The Phosphoform of the Regulatory Subunit RII of Cyclic AMP-Dependent Protein Kinase Possesses Intrinsic Topoisomerase Activity

Andreas I. Constantinou, Stephen P. Squinto, and Richard A. Jungmann
Department of Molecular Biology and The Cancer Center
Northwestern University Medical School
Chicago, Illinois 60611

Summary

The phosphoform of the type II regulatory subunit (phospho-RII-cAMP) of cAMP-dependent protein kinase from rat liver was found to possess intrinsic topoisomerase activity towards several DNA substrates such as 3x174, pBR322, SV40, and M13. Like the type I topoisomerasers from several eukaryotic cells, phospho-RII-cAMP can relax both positive and negative superhelical turns of 3x174 DNA. Topological isomers with a decreasing number of superhelical turns can be identified as transient products. Conditions under which phospho-RII-cAMP relaxes superhelical 3x174 DNA lead to transient formation of a DNA-phospho-RII-cAMP complex via DNA strand breakage and covalent attachment of the DNA to a tyrosine residue of phospho-RII-cAMP via a phosphodiester bond. The topoisomerase activity of phospho-RII-cAMP depends on the presence of cAMP and is altered by changes in the degree of phosphorylation of RII. Both dephosphorylation and removal of cAMP from phospho-RII-cAMP abolish its topoisomerase activity.

Introduction

Cyclic adenosine 3',5'-monophosphate (cAMP or cyclic AMP) is an important metabolic effector molecule that regulates diverse biochemical events in both prokaryotes and eukaryotes. In prokaryotes, cAMP acts as an allosteric effector that, after interaction with a specific gene activator protein (CAP), binds to specific gene sequences, leading to the transcription of several catabolite-sensitive operons (Peterkosky, 1976; Pastan and Adhya, 1976). In eukaryotes, our understanding of the involvement of cAMP in the control of gene transcription is limited. However, there is evidence that a number of cAMP-induced proteins are regulated at the level of transcription by as yet unknown mechanisms (Maurer, 1981; Lamers et al., 1982; Murdoch et al., 1982; Jungmann et al., 1983; Evans and McKnight, 1984; Hashimoto et al., 1984; Sasaki et al., 1984). So far, cAMP-dependent protein kinase is the only recognized mediator of cAMP action in eukaryotic cells and it has, in fact, been suggested that all of the intracellular effects of cAMP are mediated via cAMP-dependent protein kinase (Kuo and Greengard, 1969).

Cyclic AMP-dependent protein kinase exists as two isoenzymes (types I and II), both of which consist of a tetrameric holoenzyme composed of two regulatory and two catalytic subunits. The two isoenzymes differ in the characteristics of their regulatory subunits (RI and RII, respectively), whereas the catalytic subunit (C) of both isoenzymes appears to be identical in structure and function (Rubin and Rosen, 1973; Krebs and Beavo, 1978; Flood and Corbin, 1982). The physiological significance of protein phosphorylation by the catalytic subunit is well established (Rubin and Rosen, 1973; Krebs and Beavo, 1979; Cohen, 1982; Ingelbritsen and Cohen, 1983), but the only recognized function of the regulatory subunits is the inhibition of catalytic subunit activity (Rubin and Rosen, 1974). There are, however, an increasing number of observations that suggest selective functional roles for the regulatory subunits in addition to their inhibitory effect on the catalytic subunit. These observations include the differential expression of the type I and type II isoforms during development (Jungmann and Russell, 1977) and at specific phases during cell-cycle progression (Costa et al., 1978), the dibutyryl cAMP-mediated modulation of regulatory subunit levels without a concomitant change of catalytic subunit concentration (Prashad et al., 1979; Walter et al., 1979; Liu et al., 1981), and the interaction of the regulatory subunits with a number of cellular proteins (Hathaway et al., 1981; Vallee et al., 1981; Miller et al., 1982; Lohmann et al., 1984).

At the nuclear level, the mechanism of control of gene transcription by cAMP and cAMP-dependent protein kinase remains to be elucidated. It has been proposed that the effects of cAMP on gene transcription involve changes in chromosomal protein phosphorylation by the catalytic subunit, and these phosphorylative modifications have been correlated with altered transcriptional activity (Jungmann and Kranias, 1977; Johnson, 1977; Rosenfeld and Barrieux, 1979). However, an effect of cAMP mediated by the regulatory subunits acting at the gene level must also be seriously considered. There are several experimental findings that seem compatible with a gene-regulatory function of either RI or RII. First, a modulation of the levels of nuclear regulatory subunits RI and RII has been observed at times of increased transcriptional activity (Jungmann et al., 1979; Laks et al., 1981; Squinto et al., 1985). Second, although its functional significance is not known, structural homology exists between RII and the prokaryotic cAMP-catabolite gene activator protein (CAP) (Weber et al., 1982). Third, Wynshaw-Boris et al. (1983) have recently identified a cAMP-responsive sequence of the phospho-enolpyruvate carboxykinase gene and localized it to the 5' end of the gene. However, it remains to be established whether or not the regulatory subunits are capable of a direct functional interaction with cAMP-regulated eukaryotic gene sequences.

In recent years, attention has been focused on enzymes (topoisomerases) that alter the degree of DNA supercoiling (Liu, 1980; Gellert, 1981). Although the biological functions of this group of enzymes appear diverse and are in most cases still unknown, it is interesting to consider that in eukaryotes, structural and functional modifications of
Figure 1. Relaxation of Negatively Supercoiled DNA Templates by Phospho-Rll-cAMP

Phospho-Rll-cAMP (60 ng) was incubated under relaxing conditions with various supercoiled DNA templates (1 μg each), and the DNA products were resolved by agarose gel electrophoresis. The DNA relaxing and agarose gel electrophoresis conditions are described in Experimental Procedures.

(A) Lanes 1 and 2, 4x174 RFI and RFII DNA standards, respectively; lanes 3 through 7, 4x174 RFI DNA incubated in the presence of phospho-Rll-cAMP (lane 3); calf thymus topoisomerase I (20 units; 1 unit catalyzes the conversion of 0.5 μg of 4x174 RFI DNA to the relaxed form in 30 min at 37°C) (lane 4); phospho-Rll-cAMP that had been preincubated for 1 hr at room temperature with a 1:100 dilution of affinity-purified anti-Rll antisum (lane 5); rat liver regulatory subunit RII (60 ng) (lane 6); rat liver catalytic subunit C (60 ng) (lane 7). (B) Lanes 1 and 2, plasmid pBR322 RFI DNA incubated in the absence and presence of phospho-Rll-cAMP. Lanes 3 and 4, SV40 RFI DNA incubated in the absence and presence of phospho-Rll-cAMP. Lanes 5 and 6, M13mp8 RFI DNA incubated in the absence and presence of phospho-Rll-cAMP, supercoiled DNA, 1 rel, closed, circular DNA; II, nicked circular DNA.

In this report, we provide the first evidence for a functional interaction of the phosphoform of the regulatory subunit RII of cAMP-dependent protein kinase with DNA and demonstrate that RII exhibits superhelical DNA relaxing activity similar to eukaryotic type I topoisomerases.

Results

Evidence that Phospho-Rll-cAMP Possesses Topoisomerase Activity

The highly purified phosphoform of the regulatory subunit RII cAMP (phospho-Rll-cAMP) from rat liver is capable of converting superhelical DNA to its relaxed isomer. To demonstrate this, we have chosen experimental conditions that were identified previously to be optimal for the expression of various eukaryotic type I topoisomerases (Keller, 1975; Champoux and McConaughy, 1976; Liu, 1983). Resolution of the superhelical and relaxed DNA isomers by agarose gel electrophoresis provides an efficient method of assessing the relative amounts of the different topological forms of DNA. Figure 1 shows a typical set of DNA relaxing activity experiments using a number of different circular duplex DNA templates. Incubation of negatively supercoiled 4x174 RFI DNA with phospho-Rll-cAMP at a protein/DNA molar ratio of 20:1 resulted in a conversion of the faster-migrating supercoiled form to the slower-migrating relaxed form (Figure 1A, cf. lanes 1 and 3), similar to the action of calf thymus DNA topoisomerase I (Figure 1A, cf. lanes 1 and 4). Preincubation of phospho-Rll-cAMP with a 1:100 dilution of an affinity-purified polyclonal anti-RII antibody abolished the relaxing activity of the regulatory RII subunit (Figure 1A, lane 5). Preincubation of calf thymus DNA topoisomerase I with anti-RII antisum as well as preincubation of either phospho-Rll-cAMP or calf thymus DNA topoisomerase I with preimmune serum (1:100 dilution) did not affect their DNA relaxing activity (data not shown). The regulatory subunit RII and the catalytic subunit C of cAMP-dependent protein kinase were ineffective in relaxing superhelical DNA (Figure 1A, lanes 6 and 7, respectively).

Under identical experimental conditions, phospho-Rll-cAMP catalyzed the conversion of supercoiled pBR322, SV40, and M13 DNAs (Figure 1B, lanes 1, 3, and 5, respectively) to their relaxed isomeric forms (Figure 1B, lanes 2, 4, and 6, respectively).

Addition of increasing amounts of phospho-Rll-cAMP resulted in an increased conversion of supercoiled 4x174 RFI DNA to the relaxed form (Figures 2A and 2B). At a protein/DNA molar ratio of 0.03 (0.42 ng of phospho-Rll-cAMP (7 fmol)/1.0 μg of DNA) and 30 min of incubation at 25°C, we observed a 50% conversion of the supercoiled form to the relaxed isomers (Figure 2A, lane 3 and Figure 2B).

Time Course of Relaxing Activity and Effect of Na₂EDTA

The time course of the relaxing activity of phospho-Rll-cAMP with supercoiled 4x174 RFI as substrate and the effect of Na₂EDTA on the relaxing reaction are shown
Figure 2. Effect of Phospho-Rll-cAMP Concentration on the Relaxation of \( \lambda \) X74 RFI DNA
(A) Phospho-Rll-cAMP was serially diluted and assayed for DNA relaxing activity using 1.0 \( \mu \)g of \( \lambda \) X74 RFI DNA as described in Experimental Procedures. All reactions contained \( \lambda \) X74 DNA with the following amounts of phospho-Rll-cAMP: none (lane 1); 5.4 fmol (lane 2); 7.6 fmol (lane 3); 10.8 fmol (lane 4); 12.9 fmol (lane 5); 13.5 fmol (lane 6); 18.0 fmol (lane 7); 36 fmol (lane 8). (B) Quantitative assessment of the decrease of supercoiled \( \lambda \) X74 RFI DNA as a function of increasing phospho-Rll-cAMP concentration. Lanes 1–8 in A were scanned with a Zeinah soft laser densitometer integrated with a Hewlett-Packard computer model 3390A to calculate automatically the percentage of supercoiled DNA (I) remaining in the reaction mixture.

Figure 3. Time Course of Relaxation of Negatively Supercoiled \( \lambda \) X74 RFI DNA by Phospho-Rll-cAMP in the Absence and Presence of \( Na_2EDTA \)
(A) Five micrograms of \( \lambda \) X74 RFI DNA were incubated with 4 ng of phospho-Rll-cAMP in a total volume of 50 \( \mu \)l and in the absence (lanes 1 through 5) or presence of 10 mM \( Na_2EDTA \) (lanes 6 through 10). Incubations were carried out for 0 min (lanes 1 and 6); 1 min (lanes 2 and 7); 3 min (lanes 3 and 8); 7 min (lanes 4 and 9); and 15 min (lanes 5 and 10). Aliquots containing 58 \( \mu \)g of DNA were electrophoresed in 1% agarose gels using Tris-chloroquine buffer (50 mM Tris-phosphate, pH 7.2; 1 mM \( Na_2EDTA \); and 50 \( \mu \)g/ml of chloroquine phosphate) at 35 V (38 mA) for 19 hr. The chloroquine-containing agarose gels were stained in an aqueous solution of ethidium bromide (1 \( \mu \)g/ml) for 2 hr. The DNA bands were visualized with UV light and photographed as described in Experimental Procedures. (B) Quantitative assessment of relaxed \( \lambda \) X74 (I rel) as a function of incubation time. Lanes 1 through 10 in A were scanned with a Zeinah soft laser densitometer to determine the percentage of relaxed DNA (I rel) formed.

Figure 3. In these experiments, the agarose gels were prepared with chloroquine, an intercalating agent, which allows separation of the nicked form of DNA (II) from the fully-relaxed isomer (I rel) (Shure et al., 1979). At a protein/DNA molar ratio of 0.05, complete relaxation of \( \lambda \) X74 DNA was achieved in less than 7 min (Figure 3A, lane 4 and Figure 3B). In the presence of 10 mM \( Na_2EDTA \), the rate of the relaxing activity of phospho-Rll-cAMP decreased initially but was not abolished (Figure 3B), and complete conversion of supercoiled \( \lambda \) X74 RFI to the relaxed isomer was observed at 15 min in the absence or presence of \( Na_2EDTA \) (Figure 3B). The presence of ATP (1 mM) in the reaction mixture had no stimulatory effect on the topoisomerase activity of phospho-Rll-cAMP (data not shown). The nicked form of DNA remained constant throughout the incubation periods. The high electrophoretic resolution obtained in the presence of chloroquine allowed us to visualize several isomeric intermediates migrating between fully-supercoiled and fully-relaxed DNA (Figure 3A).

Relaxation of Positively Supercoiled DNA
Topoisomerases from eukaryotic sources can remove both negative and positive superhelical turns from DNA (Champoux and Dulbecco, 1972; Keller, 1975). To test whether phospho-Rll-cAMP is capable of relaxing posi-
Figure 4. Relaxation of Positively Supercoiled 4X174 DNA by Phospho-Rli-cAMP and Calf Thymus DNA Topoisomerase I

The phospho-Rli-cAMP was then inactivated by heating at 65°C for 10 min. To generate positive supercoils, ethidium bromide (2 μg/ml) was subsequently added to the relaxed DNA (Wang, 1975). This sample was divided into two equal aliquots. Ninety nanograms of the phospho-Rli-cAMP was added to one aliquot, and 20 units of calf thymus DNA topoisomerase I was added to the other. Incubation was carried out for 30 min at 37°C. After incubation, ethidium bromide was removed by phenol followed by chloroform extraction. Samples containing 0.6 μg of DNA were electroforesed on 0.8% agarose gel as described in Experimental Procedures. The net gain of negative supercoils (I) generated by either phospho-Rli-cAMP (lane 3) or calf thymus DNA topoisomerase I (lane 4) from the positively supercoiled form is shown.

Identification of Phospho-Rli-cAMP–Oligonucleotide Complex

Topoisomerase I has been shown to relax supercoiled DNA that is relaxed covalently closed circular 4X174 DNA generated by incubation of negatively supercoiled DNA with phospho-Rli-cAMP (Figure 4, lanes 1 and 2). The resulting intercalation of the dye with the DNA has been shown to generate positive turns (Wang, 1975). If phospho-Rli-cAMP, like other eukaryotic topoisomerases, can remove these positive turns, the DNA product will show a net gain of negative supercoils after removal of the dye (Osheroff et al., 1983). The negative supercoils can be detected by the increased electrophoretic mobility of the DNA (I) products. Figure 4 shows that both phospho-Rli-cAMP (Figure 4, lane 3) and calf thymus DNA topoisomerase I (Figure 4, lane 4) are capable of relaxing positively supercoiled 4X174 DNA.

Figure 5. Formation of a Covalent Complex between 32P-Labeled 4X174 RFII DNA and Phospho-Rli-cAMP

The phospho-Rli-cAMP–4X174 RFII DNA complex was formed, and DNA was digested with micrococcal nuclease as described in Experimental Procedures.

(A) The reaction products were analyzed in the presence of 5% polyacrylamide gel electrophoresis and absence (■) of phospho-Rli-cAMP were resolved on a Bio-Gel P-60 gel (Bio-Rad) equilibrated in 30 mM potassium phosphate, pH 5.5, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, and 0.15 M KCl. Consecutive fractions of 150 μl were collected, and 15 μl of each fraction was assayed for 32P-radioactivity.

(B) Electrophoretic analysis of reaction products (Laemmli, 1972) separated on Bio-Gel P-60. Lanes 1 through 5, Coomassie blue-stained gels. Lane 1, pyruvate kinase (M, 58,000); glucose dehydrogenase (M, 53,000), and fumarase (M, 49,000); lane 2, pooled fractions 6–11 (Peak I from Bio-Gel P-60); lane 3, 5 μg of rat liver Rli (M, 55,000); lane 4, 50 μg of micrococcal nuclease; lane 5, pooled fractions 12–16 (Peak II) from Bio-Gel P-60.

(C) Autoradiograph of 32P-labeled proteins resolved by SDS-polyacrylamide gel electrophoresis. Lane 1, 32P-labeled peptide resolved under peak I from the Bio-Gel P-60 column; lanes 2 and 3, B-azido [32P] cAMP photolabeled peptide resolved under peak I. In the experiments shown in lanes 2 and 3, phospho-Rli-cAMP–DNA complex was formed using unlabeled 4X174 RFII DNA. Subsequently, photolabeled labeling of the nonradioactive peptide in peak 1 was carried out as described by us (Leves et al., 1981) in the absence (lane 2) and presence (lane 3) of 10 μM cAMP.

incubated with phospho-Rli-cAMP, allowing transfer of a [32P]phosphate from the DNA to the regulatory subunit. After trapping the 32P-labeled DNA–protein complex by add-

Figure 4. Relaxation of Positively Supercoiled 4X174 DNA by Phospho-Rli-cAMP and Calf Thymus DNA Topoisomerase I

Four micrograms of negatively supercoiled replicative form of 4X174 (I) (lane 1) was first fully relaxed to form I rel with 60 ng of phospho-Rli-cAMP (lane 2). Relaxation was carried out under the conditions described in the legend to Figure 1. The phospho-Rli-cAMP was then inactivated by heating at 65°C for 10 min. To generate positive supercoils, ethidium bromide (2 μg/ml) was subsequently added to the relaxed DNA (Wang, 1975). This sample was divided into two equal aliquots. Sixty nanograms of phospho-Rli-cAMP was added to one aliquot, and 20 units of calf thymus DNA topoisomerase I was added to the other. Incubation was carried out for 30 min at 37°C. After incubation, ethidium bromide was removed by phenol followed by chloroform extraction. Samples containing 0.6 μg of DNA were electrophoresed on 0.8% agarose gel as described in Experimental Procedures. The net gain of negative supercoils (I) generated by either phospho-Rli-cAMP (lane 3) or calf thymus DNA topoisomerase I (lane 4) from the positively supercoiled form is shown.

Identification of Phospho-Rli-cAMP–Oligonucleotide Complex

Topoisomerase I has been shown to relax supercoiled DNA by introducing a single-strand break into the DNA followed by reclosure of the break (Wang, 1981). The DNA strand breakage by eukaryotic topoisomerase I involves formation of a phosphodiester bond between a tyrosine residue of the enzyme and the terminal 3' phosphate at the position of the nick (Champoux, 1981). To test whether phospho-Rli-cAMP forms a similar transient DNA–protein complex, 32P-labeled 4X174 RFII DNA was incubated with phospho-Rli-cAMP, allowing transfer of a [32P]phosphate from the DNA to the regulatory subunit. After trapping the 32P-labeled DNA–protein complex by add-
Figure 6. Identification of \(^{32}P\)-phosphothreonine
Fifty microliters of peak I (see Figure 5A) containing approximately 200,000 cpm of \(^{32}P\) were treated with proteinase K (10 \(\mu\)g/ml) overnight at 37°C. HCl was added to a final concentration of 6 N, and the mixture was heated for 30 min at 100°C. The mixture was hydrolyzed, and the residue was resuspended in 25 \(\mu\)l of water. Standard phosphoserine (P-SER), phosphothreonine (P-THR), and phosphotyrosine (P-TYR) were added. Phosphoamino acids were resolved by high voltage electrophoresis on Whatman 3MM paper in 2.5% formic acid, 78% acetic acid buffer (pH 1.8). Electrophoresis was carried out for 18 hr at 450 V. \(^{32}P\)-radiolabeled phosphoamino acids were visualized by ninhydrin staining and autoradiography.

Figure 7. Effect of cAMP, Phosphorylation, and Reconstituted Holoenzyme on the DNA-Relaxing Activity of the Regulatory Subunit RII
Cyclic AMP-free phospho-RII, dephospho-RII-cAMP, cAMP-free dephospho-RII, as well as reconstituted holoenzyme were prepared and examined for their ability to relax supercoiled \(\phi\)X174 RF1 DNA. Protocols for the removal of bound cAMP, dephosphorylation, rephosphorylation of RII, and reconstitution of holoenzyme from phospho-RII-cAMP and the catalytic subunit are described in Experimental Procedures. Relaxation of 1 \(\mu\)g of \(\phi\)X174 RF1 DNA was carried out as described in Experimental Procedures using 40 ng of each form of RII. (A) Electrophoretic analysis of \(\phi\)X174 DNA on a 0.9% agarose gel; (B) electrophoretic analysis of \(\phi\)X174 DNA on a 1% agarose gel containing chloroquine under the conditions described in the legend to Figure 6. Lanes 1, \(\phi\)X174 RF1 DNA; lanes 2, \(\phi\)X174 RF1 DNA and phospho-RII-cAMP; lanes 3, \(\phi\)X174 RF1 DNA and phospho-RII; lanes 4, \(\phi\)X174 RF1 DNA and phospho-RII that had been preincubated for 60 min at room temperature with 1 mM cAMP prior to the DNA relaxation reaction; Unbound cAMP was removed by dialysis for 4 hr at 4°C against 10 mM Tris-HCl (pH 7.4), 0.1 mM EGTA; lanes 5, \(\phi\)X174 RF1 DNA and dephospho-RII-cAMP; lanes 6, \(\phi\)X174 RF1 DNA and dephospho-RII-cAMP that had been dephosphorylated; lanes 7, \(\phi\)X174 RF1 DNA and dephospho-RII; lanes 8, \(\phi\)X174 RF1 DNA and reconstituted cAMP-dependent protein kinase holoenzyme.

Identification of \(\text{\textit{\textbf{O}}}\)-Phosphothreonine Linkage
An aliquot of the \(^{32}P\)-labeled 56,000 dalton protein resolved under peak I (Figure 5A) was digested with proteinase K. The products of the digestion were subsequently acid hydrolyzed, and the hydrolyzed products were resolved by high voltage paper electrophoresis (Tse et al., 1980). Figure 6 indicates that the \(^{32}P\)-radioactivity comigrated with \(\text{\textit{\textbf{O}}}\)-phosphothreonine, thus identifying tyrosine as the \(^{32}P\)phosphate acceptor amino acid at the point of linkage of RII to the \(^{32}P\)-labeled DNA.

Regulation of the Regulatory Subunit RII Topoisomerase Activity
It is known that in response to increased intracellular...
CAMP levels, the type II CAMP-dependent protein kinase holoenzyme dissociates into catalytic subunit dimers (Flockhart and Corbin, 1982). In the process, each RII binds two molecules of CAMP (Builder et al., 1980). Furthermore, RII can undergo autophosphorylation by the catalytic subunit (Rosen and Erlitchman, 1975; Flockhart et al., 1982). To identify whether CAMP binding and/or phosphorylation of RII are required for the expression of topoisomerase I activity by RII, the following experiments were carried out. In addition to the phosphorylated form of RII-CAMP, the dephosphoforms of RII-CAMP (dephospho-RII-CAMP) and CAMP-free RII (dephospho-RII) were prepared, and their topoisomerase activities were assessed using negatively supercoiled 4X174 RFI DNA. In this series of experiments DNA was separated on agarose gels in the absence (Figure 7A) or presence (Figure 7B) of chloroquine. Both removal of CAMP from phospho-RII-CAMP (cf. lanes 2 and 3 in Figures 7A and 7B) as well as dephosphorylation of phospho-RII-CAMP (Figures 7A and 7B, cf. lanes 2 and 5) resulted in a complete loss of topoisomerase activity. Addition of CAMP to phospho-RII (Figures 7A and 7B, cf. lanes 3 and 4) and rephosphorylation of dephospho-RII-CAMP with catalytic subunit (Figures 7A and 7B, cf. lanes 5 and 6) restored the relaxing activity of the regulatory subunit. Dephosphorylated RII from which CAMP had been removed was inactive as topoisomerase (Figures 7A and 7B lane 7). Addition of the catalytic subunit to phospho-RII-CAMP under conditions that cause the reassociation of catalytic and regulatory subunits reconstituting protein kinase holoenzyme (Hofmann, 1980) also abolished the topoisomerase activity of phospho-RII-CAMP (Figures 7A and 7B lane 8).

Discussion

Mechanism of DNA Relaxation by the Regulatory Subunit RII

We have demonstrated that the phosphoform of the regulatory subunit RII of CAMP-dependent protein kinase from rat liver exhibits active DNA relaxing activity towards several DNA templates such as 4X174, pBR322, SV40, and M13. Several criteria indicate that the relaxation of DNA by phospho-RII-CAMP is mechanistically and functionally comparable to other eukaryotic DNA topoisomerases. Characterization of the DNA-relaxing properties indicate that phospho-RII-CAMP is capable of converting the negative as well as the positive superhelical forms of 4X174 DNA to their relaxed forms. Relaxation occurs in the absence of ATP via formation of a transient phospho-RII-CAMP-oligonucleotide complex and covalent attachment of the DNA to a tyrosine residue of the regulatory subunit probably through a phosphodiester bond. Phospho-RII-CAMP does not alter the concentration of nicked circles (form II) during the process of relaxation of supercoiled DNA, indicating that topoisomerization of DNA by phospho-RII-CAMP occurs by way of a concerted breaking and resealing mechanism. These findings together with the identification of several transient topological isomers (see Figures 1 through 3) indicate that the mechanism of DNA relaxation by phospho-RII-CAMP is similar to the one reported for several other eukaryotic topoisomerases (Wang, 1971; Champoux and Durbec, 1972; Champoux, 1978; Durnford and Champoux, 1978; Champoux, 1981).

Stoichiometry of DNA Relaxation Reaction

The total untwisting activity of phospho-RII-CAMP was determined by carrying out the relaxation at a relatively high DNA concentration. Since 4X174 DNA contains approximately 30 supercoils per molecule and eukaryotic topoisomerase catalyzes the removal of one supercoil per strand passage, about 30 molecules of phospho-RII-CAMP would be required for complete relaxation of one molecule of 4X174 DNA, assuming that each molecule of phospho-RII-CAMP catalyzes a single event. We found that at a phospho-RII-CAMP to 4X174 DNA molar ratio of 0.03, half of the DNA became relaxed within 30 min. Complete relaxation of the DNA template was obtained in less than 7 min at a protein/DNA molar ratio of 0.05. Thus, the specific topoisomerase activity exhibited by phospho-RII-CAMP is comparable with or higher than the activities measured for several previously identified topoisomerases from HeLa cells (Liu, 1983), wheat germ (Dynan et al., 1981), yeast (Durnford and Champoux, 1978), Novikoff hepatoma cells (Durban et al., 1983), mouse L cells (Vosberg and Vinograd, 1976), and rat liver nuclei and mitochondria (Champoux and McConaughy, 1976; Fairfield et al., 1979). The relaxation of negatively supercoiled DNA by phospho-RII-CAMP at this relatively low protein/DNA molar ratio indicates that the regulatory subunit acts catalytically.

Specificity of Topoisomerase Activity of the Regulatory Subunit RII

To rule out the possibility that the topoisomerase activity exhibited by phospho-RII-CAMP is due to a contamination by cellular topoisomerase that was inadvertently copurified, the following evidence can be cited: as judged by analytical SDS-polyacrylamide gel electrophoresis, only a single protein band of M, = 56,000 corresponding to RII was observed, even when a relatively high amount of protein was loaded onto the gel; a highly specific antisemur against rat liver RII inhibited topoisomerase activity of phospho-RII-CAMP. Contamination of the RII antigen used for antisemur production with cellular topoisomerase is unlikely, since evaluation of the specificity of the antisemur by a number of different methods did not reveal any contaminating antigen (Kuettel et al., 1985); the relatively high specific topoisomerase activity exhibited by phospho-RII-CAMP argues against a minor contaminant; cAMP regulation of phospho-RII-CAMP topoisomerase activity is not compatible with a contaminating cellular topoisomerase; and addition of the catalytic subunit to phospho-RII-CAMP under conditions that allow holoenzyme reconstitution abolished the topoisomerase activity of phospho-RII-CAMP.

Regulation of the Topoisomerase Activity of the Regulatory Subunit RII

It is known that the type II CAMP-dependent protein kinase catalyzes the incorporation of phosphate from ATP to ser-
ine 95 of its regulatory subunit RII. This reaction appears to be an autophosphorylation by the catalytic subunit of the type II kinase (Rosen and Erlichman, 1975; Flockhart and Corbin, 1982). Additionally, several other phosphorylation sites in RII can be phosphorylated in vitro and in vivo by casein kinase II and by glycogen synthase kinases 3 and 5 (Carmichael et al., 1982; Hemmings et al., 1982). Autophosphorylation of RII at serine 95 modifies its function in that it facilitates dissociation of the type II holoenzyme by cAMP (Rangel-Alldao and Rosen, 1977).

We have convincingly demonstrated that rat liver regulatory subunit RII expresses topoisomerase activity only when its cAMP binding sites are occupied by cAMP and when it is in a phosphorform. However, our data do not indicate whether one or both of the cAMP binding sites of RII must be occupied and which of the RII phosphorylation sites needs to be modified. Based on our findings, it is conceivable that under in vivo conditions hormonal modulation of intracellular cAMP levels followed by phosphorylation/dephosphorylation of RII are processes instrumental in regulating the topoisomerase activity of RII.

Conclusive evidence exists indicating the importance of protein phosphorylation/dephosphorylation as a major mechanism of regulating enzyme activity in mammalian cells (Krebs and Beavo, 1979; Flockhart and Corbin, 1982). More specifically, Durban et al. (1983) have demonstrated activation of Novikoff hepatoma cell topoisomerase I activity after phosphorylation of a seryl residue by a nuclear protein kinase. Furthermore, Sander et al. (1984) have identified a cyclic nucleotide- and Ca²⁺-independent protein kinase activity that is tightly associated with Drosophila type II topoisomerase and that catalyzes the phosphorylation of the Drosophila topoisomerase. Phosphorylation of the calf thymus topoisomerase I by a tyrosine kinase inactivates the topoisomerase activity (Tse-Dinh et al., 1984). Based on these findings, it will be of interest to determine whether regulation of cellular topoisomerase activity through covalent phosphorylative modification is a general functional phenomenon.

The precise functional and biological significance of the topoisomerase activity exhibited by phospho-RII-cAMP is unknown and remains to be fully investigated, particularly in eukaryotic systems. Expression of DNA-relaxing activity by phospho-RII-cAMP in hormonally- and cAMP-stimulated cells, however, may represent a key step in the mechanism of gene control by cAMP and type II cAMP-dependent protein kinase. Topoisomerase action has been implicated in DNA replication, transcription, condensation/decondensation of DNA and DNA recombination events (Liu, 1980; Geller, 1981). Given the complexity and largely unknown action of cAMP at the nuclear level, further investigations related to the newly identified topoisomerase activity of the regulatory subunit RII appear to be a promising approach to define the role of cAMP in gene expression.

Experimental Procedures

Materials

The regulatory subunits RI and RII as well as the catalytic subunit C of cAMP-dependent protein kinase were purified to homogeneity from rat liver (Dills et al., 1979; Sugden et al., 1978). Antibody against rat RII was raised in rabbits and has been fully characterized as described by us (Kuetter et al., 1985). [β-³²P]ATP (400 Ci/mmol; 1 Ci = 37 Gbq) and [γ-³²P]ATP (3000 Ci/mmol) were purchased from New England Nuclear (Berkeley, CA). [³²P]cAMP (90 Ci/mmol) was from ICN. Potato acid phosphatase was from Sigma Chemical Co. A nick translation kit, calf thymus topoisomerase I, SV40 DNA (>90% supercoiled), and M13mp8 RF DNA were purchased from BRL. [α-³²P]dCTP (500 Ci/mmol) was from Amersham. Plasmid pBR325 RF DNA (85% supercoiled) was from Pharmacia LKB Biocleased. Other chemical reagents were analytical grade.

Assay of Topoisomerase Activity

For the relaxation of negatively supercoiled DNA, reaction mixtures (total volume 20 μl) contained 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, 0.1 mM Na₃EDTA, 30 μg/ml of bovine serum albumin, with protein and DNA in the concentrations given in the figure legends. After incubation for 30 min at 25°C, the reaction was terminated by the addition of 4 μl of 0.1 M Na₃EDTA, 0.5% sodium dodecyl sulfate, 60% sucrose, 0.05% bromophenol blue, and 0.05% xylene cyanol.

Gel Electrophoresis

Aliquots containing 0.6 μg of DNA were analyzed on 0.8% agarose gels. The aliquots were placed into the wells of the agarose gel and electrophoresed at 40 volts (15 mA) for 16 to 18 hr in an electrophoresis buffer containing 50 mM Tris borate (pH 8.3), 1 mM Na₃EDTA (Stein-glanz et al., 1981). Under these experimental conditions the fully-relaxed isomer of DNA (I) comigrates with nicked, circular DNA (II) and is separated from the faster migrating fully-supercoiled isomer of DNA (II). After electrophoresis, the gels were stained for 30 min in 1 μg/ml of ethidium bromide and destained for 30 min in 1 mM MgSO₄. The gels were photographed under UV illumination using type 57 Polaroid film.

Removal of cAMP from Phospho-RII-CAMP

Cyclic AMP was removed from phospho-RII-cAMP by treatment with 8 M urea for 30 min at 0°C (Hofmann, 1980). Urea and cAMP were removed by Sephadex G-25 gel filtration. The pooled protein peak from the G-25 column was dialyzed extensively against 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.2 mM EDTA, 0.2 mM benzamidine, 15 mM 2-mercaptoethanol.

De-phosphorylation and Rephosphorylation of Phospho-RII-CAMP

De-phosphorylation of either phospho-RII-cAMP or phospho-RII (6 μg) was carried out in 50 mM Mes/Tris buffer (pH 6.5), containing 2.0 mM benzamidine and 10 μg of potato acid phosphatase in a total reaction volume of 0.2 ml (Rymond and Hofmann, 1982). The de-phosphorylated subunit was separated from potato acid phosphatase by gel filtration through Bio-Gel P-60 in 10 mM Tris-HCl (pH 7.5) and 1 mM Na₃EDTA.

The de-phosphophorm of RII was rephosphorylated in a reaction mixture containing 0.1 M NaCl, 2 mM MgCl₂, 50 mM Tris-HCl (pH 7.4), 6.7 x 10⁵ M [γ-³²P]ATP, 6 μg of phospho-RII, and 6 μg of catalytic subunit in a total reaction volume of 0.25 ml. The reaction was started by the addition of catalytic subunit. After 10 min at 30°C, the reaction was terminated by placing the mixture on ice. Phospho-RII was purified by affinity chromatography on 8-(8-aminophenylamino)-cAMP-Sepharose 4B as described by Dills et al. (1979).

Holoenzyme Reconstitution

Cyclic AMP-dependent protein kinase holoenzyme was reconstituted following a slightly modified procedure of Hofmann (1980). Accordingly, rat liver phospho-RII-cAMP (25 μg) and catalytic subunit (25 μg) were incubated in a buffer containing 100 mM ATP, 10 mM MgCl₂, and 10 mM Tris-HCl (pH 7.5) for 30 min at 30°C. The reaction products were dialyzed for 24 hr at 4°C against 10 mM Tris-HCl (pH 7.5), 20 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 0.05 mM EGTA, and 4 mM EDTA.

Formation of the Protein-DNA Covalent Complex

[α-³²P]dCTP (5 μg) was nick translated using the BRL nick translation kit and 5 μl of [α-³²P]dATP in a total reaction volume of 220 μl. The [³²P]-labeled nicked [α-³²P]dCTP DNA was denatured for 10 min at
37°C with KOH (50 mM final concentration). The solution was neutralized by the addition of 0.1 volume of 1 M Tris-HCl (pH 8.0) and 1 M HCl (1:1, v/v). The relaxation reaction mixture (623 μl total reaction volume) containing 10 mM Tris-HCl (pH 8.0), 20 mM NaCl, 50 μg/ml bovine serum albumin, 1 μg of denatured 4X174 RFI DNA, and 1.8 μg of phospho-Rsi-CAMP was incubated for 30 min at 37°C. To trap the protein–DNA complex (intermediate), KOH was added again at a final concentration of 50 mM (Tse et al., 1986). The mixture was neutralized with 1 M Tris-HCl (pH 8.0) and 1 M HCl. DNA was digested for 16 hr at 37°C with micrococcal nuclease (30 units/ml) in the presence of 1 mM CaCl₂.

Acknowledgments

This work was funded by the National Institutes of Health grant GM 23895, a grant from an American Heart Association, and by the Research and Education Fund, Northwestern Memorial Hospital. Andrew I. Constantinos and Stephen P. Squinto are recipients of National Research Service Awards from the NIH. We gratefully acknowledge the assistance of Lynda Kern in the purification of rat liver RII and of Lynn Squinto in the photographic work.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 22, 1985; revised June 11, 1985.

References


