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Protein kinase C phosphorylates DNA topoisomerase I

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The induction of mammalian cell proliferation requires the expression of a specific set of genes. Tumor promoters stimulate cell growth by activating the Ca^{2+} and phospholipid-dependent protein kinase, protein kinase C (PKC). DNA topoisomerase I, a nuclear enzyme involved in transcription, was phosphorylated by activated PKC in vitro. Phosphorylation by PKC stimulated the DNA relaxation activity of topoisomerase I two- to three-fold. Therefore, DNA topoisomerase I is a substrate for PKC-mediated activation by phosphorylation and may serve as a nuclear target of mitogenic signals generated by tumor promoters in vivo.

Protein kinase C; Topoisomerase I, DNA-; Protein phosphorylation; Mitogenic signal transduction; Nuclear target

1. INTRODUCTION

The tumor-promoting phorbol ester TPA, and other mitogens, induce the expression of a set of genes involved in cellular growth and transformation. PKC, the serine and threonine-specific, Ca^{2+}- and phospholipid-dependent, diacylglycerol-activated kinase, is the major receptor for TPA and is stimulated by treatment with ligands that induce the turnover of inositol phospholipids [1–3]. PKC is thought to regulate numerous metabolic events in the cell, including transmembrane signal transduction, gene expression and cell growth [3,4].

Topoisomerase I transiently nicks a single DNA strand, catalyzing topological manipulations such as the relaxation of supercoiled DNA molecules [5–7]. DNA topoisomerase I is required for rRNA synthesis [8–10] and is associated with transcriptionally active genes [11–19], where it is probably involved in resolving local supercoiling caused by the transcriptional machinery [20–23]. The activity of topoisomerase I increases during cell proliferation [24–26].

Several in vitro substrates for PKC have been identified, including the nuclear enzymes DNA topoisomerase II [27,28], DNA methyltransferase [29], DNA polymerase α [30] and RNA polymerase II [31] as well as the nuclear factor CREB [32]. We report here that PKC also phosphorylates DNA topoisomerase I in vitro resulting in a two- to three-fold increase in topoisomerase I activity and suggest a role for nuclear PKC in the signal transduction pathway for the induction of cell proliferation.

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Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; FPLC, fast protein liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid

2. MATERIALS AND METHODS

2.1. *Enzymes*

Topoisomerase I was purified to near homogeneity from HeLa cells essentially as described previously [7]. PKC was purified from mouse brain using DE52 DEAE-cellulose (Whatman) and FPLC Mono Q (Pharmacia) column chromatography as described previously [33], except chromatography in the presence of ATP was not performed and the column buffers contained 0.1% Triton X-100.

2.2. *Phosphorylation reaction*

Reactions contained 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin A, 1 mM PMSF, 0.5 mM Na_3VO_4, 10 mM *p*-nitrophenyl phosphate, and either 0.1 mM $CaCl_2$, 25 μg/ml PS and 100 ng/ml TPA or 2 mM EGTA, in 30 μl. Reactions were either in the presence or absence of 0.016 units PKC (90 units/mg) and 25 ng topoisomerase I. One unit was defined as the amount of PKC required to transfer 1 nmol of $^{32}P_i$ from [γ-^{32}P]ATP to histone (calf thymus, type III-S, Sigma) in 15 min at 30°C. The reaction was initiated by adding 0.1 μM [γ-^{32}P]-ATP (22 μCi; ICN), incubated at 30°C for 15 min, and terminated by placing in an ice-water bath, adding 30 μl 2 × Laemmli's sample loading buffer [34] and heating in a boiling water bath for 5 min. Samples were electrophoresed on a 7% SDS-polyacrylamide gel for 14 h at 70 V (4.6 V/cm). Gels were fixed, dried and exposed to Konica type A film with intensifying screens at −70°C for 1 day.

2.3. *Immunoprecipitation*

The phosphorylation reaction (30 μl) was terminated by placing in an ice-water bath and adding 2 μl 0.1 M EGTA, pH 7.5, and was then diluted with 1 ml 10 mM sodium phosphate, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 0.5 mM Na_3VO_4, and 1 mM PMSF. This was added to 20 μl Protein A-Sepharose CL-4B (Pharmacia) loaded with 6 μg anti-topoisomerase antibody [35] and incubated with gentle agitation at 4°C for 16 h. The supernatant (flow-

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through) was collected by acid precipitation with 20% TCA and resuspended in 30 μl buffer A. The pellet (immunoprecipitate) was washed essentially as described previously [36] and resuspended in 30 μl 2 × Laemmli's sample loading buffer [34]. Electrophoresis was performed as described above.

2.4. *Topoisomerase I assay*

Topoisomerase I was phosphorylated by PKC essentially as described above, except the reaction was terminated by placing in an ice-water bath and adding 1.5 μl 0.1 M EGTA, pH 7.5. Reactions were diluted in 6% polyethylene glycol 8000, 20 mM Tris-HCl, pH 7.5, 1 mM 2-mercaptoethanol, and 10 μg/ml bovine serum albumin. DNA relaxation activity was measured essentially as previously described [7,35]; the reaction was initiated by addition of diluted phosphorylation reaction (0.5 to 2.5 ng topoisomerase I).

3. RESULTS

Human DNA topoisomerase I was incubated with murine PKC in vitro to determine if phosphorylation mediated by PKC modulates topoisomerase I activity. Topoisomerase I was purified as a single polypeptide with a molecular mass of 100000 Da (data not shown). PKC, a heterogeneous family of proteins with an average molecular mass of 77000 Da, was activated by Ca^{2+}, PS and TPA resulting in its autophosphorylation (fig.1, lane 2). The phosphorylation of PKC in the absence of the activators Ca^{2+}, PS and TPA was less than 10% of the phosphorylation in their presence (fig.1, lane 1).

The topoisomerase I preparation contained no autophosphorylating activity in the presence or absence of Ca^{2+}, PS and TPA (fig.1, lanes 3,4). However, topoisomerase I was significantly phosphorylated by activated PKC in vitro (fig.1, lane 6). The identity of the phosphorylated protein was confirmed by immunoprecipitation from the PKC reaction with an anti-topoisomerase I antiserum [35] (fig.2). The possibility that one of the ten other phosphoproteins in the PKC fraction is a kinase that is activated by PKC and phosphorylates topoisomerase I was eliminated by phosphorylation of topoisomerase I by three distinct PKC subspecies isolated by fractionating the protein kinase preparation on hydroxyapatite column chromatography (Samuels, D.S. and Shimizu, N., manuscript in preparation).

Phosphorylation of DNA topoisomerase I by PKC resulted in a two- to three-fold increase in topoisomerase I activity as assayed by the conversion of supercoiled form I DNA molecules to their relaxed topoisomers. The relaxed form I topoisomers migrated slower than the supercoiled molecules during agarose gel electrophoresis due to their decreased compactness. The effect of PKC-mediated phosphorylation was tested after preincubation of topoisomerase I in the absence or presence of PKC in the phosphorylation reaction.

Approximately 1.4 ng of untreated topoisomerase I relaxed half of the supercoiled plasmid while less than 0.7 ng of topoisomerase I phosphorylated by PKC was

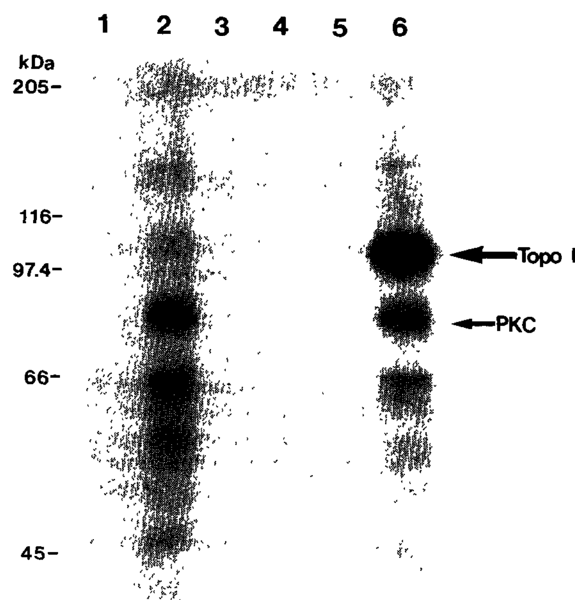

Fig.1. Phosphorylation of DNA topoisomerase I by PKC. Reactions contained either $CaCl_2$, PS and TPA (lanes 2,4,6) or EGTA (lanes 1,3,5) and were in the presence (lanes 1,2,5,6) or absence (lanes 3,4) of PKC and the presence (lanes 3–6) or absence lanes 1,2) of DNA topoisomerase I (Topo I).

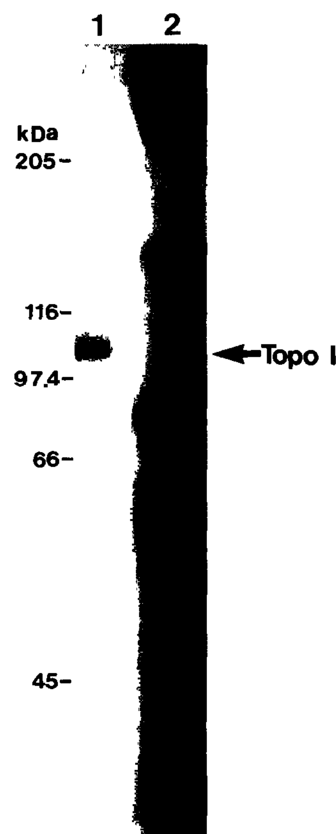

Fig.2. Immunoprecipitation of phosphorylated DNA topoisomerase I. Immunoprecipitate using anti-DNA topoisomerase I antibody (lane 1) and flow-through (lane 2) from the PKC phosphorylation reaction with DNA topoisomerase I (Topo I).

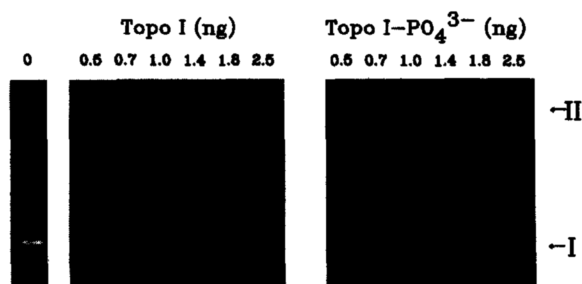

Fig.3. Relaxation activity of phosphorylated DNA topoisomerase I. Reactions contained DNA topoisomerase I (0.5 to 2.5 ng) preincubated in the phosphorylation reaction without PKC (Topo I) or with PKC (Topo I-PO_4^{3-}). DNA topoisomerase I converted supercoiled form I plasmid DNA (I) to relaxed form I plasmid DNA which migrated through agarose gel electrophoresis at the same position as form II plasmid DNA (II).

required to relax the same amount of DNA (fig.3). This trend persisted at other dilutions indicating that inhibition of untreated topoisomerase I by ATP not expended in the reaction or PS and TPA not bound to PKC did not significantly contribute to the difference in activity of topoisomerase I preincubated with and without PKC. PKC had no intrinsic topoisomerase I activity (data not shown).

4. DISCUSSION

Topoisomerase I is most likely regulated in vivo by phosphorylation [37,38]. Its activity [24–26], but not the amount or stability of the protein [39], varies during the cell cycle. Topoisomerase I is isolated from Novikoff hepatoma cells and *Xenopus laevis* ovaries as a phosphoprotein and treatment of it with alkaline phosphatase decreases or abolishes its DNA relaxation activity [38,40]. In addition, it is phosphorylated by casein kinase II in vitro resulting in the stimulation of its activity approximately three-fold [38,40,41].

DNA topoisomerase I is a nuclear enzyme; PKC has also been detected in the nucleus [42–52]. Preliminary results indicate that a topoisomerase I-like phosphoprotein is present in the nucleus of mouse fibroblasts from 10 to 75 min after TPA treatment (Samuels, D.S. and Shimizu, N., unpublished results), shortly after the appearance of activated PKC in the nucleus [42,45,46,49,52–59], when early genes required for DNA synthesis, such as c-*fos* and c-*myc*, are expressed.

Topoisomerase I is likely to be a physiologically important substrate for PKC because of its role in transcription and the effect phosphorylation has on its DNA relaxation activity. This post-translational regulation may be a key event in the induction of gene expression by tumor promoters. Several other nuclear proteins are also substrates for PKC in vitro [27–32]. Histones H2B, H4 and H1° [53,55], nuclear matrix proteins NP80 and NP33 [54], lamin B [56,57] as well as the nuclear proteins encoded by c-*erb*A [58] and *ets*-2 [59] are phosphorylated in response to TPA treatment in vivo. Thus, PKC could prove to be an essential kinase in the control of nuclear processes during, and in preparation for, cell growth and differentiation.

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