase II as well as that of phosphorylated topoisomerase II that subsequently was dephosphorylated by treatment with alkaline phosphatase (□). Hydrolysis rates were assigned using a linear regression least-squares analysis. Data are typical of two independent experiments.

Effects of Protein Kinase C Mediated Phosphorylation on Reaction Steps of the Topoisomerase II Catalytic Cycle That Precede ATP Hydrolysis. The correlation between protein kinase C mediated stimulation of ATPase activity and enhancement of overall catalysis suggests that this kinase regulates topoisomerase II function by the same mechanism as does casein kinase II. However, in order to prove that ATP hydrolysis is the control point for regulation of enzyme activity by phosphorylation, it is necessary to show that protein kinase C does not stimulate enzyme function at any reaction step that precedes ATPase in the catalytic cycle of topoisomerase II. Therefore, the effects of protein kinase C mediated phosphorylation on enzyme·DNA binding (step 1 in Figure 1), prestrand passage DNA cleavage/religation (step 2), DNA strand passage (step 3), and poststrand passage DNA cleavage/religation (step 4) were examined. Results of these studies are shown in Figure 4. As described in the following sections, modification had no effect on any of these reaction steps.

Step 1: Enzyme·DNA Binding. The initial step of the topoisomerase II catalytic cycle is the recognition and binding of DNA by the enzyme (Osheroff et al., 1991). An electrophoretic mobility shift assay was employed to examine the effect of protein kinase C mediated phosphorylation on the affinity of topoisomerase II for negatively supercoiled DNA. As seen in Figure 4A, modification did not alter enzyme·DNA binding.

Previous studies indicate that topoisomerase II can discern the topological state of its nucleic acid substrate and binds to negatively supercoiled DNA ~3–4-fold more efficiently than to nicked molecules (Osheroff & Brutlag, 1983; Osheroff, 1986). As determined by a series of competition binding experiments (not shown), phosphorylation had no effect on the ability of the enzyme to distinguish different topological states of DNA.

Step 2: Prestrand Passage DNA Cleavage/Religation. Following DNA binding, topoisomerase II establishes a prestrand passage DNA cleavage/religation equilibrium (Osheroff et al., 1991). Levels of double-stranded DNA cleavage at equilibrium can be determined by monitoring the conversion of covalently-closed circular nucleic acids to linear molecules (Figure 4B, inset). Modification had little effect on the DNA cleavage/religation equilibrium of topoisomerase II. As determined in four independent experiments, levels of double-stranded DNA cleavage generated by the phosphorylated enzyme were 94 ± 5% of those generated by mock-phosphorylated topoisomerase II.

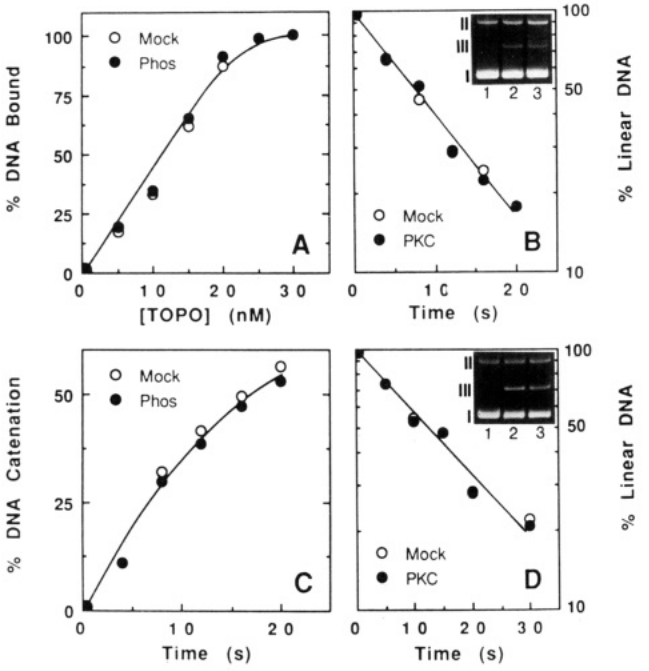

FIGURE 4: Effects of protein kinase C mediated phosphorylation on the steps of the topoisomerase II catalytic cycle that precede ATP hydrolysis. All experiments compare the activities of mock-phosphorylated (○) and phosphorylated (●) topoisomerase II and are the averages of two independent experiments. Panel A shows results of enzyme·DNA binding (step 1) assays. Panel B shows results of prestrand passage DNA religation assays. The agarose gel in the inset shows levels of prestrand passage DNA cleavage (step 2) mediated by topoisomerase II at equilibrium. Lane 1, no topoisomerase II; lanes 2 and 3, DNA cleavage mediated by phosphorylated and mock-phosphorylated enzyme, respectively. The positions of negatively supercoiled (form I), nicked (form II), and linear (form III) DNA are indicated. Panel C shows results of nonturnover DNA catenation assays. This assay monitors enzyme-mediated reaction steps up to and including the DNA strand passage event (step 3). Panel D shows results of poststrand passage DNA religation (step 4) assays. The agarose gel shown in the inset was run in the presence of ethidium bromide and shows levels of poststrand passage DNA cleavage mediated by topoisomerase II. Lane assignments are as in panel B (inset). The average standard errors for the data shown in panels A, B, C, and D are less than 6%, 2%, 5%, and 2%, respectively.

Since levels of nucleic acid breakage generated in the above assay reflect the ratio of the forward rate of DNA cleavage versus the reverse rate of DNA religation, it is possible that phosphorylation could have a pronounced, but coordinated effect on both reaction rates without disturbing the overall DNA cleavage/religation equilibrium. Therefore, in order to conclude that phosphorylation does not stimulate the rate of DNA cleavage, it is necessary to uncouple the equilibrium. The only assay systems currently available that uncouple DNA cleavage and religation are those that monitor the ability of topoisomerase II to rejoin nucleic acid breaks (Osheroff & Zechiedrich, 1987; Hsiang & Liu, 1989; Hsiang et al., 1989; Zechiedrich et al., 1989; Robinson & Osheroff, 1991). The effects of modification on the apparent first-order rate of enzyme-mediated DNA religation are shown in Figure 4B. Identical rates were observed for the protein kinase C and mock-phosphorylated enzymes. Taken together with the results of equilibrium assays (Figure 4B inset), this latter finding provides strong evidence that phosphorylation does not enhance the rate of DNA cleavage mediated by topoisomerase II prior to strand passage.

Step 3: *Double-Stranded DNA Passage.* While ATP binding induces topoisomerase II to pass an intact double helix through the transient double-stranded DNA break generated in the preceding reaction step, hydrolysis is necessary for enzyme recycling (Osheroff et al., 1983; Osheroff, 1986). Consequently, in the presence of nonhydrolyzable ATP analogs, each topoisomerase II homodimer is able to carry out only a single round of DNA strand passage. Therefore, by employing a nonturnover DNA catenation assay in which ATP is replaced by APP(NH)P, it is possible to monitor the rate of enzyme catalysis through the DNA strand passage event. Since phosphorylation affected neither the enzyme·DNA binding nor the DNA cleavage/religation step of the enzyme's catalytic cycle, changes in the rate of nonturnover DNA catenation should be indicative of alterations in the rate of DNA strand passage. As seen in Figure 4C, the rate of nonturnover DNA catenation by protein kinase C modified topoisomerase II was the same as that of the mock-phosphorylated enzyme. Thus, it appears that phosphorylation does not alter the ability of topoisomerase II to carry out its DNA strand passage event.

Step 4: *Poststrand Passage DNA Cleavage/Religation.* Following DNA strand passage, topoisomerase II once again establishes a DNA cleavage/religation equilibrium (Osheroff et al., 1991). This poststrand passage equilibrium can be isolated from prestrand passage events by the inclusion of APP(NH)P in assays (Osheroff, 1986; Robinson & Osheroff, 1991). Although the kinetic pathways utilized by the enzyme to establish the pre- and poststrand passage DNA cleavage/religation equilibria appear to be identical, covalent enzyme·DNA cleavage complexes generated after strand passage are 4–5-fold more stable than their prestrand passage counterparts (Robinson & Osheroff, 1991).

Phosphorylation had little effect on levels of poststrand passage nucleic acid breakage (Figure 4D inset). As determined in four independent experiments, the level of DNA cleavage generated by modified topoisomerase II was 94 ± 5% of that observed for the mock-phosphorylated enzyme. Moreover, apparent first-order rates of poststrand passage DNA religation were unaltered by modification (Figure 4D). Therefore, protein kinase C does not appear to alter the rate of either poststrand passage DNA cleavage or religation.

The studies described above provide strong evidence that protein kinase C does not enhance the rate of enzyme catalysis at any reaction step that precedes ATP hydrolysis. Taken together with the results of ATPase assays, it is concluded that ATP hydrolysis is the control point for regulation of topoisomerase II activity by protein kinase C mediated phosphorylation.

ATPase Activity of Topoisomerase II following Simultaneous Phosphorylation by Casein Kinase II and Protein Kinase C. Casein kinase II and protein kinase C can phosphorylate topoisomerase II simultaneously in an additive manner (DeVore et al., 1992). However, the overall catalytic activity of the doubly-modified enzyme is no higher than that of topoisomerase II that had been phosphorylated by either kinase alone (DeVore et al., 1992). Results in the preceding sections indicate that protein kinase C activates topoisomerase II by the same mechanism as previously described for casein kinase II (Corbett et al., 1992) (i.e., by stimulating rates of ATP hydrolysis). If this conclusion is correct, simultaneous phoshorylation should not increase rates of ATP hydrolysis beyond that of topoisomerase II modified by either protein kinase C or casein kinase II. As shown in Figure 5, this was indeed the case. Similar ATPase activity was observed for the enzyme following phosphorylation by protein kinase C (which incorporated ~1.7 phosphates/topoisomerase II homodimer), casein kinase II (~1.9 phosphates/homodimer),

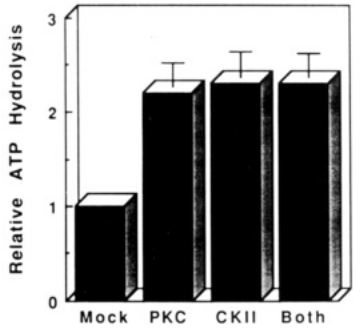

FIGURE 5: ATPase activity of topoisomerase II phosphorylated simultaneously by protein kinase C and casein kinase II. Results compare the relative level of ATP hydrolyzed by mock-phoshorylated topoisomerase II (Mock) to levels observed for enzyme phosphorylated with protein kinase C (PKC), casein kinase II (CKII), or both kinases simultaneously (Both). The level of ATP hydrolyzed by mock-phosphorylated topoisomerase II was set to 1. Data are the averages of three independent assays. Standard deviations are represented by the error bars.

or both kinases (~3.8 phosphates/homodimer). This finding supports the conclusion that these two protein kinases share a common mechanism for the regulation of topoisomerase II.

Partial Proteolytic Mapping of Phosphorylated Topoisomerase II. Peptide mapping and deletion studies indicate that sites of casein kinase II mediated phosphorylation are located in the C-terminal domain of topoisomerase II (Cardenas et al., 1992). This ~350 amino acid region has no counterpart in the primary structure of prokaryotic type II topoisomerases (Wyckoff et al., 1989) and is believed to have a regulatory function in the eukaryotic enzyme (Cardenas et al., 1992).

Previous peptide mapping (Cardenas et al., 1992) and enzymatic studies (DeVore et al., 1992) demonstrated that sites in topoisomerase II phosphorylated by protein kinase C are distinct from those modified by casein kinase II. In order to characterize relationships between sites modified by these two kinases, topoisomerase II that had been phosphorylated by casein kinase II, protein kinase C, or both kinases was subjected to partial proteolytic mapping. Four proteases with different cleavage specificities were employed for these experiments, including chymotrypsin (specific for aromatic amino acids), elastase (neutral aliphatic amino acids), trypsin (lysine and arginine), and V8 protease (glutamic acid). As seen in Figure 6, maps for any given protease were virtually identical. This indicates that sites phosphorylated by protein kinase C and casein kinase II are in relatively close proximity to one another. The upper limit for the distance between phosphorylation sites can be approximated by determining the molecular mass of the smallest common proteolysis products. Since the smallest common phosphopeptides are ~6–7 kDa in mass, this places the sites of protein kinase C and casein kinase II modification within ~50 amino acids of each other. Furthermore, since sites of casein kinase II phosphorylation have been localized to the final 200 amino acids of topoisomerase II (Cardenas et al., 1992), the above results indicate that the site(s) modified by protein kinase C also is (are) located in the C-terminal regulatory domain of the enzyme.

DISCUSSION

Topoisomerase II exists in the eukaryotic cell as a phosphoprotein (Rottman et al., 1987; Ackerman et al., 1988; Heck et al., 1989; Kroll & Rowe, 1991; Takano et al., 1991; Cardenas et al., 1992; Saijo et al., 1992). Of the numerous

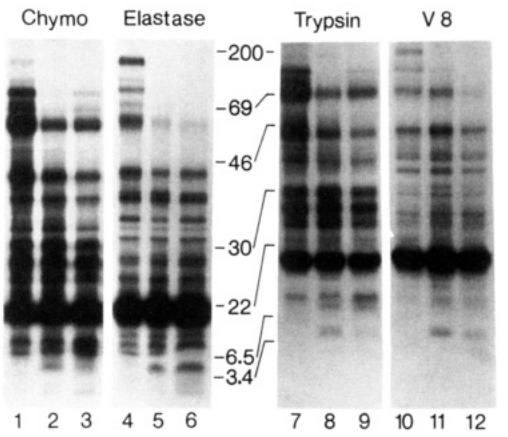

FIGURE 6: Partial proteolytic maps of phosphorylated topoisomerase II. Topoisomerase II was phosphorylated with casein kinase II (lanes 1, 4, 7, and 10), protein kinase C (lanes 2, 5, 8, and 11), or a combination of both kinases (lanes 3, 6, 9, and 12). Phosphorylated topoisomerase II was partially digested with chymotrypsin (lanes 1–3), elastase (lanes 4–6), trypsin (lanes 7–9), or V8 protease (lanes 10–12). Digests were normalized for incorporated radioactivity prior to electrophoresis on 12.5% polyacrylamide gels. Autoradiograms of the gels are shown. Marker proteins and their molecular masses in kilodaltons are myosin, 200; bovine serum albumin, 69; ovalbumin, 46; carbonic anhydrase, 30; trypsin inhibitor, 22; aprotinin, 6.5; and insulin B chain, 3.4.

kinases that have been described to date, only casein kinase II (Ackerman et al., 1988; Cardenas et al., 1992) and protein kinase C (Rottman et al., 1987; Constantinou et al., 1989; Matthes et al., 1990; Müller et al., 1990; Song et al., 1992) have been implicated in the physiological modification of the enzyme. Since both kinases have been found in the nucleus as well as the cytosol (Edelman et al., 1987; Halsey et al., 1987; Thomas et al., 1988; Leach et al., 1989; Stabel & Parker, 1991; Yu et al., 1991; Krek et al., 1992), topoisomerase II should be accessible to either under a variety of growth conditions. In vitro, phosphorylation by casein kinase II (Ackerman et al., 1985; Corbett et al., 1992) or protein kinase C (Sahyoun et al., 1986; Rottman et al., 1987; DeVore et al., 1992) stimulates the overall catalytic activity of topoisomerase II ~2–3-fold. Results of the present work indicate that protein kinase C activates the enzyme by specifically stimulating its ATPase activity. Taken together with a previous mechanistic study on the activation of topoisomerase II by casein kinase II (Corbett et al., 1992), this finding strongly suggests that these two disparate protein kinases regulate the catalytic function of the enzyme by a common mechanism. Moreover, ATP hydrolysis appears to be the control point for the modulation of topoisomerase II activity by phosphorylation.

It is not known whether modification stimulates ATPase activity by increasing the catalytic constant for ATP hydrolysis or by enhancing the binding affinity of the enzyme for its nucleoside triphosphate cofactor. However, the former seems to be the most likely, as a change in the binding constant for ATP probably would alter the rate of the DNA strand passage event mediated by topoisomerase II.

The physiological regulation of DNA topology by topoisomerase II requires the coordinated function of all the steps of the enzyme's catalytic cycle (see Figure 1) (Wang, 1985; Osheroff, 1989; Sutcliffe et al., 1989; Osheroff et al., 1991). In contrast, the reported roles of the type II enzyme in maintaining chromosome structure (Berrios et al., 1985; Earnshaw & Heck, 1985; Earnshaw et al., 1985; Gasser et al., 1986; Gasser & Laemmli, 1986) and mediating nucleic acid recombination (Bae et al., 1988; Dillehay et al., 1989; Sperry et al., 1989; Wang et al., 1990; Gale & Osheroff, 1992) rely

specifically on the enzyme·DNA binding and DNA cleavage/religation reaction steps, respectively. ATP hydrolysis is downstream from these two reaction steps and is a prerequisite for the limiting events of the topoisomerase II catalytic cycle, namely, dissociation of the enzyme from its nucleic acid product and reinitiation of a new round of catalysis (i.e., enzyme turnover) (Osheroff et al., 1991). Therefore, by enhancing enzyme activity specifically at the ATP hydrolysis step, casein kinase II and protein kinase C have the potential to regulate the topoisomerase II mediated interconversion of DNA topoisomers without significantly affecting the other cellular functions of the enzyme.

Sites of casein kinase II (Cardenas et al., 1992) and protein kinase C mediated phosphorylation appear to be located in the C-terminal regulatory domain of topoisomerase II. It is unclear why phosphorylation of different and/or multiple sites in the enzyme should have the same effect on catalytic activity. One possibility is that any modification in the C-terminal domain stabilizes a more active conformation of topoisomerase II. Such a mechanism has been proposed for the regulation of some prokaryotic transcription factors (Bourret et al., 1991). Alternatively, the C-terminal domain may be autoinhibitory in nature, and phosphorylation disrupts its interaction with the ATPase domain of the type II enzyme. A number of protein kinases appear to employ a regulatory mechanism of this latter type (Smith et al., 1990; Soderling, 1990). Ultimately, a crystal structure of topoisomerase II probably will be required before it can be determined whether either of the above two possibilities has credence.

Considerable evidence indicates that casein kinase II and protein kinase C are stimulated by a number of growth-related processes (Krebs et al., 1988; Carpenter & Cohen, 1990; Ralph et al., 1990; Stabel & Parker, 1991; Tuazon & Traugh, 1991; Cadena & Gill, 1992). Moreover, both kinases have been implicated in signal transduction pathways that link mitogenic events at the cell surface to alterations in nucleic acid metabolism in the nucleus. Since topoisomerase II is intimately involved in most DNA processes, it is not surprising that the activity of this enzyme is elevated in rapidly proliferating, mitogen-treated, or virally transformed cells (Duget et al., 1983; Miskimins et al., 1983; Taudou et al., 1984; Heck & Earnshaw, 1986; Bodley et al., 1987; Nelson et al., 1987; Crespi et al., 1988; Genovese et al., 1988; Hsiang et al., 1988; Holden et al., 1990). Increases in topoisomerase II activity parallel increases in enzyme phosphorylation over the cell cycle (Chow & Ross, 1987; Heck et al., 1988, 1989; Cardenas et al., 1992; Saijo et al., 1992). Therefore, it is likely that phosphorylation accounts for at least some of the enhancement of enzyme activity that accompanies cell proliferation. The finding that casein kinase II and protein kinase C both enhance the catalytic activity of topoisomerase II, and do so by the same mechanism, suggests the existence of redundant regulatory pathways that ensure the appropriate activation of topoisomerase II in response to a variety of mitogenic signals.

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