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The Identification of Type II DNA Topoisomerase-Associated Protein Kinase Activity from Regenerating Rat Liver

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We have found a protein kinase activity that is tightly associated with type II DNA topoisomerase purified from regenerating rat liver. The activities of protein kinase and topoisomerase II were not separable when the enzyme was subjected to analytical chromatographies (Hydroxyapatite, phosphocellulose, and double strand DNA cellulose) and glycerol gradient sedimentation. The kinase activity from purified rat topoisomerase II was also inactivated by the topoisomerase II inhibitors such as N-ethylmaleimide or novobiocin. The evidences, however, do not rule out a possibility that the kinase activity resides in a polypeptide other than the topoisomerase II protein. The topoisomerase II-associated protein kinase required Mg^{++} for its activity, and this requirement was not substituted by any other mono- or divalent ions. Histone H1 act as a strong stimulator and a good substrate for the kinase activity and other histones and α-casein could not substitute the effect of histone H1.

KEY WORDS: Topoisomerase II, Protein kinase, Regenerating rat liver, Novobiocin, N-ethylmaleimide

DNA topoisomerases are a class of enzyme which catalyze the transient breakages of phosphodiester bond of DNA backbone to permit the crossing of DNA strands. The enzymes are classified as two types; the type I topoisomerase break and rejoin a single DNA strand at a time; the type II topoisomerase break and rejoin both strands of a double stranded DNA in concert (Wang, 1985).

Type II topoisomerases (topo II) have been isolated from various mammalian sources such as HeLa cells (Miller *et al.*, 1981), mouse (Colwill and Sheinin, 1983), and calf thymus (Halligan *et al.*, 1985). The enzyme is essential for the viability of eukaryotic cells and is involved in many aspects of nucleic acid metabolism including DNA replication (Noguchi *et al.*, 1983; Jazwinski and Edelman, 1984), transcription (Rowe *et al.*, 1986), and chromosome segregation (Uemura *et al.*, 1987). At present, very little is understood about the physiological roles of topo II in eukaryotic cells despite of its importance. Their activity is increased over 10-fold during cell proliferation (Fairman and Brutlag, 1987). During the interphase of the cell cycle, the enzyme is known as a major polypeptide component of the nuclear matrix (Earnshaw and Heck, 1985; Earnshaw *et al.*, 1985).

Phosphorylation and dephosphorylation events have long been shown to alter the functions of many enzymes (Cohen, 1976) and structural proteins (Gerace and Blobel, 1980; Ottaviano and Gerace, 1985). In our studies on topo II purified from regenerating rat liver, we found a protein kinase activity which is tightly associated with the topo II activity. It will be of interest to investigate

the kinase activity of eukaryotic topo II as well as regulation of topo II activity by phosphorylation.

MATERIALS AND METHODS

Topoisomerase II and DNAs: Topoisomerase II was purified from regenerating rat liver as described (Manuscript submitted). Supercoiled plasmid DNAs and single strand f1 phage DNA were prepared by the procedures of Sambrook *et al.* (1989).

Protein substrate for kinase assay: Casein and calf thymus histone H1 were obtained from Sigma and dephosphorylated at alkaline pH and high temperature according to Reimann *et al.* (1971). They were then neutralized and stored at -20°C at a concentration of 50 mg/ml.

Assay for topoisomerase II activity: Topological activity of purified topo II was assayed as described by Baldi *et al.* (1980). The enzyme had a specific activity of 1.6×10^5 units/mg, in which 1 unit of activity is defined as the amount of enzyme required to catenate 500 ng of ϕX174 form I DNA under the standard assay condition.

Assay for protein kinase activity:

Acid precipitation: The activity of protein kinase was assayed as described by Sander *et al.* (1984). Reaction mixture (20 μl) consist of 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, and 1 μM γ^{32}P-ATP (0.1 - 0.25 Ci/mmol; 1 Ci = 37 GBq, NEN). Protein substrate was 20 μg of histone H1, 1 mg of casein per ml or as indicated. The reaction was initiated by addition of enzyme (2 μl from chromatography fractions or 2 μg of purified enzyme) and incubated at 30°C for 10 to 20 min. The reaction was terminated by addition of 1ml of 10% trichloroacetic acid (TCA) and 0.1 M sodium pyrophosphate. Precipitates were then collected on Whatman GF/C filter by using an aspirator manifold. Each filter was washed with 25 ml of cold 10% TCA and 0.1 M sodium pyrophosphate, rinsed with ethanol, dried, and assayed for radioactivity.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel analysis: Assay condition was the same as above, except (γ^{32}P)-ATP was at a specific activity of 1.0-2.5 Ci/mmol. The reaction was terminated by the addition of 80 μl of phosphate buffered saline (0.137 M NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.09 mM Na_2HPO_4 and 2.5 mM Na_3EDTA). Samples were then brought to 10-20% TCA and precipitated by centrifugation. Precipitates were washed once with acetone, and SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). Gels were dried and exposed to X-ray film at -70°C.

RESULTS

When purified topo II was incubated in the presence of γ^{32}P-ATP, ^{32}P-label was transferred to a protein species whose mobility in SDS-polyacrylamide gel was identical to that of the purified topo II bands (Fig. 1). Since the ability to catalyze autophosphorylation is the property of

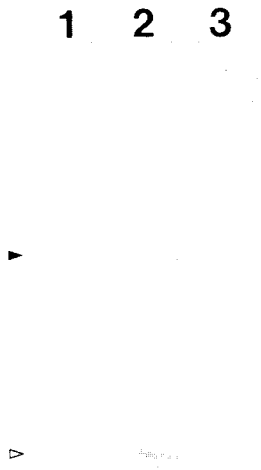

Fig. 1. Protein kinase activity of rat topo II. The kinase activity was assayed as described in Materials and Methods. After 20 min reaction with either histone H1 or α-casein, the reaction products were electrophoresised on a 10% SDS-polyacrylamide gel. Thereafter, the gels were dried and subjected to the autoradiography. Closed triangles indicate the autophosphorylated peptide bands of the purified rat topo II. Open triangle represents the phosphorylated histone H1 band.

several known protein kinases (Katoh and Fujisawa, 1991), it is possible that the phosphorylated bands are autophosphorylated topo II fragments. Since histone phosphorylation has been closely correlated with chromatin condensation (Pardee, 1978) the purified enzyme was tested for its ability to phosphorylate histones. Table 1 and Fig. 1 show that histone H1 is a strong stimulator and also a good substrate for the kinase activity. Other histones and α-casein could not substitute histone H1. During the preparation of topo II, all the active fractions from each purification step (ammonium sulfate fractionation, hydroxyapatite, phosphocellulose, and double-stranded DNA cellulose chromatographies and Sephacryl S-300 gel filtration) were tested for the activity of protein kinase. Very interestingly, the two activities were not separable (data not shown). Fig. 2 shows the results obtained from the parallel assays for topo II (relaxation activity) and protein kinase with the fractions from glycerol gradient (20-50%) sedimentation analysis of purified rat topo II. The two activities also could not be separated. As shown in Table 2, the protein kinase activity required $MgCl_2$, and this requirement was not substituted by another monovalent or divalent ions such as KCl, NaCl, $CaCl_2$, and $MnCl_2$. The single strand DNA (ssDNA) inhibited the kinase activity to about 50%, whereas the double strand DNA (dsDNA) had little

Table 1. Effects of histones and topo II inhibitors on protein kinase activity of purified enzyme.

Assay condition	% Maximal activity
Standard	100.0
No histones	17.0
+ H2A, 1mg/ml	18.3
+ H2B, 1mg/ml	15.7
+ H3, 1mg/ml	16.0
+ H4, 1mg/ml	20.5
N-ethylmaleimide, 5 mM	23.1
Novobiocin, 5 mM	8.8

Acid precipitation assays were carried out as described in the Materials and Methods. The values are the average of several determinations. Additions to assay are indicated. Histone H1 concentration was 1 mg/ml throughout, unless indicated otherwise.

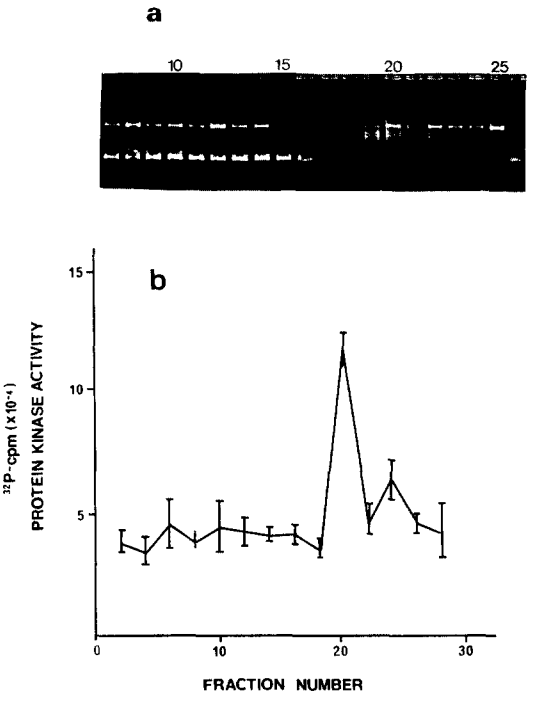

Fig. 2. Glycerol gradient sedimentation analysis of topo II-associated protein kinase activity. About 2 μg of purified topo II was subjected to a glycerol density gradient (20-50%) by centrifugation at 100,000 × g for 20 hours. Total 28 fractions were collected from the bottom of the Beckman SW 50.1 tube, and were subjected to the assays of the topological activity (a) and the protein kinase activity (b) in a parallel reaction groups. The topological reactions were carried out in a condition for the relaxation activity. The kinase activity was assayed as described in materials and methods. The kinase activity was expressed as the radioactivity of ^{32}P-cpm incorporated into the acid-precipitable macromolecules.

or slight enhancing effect. The protein kinase activity of the rat topo II was inhibited by novobiocin, an inhibitor for topo II. In addition, the protein kinase activity was also blocked when another topo II inhibitor, N-ethylmaleimide (NEM) was treated (Fig. 3).

DISCUSSION

The purified topo II from regenerating rat liver showed protein kinase activity as well as topoisomerase II activities. These two activities

Table 2. Effectors and inhibitors for the protein kinase activity of the purified enzyme.

Assay condition	% Maximal activity
Standard	100.0
No $MgCl_2$	6.5
+ 10 mM $CaCl_2$	39.3
+ 10 mM $MnCl_2$	19.6
cAMP	
1 μM	18.4
NaCl	
50 mM	13.8
100 mM	9.3
KCl	
50 mM	7.4
200 mM	6.0
DNA (15 μg/ml)	
single stranded	48.4
double stranded	112.7

Acid precipitation assays were carried out with histone H1 as substrate. The values are the average of several determinations. Additions to assay are indicated.

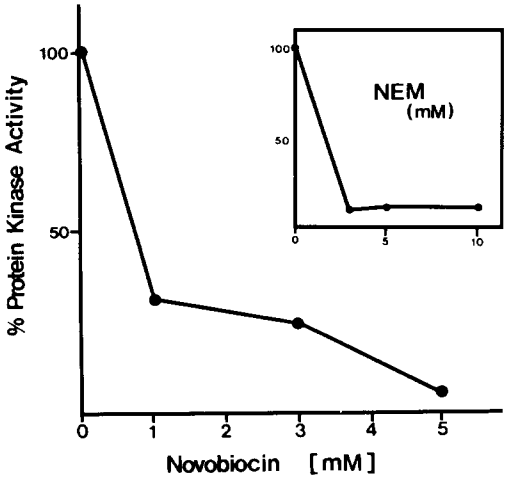

Fig. 3. The effects of novobiocin and N-ethylmaleimide on the kinase activity. In the presence of varying concentration of inhibitors, the protein kinase activity was assayed in the standard reaction buffer containing 20 μg of histone H1. The activity was expressed as the percentage of that obtained through the control assay without any inhibitor.

were not separated throughout all purification steps and analytical glycerol gradient sedimentation. Furthermore, protein kinase activity of the enzyme was blocked by the topo II inhibitor, novobiocin. These results suggest that the protein kinase activity of purified topo II is an intrinsic property of topo II. Sander *et al.* (1984) first reported that a protein kinase activity is tightly associated with topo II in *Drosophila melanogaster*. The kinase detected in our study shares several characteristics described by Sander *et al.* (1984) i. e., parallel mobility of the kinase and topo II activities on glycerol gradient sedimentation, the common sensitivity of two enzymes to NEM (Fig. 3), the absolute requirement of $MgCl_2$, and the sensitivity to ssDNA. The important differences are that the kinase from *Drosophila* was inhibited by dsDNA and insensitive to cAMP, whereas kinase activity of rat topo II was not inhibited by dsDNA and was inhibited by cAMP. Histone H1 act as a strong stimulator and a good substrate for the kinase activity of this enzyme. Since histone phosphorylation has been closely correlated with chromatin condensation (Pardee, 1978) and topo II has been known to participate in chromatin condensation (Uemura *et al.*, 1987), the tight association of the kinase activity with topo II suggests that the two activities might be coordinately involved in the modulation of chromatin structure. Recent studies have suggested the involvement of topo II in many aspects of nucleic acid metabolism including DNA replication (Noguchi *et al.*, 1983; Jazwinski and Edelman, 1984), transcription (Rowe *et al.*, 1986), and chromosome segregation (Uemura *et al.*, 1987). Therefore, topo II seems to be regulated by a delicate control mechanism to exert directed functions in nuclear processes.

Although the kinase activity described here showed autophosphorylation activity, we have not yet determined whether this modification actually occurs *in vivo* and autophosphorylation has any effects to the enzyme activities of rat topo II. Topo II activity in different stages of cell cycle, growth and differentiation may be regulated by posttranslational modifications. Several potential modification of the enzyme have been demonstrated *in vitro* including phosphorylation. Topo II in *Drosophila* Kc cells, chicken

lymphoblastoid, mouse FM3A, HeLa cells and budding yeast were found to be phosphorylated *in vivo* (Ackerman *et al.*, 1988; Heck *et al.*, 1989; Saijo *et al.*, 1990; Kroll and Rowe, 1991; Cardenas *et al.*, 1992). Phosphorylation sites of budding yeast topo II have recently been reported (Cardenas *et al.*, 1992). The topo II activity was reported to be enhanced by phosphorylation (Ackerman *et al.*, 1985; Sahyoun *et al.*, 1986; Rottman *et al.*, 1987). It is likely that the topo II-associated protein kinase activity is important for the modulation of topo II activities *in vivo*.

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재생 쥐간에서 분리한 DNA topoisomerase II에 결합된 protein kinase 활성

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재생쥐간에서 분리한 topoisomerase II에서 protein kinase 활성이 발견되었다. topo II 활성 및 kinase 활성은 hydroxyapatite, phosphocellulose, double strand DNA cellulose chromatography 등의 순수 분리 과정 중에도 서로 분리되지 않았으며 glycerol gradient sedimentation 분석에서도 같은 분획에서 활성이 존재하였다. Kinase는 topo II 저해제인 N ethylmaleimide와 novobiocin 등에 의해 그 활성이 저해되었다. 그러나 이러한 증거들 만으로 kinase 활성이 topo II가 아닌 다른 polypeptide에 의한 것일 가능성을 완전히 배제 할수는 없다.

Topo II와 결합된 kinase 활성에는 Mg^{++}가 절대적으로 필요하였으며 다른 일가 또는 이가 이온으로는 그 효과가 대체되지 않았다. Histone H1은 kinase 활성을 증가 시키며 또 kinase에 의해 강하게 인산화된다. 이러한 효과는 다른 histone 류 및 casein 등에 의해 대체되지 않았다.