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Protein kinase C phosphorylates topoisomerase II: Topoisomerase activation and its possible role in phorbol ester-induced differentiation of HL-60 cells

(gene expression/cell cycle/tumor promoter/calmodulin)

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ABSTRACT **DNA topoisomerase II from *Drosophila* was phosphorylated effectively by protein kinase C. With a K_m of about 100 nM, the reaction was rapid, occurring at 4°C as well as at 30°C and requiring as little as 0.6 ng of the protein kinase per 170 ng of topoisomerase. About 0.85 mol of phosphate could be incorporated per mol of topoisomerase II, with phosphoserine as the only phospho amino acid produced. The reaction was dependent on Ca^{2+} and phosphatidylserine and was stimulated by phorbol esters. Calmodulin-dependent protein kinase II, but not cyclic AMP-dependent protein kinase, was also able to phosphorylate the topoisomerase. Phosphorylation of topoisomerase II by protein kinase C resulted in appreciable activation of the topoisomerase, suggesting that it may represent a possible target for the regulation of nuclear events by protein kinase C. This possibility is supported by the finding that the phorbol ester-induced differentiation of HL-60 cells was blocked by the topoisomerase II inhibitors novobiocin and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide(*m*-AMSA), but not by the inactive analog *o*-AMSA.**

Phorbol esters influence cellular functions at various levels (1, 2), presumably by activating protein kinase C (3, 4). Some cellular effects can be detected rapidly after the addition of phorbol esters. These effects include the regulation of ionic transport, release of bioactive substances, and receptor down-regulation (1, 2, 5). Other effects of phorbol esters target the genome, resulting in the regulation of DNA replication or in the modulation of gene expression. Examples of genes that appear to be induced by phorbol esters include ornithine decarboxylase in epidermal cells (6, 7), interleukin 2 and IL-2 receptor in mouse T cells and T-lymphoma cells (8–10), c-*fos* in U937 monocytes and in HL-60 cells (11), actin and vimentin in K562 erythroleukemia cells (12), and calcitonin in thyroid medullary carcinoma cells (13). Genes that seem to be repressed by phorbol esters include globin in Friend erythroleukemia cells (14), glycophorin in K562 erythroleukemia cells (15), and c-*myc* in thyroid medullary carcinoma cells (13). Moreover, phorbol esters increase the frequency of initiation of DNA replication for bacteriophage λ injected into *Xenopus* eggs (16).

The effects of phorbol esters on gene transcription generally require more time to become manifest than the effects on ionic fluxes or receptors. Thus, the regulation of c-*fos* mRNA in U937 monocytes is maximal after 30 min (11) and that of glycophorin and actin genes becomes evident after about 1 hr (12, 15), whereas the effect on c-*myc* mRNA is observed 4 hr after exposure of the cells to the tumor promoter (13). The relative delay in these responses may reflect an indirect effect of phorbol esters on gene transcription, requiring one or more intermediary steps or a cascade of reactions; alternatively, phorbol esters may alter gene transcription more directly by stimulating protein kinase C, which then may phosphorylate a polypeptide(s) involved in transcriptional regulation. In this case the delay in the appearance of the final mRNA product may be attributed to the nature of the reactions involved in gene activation, transcription, and RNA processing. Further, discrimination among events mediating the short-term and long-term effects of phorbol esters is a problem that pertains to the pleiotypic effects of growth factors in general.

The present study was designed to investigate whether the phorbol ester receptor (3, 4), protein kinase C, can directly phosphorylate cellular components that are involved in DNA metabolism, which may lead to the modulation of DNA replication and/or transcription. Topoisomerases are prokaryotic and eukaryotic enzymes that produce DNA isomers by topological interconversions in reactions like "knotting/unknotting," supercoiling/relaxation and catenation/decatenation of circular DNA (17–20). These processes basically entail cleavage and resealing of DNA with the production of enzyme–DNA covalent complexes as intermediates; type I topoisomerases produce single-strand DNA breaks, while double-strand breaks are produced by type II topoisomerases (17–20). Recent work on eukaryotic type II topoisomerases has implicated these enzymes in the regulation of DNA replication and in the transcription of specific genes. The evidence derives mainly from study of yeast topoisomerase mutants (21, 22), mapping of topoisomerase-induced DNA cleavage sites (23, 24), and use of topoisomerase II inhibitors such as novobiocin (25, 26). Further, by means of immunochemical and immunocytochemical methods, topoisomerase II has been visualized in association with the nuclear matrix of *Drosophila* embryonic cells (27) and with the "scaffold" of mitotic MSB-1 lymphoblastoid chicken cells (28). This localization may facilitate the participation of topoisomerase II in transcriptional regulation.

Regulation of topoisomerase activity itself may, therefore, result in alterations of gene expression. Phosphorylation of topoisomerases by protein kinases may represent such a regulatory mechanism. Thus, topoisomerase I from Novikoff ascites cells appears to be activated by serine/threonine phosphorylation (29), whereas calf thymus topoisomerase I is inhibited following tyrosine phosphorylation by $pp60^{src}$ (30). Moreover, purified preparations of *Drosophila* topoisomerase II contain an endogenous protein kinase, and the topoisomerase II occurs as a phosphoprotein (31). This topoisomerase is also subject to serine phosphorylation by

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Abbreviations: PBt_2, 4β-phorbol 12,13-dibutyrate; *m*/*o*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*/*o*-anisidide.

casein kinase II, leading to a 3-fold increase in enzyme activity (32).

We have examined the phosphorylation of *Drosophila* topoisomerase II by protein kinase C. The topoisomerase proved to be a high-affinity substrate which underwent very rapid phosphorylation even when subnanogram amounts of protein kinase C were used. Topoisomerase phosphorylation was accompanied by activation of the unknotting and relaxation enzyme activities. These results provide a putative target for the action of tumor promoters that may be relevant to the mechanism of transcriptional regulation for one or more genes. Consistent with this possibility is the observation that the topoisomerase II inhibitors novobiocin (33) and 4'-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) (34) prevented the phorbol ester-induced differentiation of HL-60 cells.

MATERIALS AND METHODS

[γ-^{32}P]ATP (30–40 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear; 4β-phorbol 12,13-dibutyrate (PBt_2), 4α-phorbol, and polyethylene glycol (PEG) 20,000 were obtained from Sigma; phenyl-Sepharose was supplied by Pharmacia; *m*-AMSA and *o*-AMSA were a generous gift from L. Elwell (Burroughs Wellcome).

Protein kinase C was purified to homogeneity by a rapid, high-yield method (35). In brief, 5 g of brain tissue was obtained from male Sprague–Dawley rats (150–250 g) and homogenized in a buffer containing 1 mM $CaCl_2$ and 50 μg of leupeptin/ml. Protein kinase C was released from the particulate fraction in the presence of EGTA, bound to inside-out vesicles from human erythrocytes in the presence of 1 mM $CaCl_2$, and then specifically released with EGTA. Minor contaminants were eliminated by phenyl-Sepharose chromatography, yielding about 150 μg of pure enzyme. Calmodulin-dependent protein kinase type II was purified from rat brain either in the cytoskeletal or in the solubilized form (36). Cyclic AMP-dependent protein kinase from bovine heart (Sigma) was further purified (unpublished results) by HPLC, employing sequentially a Mono Q anion exchanger followed by TSK-400 and TSK-200 gel-permeation columns in tandem (Pharmacia). Topoisomerase II was purified to homogeneity from *Drosophila melanogaster* embryos as detailed previously (37). Phage P4 DNA and plasmid pBR322 DNA were prepared as described (38, 39).

Phosphorylation assays were performed at 30°C unless indicated otherwise; assay mixtures (50 μl) contained 50 mM Tris·HCl (pH 7.7), 7 mM $MgCl_2$, 50 μM ATP, 2.5 μCi of [γ-^{32}P]ATP, and various activators of protein kinase C or other protein kinases as indicated. Phosphorylated polypeptides were analyzed by $NaDodSO_4$/PAGE in 7.5% or 10% acrylamide Laemmli gels (40); gels were stained with Coomassie blue, destained, dried, and exposed to Kodak direct-contact DEF-5 film. ^{32}P-incorporation into proteins was estimated by densitometric scanning and by direct measurement of radioactivity in dried gel slices. Phospho amino acid analysis (41) was performed on topoisomerase II after separating it from [γ-^{32}P]ATP by gel-filtration chromatography on Sephadex G-50. Protein concentration was determined by the Coomassie blue G-250 method (42). Topoisomerase II activity was measured by unknotting of knotted bacteriophage P4 capsid DNA (38), or by relaxing negatively supercoiled plasmid pBR322 (43) in the presence of 1.25 mM ATP, and the products were analyzed by

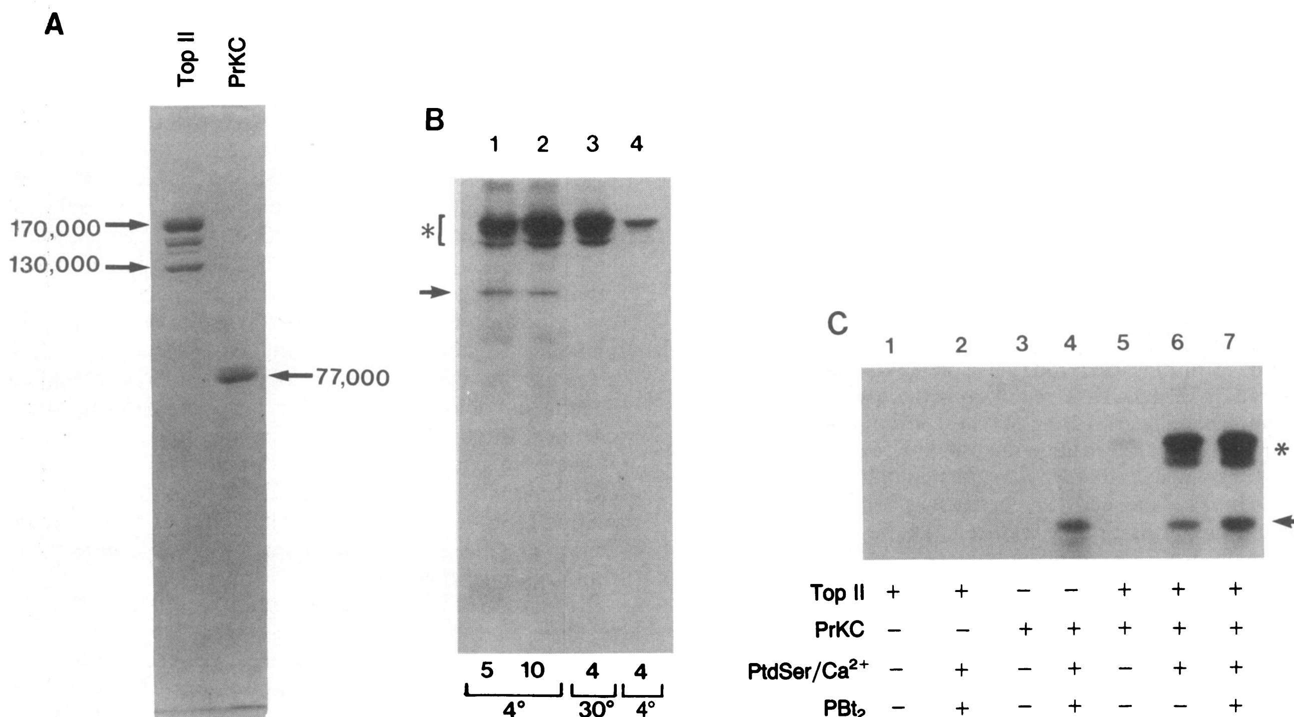

FIG. 1. Phosphorylation of topoisomerase II by protein kinase C. (*A*) Polypeptide composition of purified topoisomerase II (Top II; 4 μg) and protein kinase C (PrKC; 2 μg) analyzed by $NaDodSO_4$/7.5% PAGE. Gels were calibrated with standard molecular weight markers. M_r values of the purified polypeptides are indicated. (*B*) Topoisomerase II phosphorylation at 30°C and 4°C. The kinase reaction mixture (50 μl) contained 100 μM $CaCl_2$, 1 μg of phosphatidylserine, 50 nM PBt_2, 0.5 μg of topoisomerase II, and either 60 ng (lanes 1 and 2) or 12 ng (lanes 3 and 4) of protein kinase C. The temperature and duration (in minutes) of the assays are specified below the autoradiogram. The arrow denotes protein kinase C autophosphorylation, while the asterisk indicates topoisomerase II phosphorylation. Samples were analyzed by $NaDodSO_4$/10% PAGE. (*C*) Effect of protein kinase C activators on topoisomerase II phosphorylation. The assay mixture (30°C, 5 min) contained where indicated 1 μg of topoisomerase II (Top II), 50 ng of protein kinase C (PrKC), 1 μg of phosphatidylserine (PtdSer), 100 μM $CaCl_2$ (Ca^{2+}), and 100 nM PBt_2. Reaction products were analyzed by $NaDodSO_4$/10% PAGE followed by autoradiography. The position of protein kinase C is indicated by an arrow, and that of topoisomerase II by an asterisk.

electrophoresis in 1% agarose gels followed by ethidium bromide staining and visualization with UV light. The human promyelocytic leukemic cell line HL-60 was grown as described (44), and phorbol ester-induced differentiation was assessed by the cell-adherence method (44).

RESULTS

The purity of protein kinase C and of *Drosophila* topoisomerase II was verified by $NaDodSO_4$/PAGE (Fig. 1*A*). Protein kinase C migrates as a single polypeptide band with a M_r value of about 77,000. Topoisomerase II exists in the native form as a M_r 320,000 dimer, which is dissociated by $NaDodSO_4$/PAGE to yield a M_r 170,000 polypeptide; three other polypeptides between M_r 130,000 and M_r 170,000 also derive from the enzyme itself, probably by proteolytic cleavage (43, 45).

Topoisomerase II proved to be a very effective substrate for protein kinase C. Thus, low concentrations of the protein kinase were able to phosphorylate topoisomerase II rapidly at 30°C and more slowly at 4°C (Fig. 1*B*). Phosphorylation of topoisomerase II by protein kinase C required the presence of Ca^{2+} and phosphatidylserine and was further stimulated by 100 nM PBt_2 (Fig. 1*C*). With 5 ng of protein kinase C and 170 ng of topoisomerase II, enzyme phosphorylation was evident after 20 sec and was essentially complete by 15 min (Fig. 2*A*). The data indicate that when the reaction approached completion, about 0.85 mol of phosphate were incorporated per mol of topoisomerase II, suggesting the presence of a single phosphorylation site per subunit. Phospho amino acid analysis detected only phosphoserine (Fig. 3).

Relatively small amounts of protein kinase C were sufficient to effect phosphorylation of topoisomerase II, so that ^{32}P incorporation could be detected within 30 sec, using 0.6 ng of the kinase, and increased almost linearly when the amount of protein kinase C used was increased to 10 ng (Fig. 2*B*). Varying the topoisomerase II concentration yielded an apparent K_m value of about 100 nM (Fig. 2 *C* and *D*), representing a considerably higher affinity than that for histone H1 (apparent $K_m \approx 10$ μM).

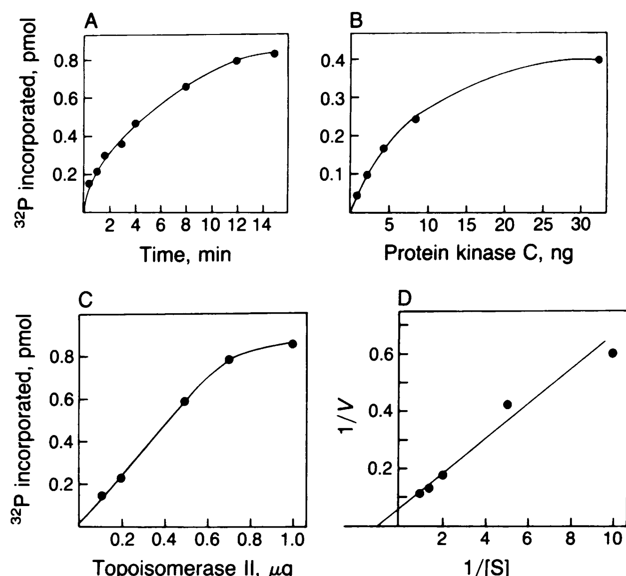

FIG. 2. Dependence of topoisomerase II phosphorylation on time (*A*), protein kinase C concentration (*B*), and substrate concentration [S] (*C* and *D*). Topoisomerase II (170 ng) was incubated with 5 ng of protein kinase C (*A*) or with various amounts of the kinase (*B*); 5 ng of the kinase was used for *C* and *D*. The reaction mixture also contained 100 μM $CaCl_2$, 20 μg of phosphatidylserine/ml, and 50 nM PBt_2; the reaction was terminated after 1 min except for *A*. Topoisomerase II phosphorylation was assessed by quantitative densitometric scanning and by determination of radioactivity in gel slices.

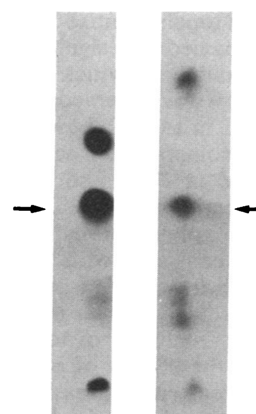

FIG. 3. Phospho amino acid analysis of topoisomerase II phosphorylated by protein kinase C (*Left*) or by calmodulin-dependent protein kinase II (*Right*). Arrows indicate the position of phosphoserine; no other phospho amino acid was detected.

The ability of other protein kinases to phosphorylate topoisomerase II was also investigated. Cyclic AMP-dependent protein kinase did not phosphorylate topoisomerase II even when high concentrations of this kinase were employed (Fig. 4*A*). However, calmodulin-dependent protein kinase type II could phosphorylate topoisomerase II appreciably, though higher concentrations of this kinase were required than of protein kinase C (Fig. 4*B*). Like protein kinase C, calmodulin-dependent protein kinase phosphorylates topoisomerase II only on a serine residue (Fig. 3). The localization of the calmodulin-dependent protein kinase to the nuclear matrix of neuronal nuclei (46) suggests that it may be able to utilize topoisomerase II as a substrate in intact cells.

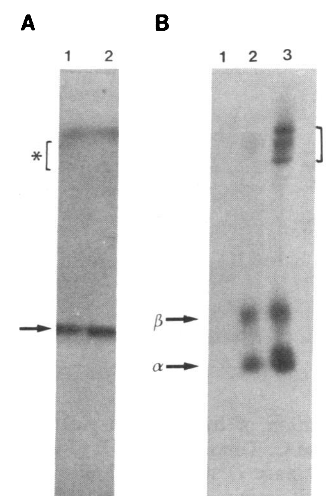

FIG. 4. Phosphorylation of topoisomerase II by cyclic AMP-dependent protein kinase (*A*) and by calmodulin-dependent protein kinase II (*B*). Reaction mixtures contained 0.3 μg of each protein kinase and 1 μg of topoisomerase II and were incubated at 30°C for 10 min, and the products were analyzed by $NaDodSO_4$/7.5% PAGE. Asterisks correspond to topoisomerase II; α and β denote the M_r 50,000 and 60,000 subunits of calmodulin-dependent protein kinase, respectively; and the arrow indicates the regulatory subunit of cyclic AMP-dependent protein kinase. Reaction mixtures contained 10 μM cyclic AMP (*A*) or 100 μM $CaCl_2$ and 1 μM calmodulin (*B*). Topoisomerase II was omitted from assays corresponding to lane 2 in *A* and in *B*. For lane 1 in *B*, kinase activators were omitted.

Phosphorylation of topoisomerase II by protein kinase C appears to be accompanied by a significant activation of the unknotting and relaxation activities of the topoisomerase (Fig. 5). The activation was observed when the enzyme was phosphorylated at 4°C or at 30°C; however, a greater degree of spontaneous topoisomerase II inactivation resulted when the reaction occurred at 30°C, and some of the apparent activation might reflect enzyme stabilization.

The effective *in vitro* phosphorylation of topoisomerase II by protein kinase C is consistent with, but does not prove, the hypothesis that topoisomerase II is a physiologic substrate for the kinase. Accordingly, it was of interest to determine whether intracellular inhibition of topoisomerase II might antagonize some of the effects of phorbol esters that are accompanied by altered gene expression. The phorbol ester-induced differentiation of the promyelocytic leukemic HL-60 cell line was, therefore, examined in the presence or absence of topoisomerase II inhibitors. Novobiocin abolished the effect of 200 nM PBt_2 (Table 1), at novobiocin concentrations that inhibit topoisomerase II *in vitro* by competition with ATP (33, 47). Novobiocin (150 μg/ml) was relatively non-toxic to the cells (<10% trypan blue uptake), did not inhibit binding of $[^3H]PBt_2$ to cells, did not inhibit protein kinase C activity, and did not block the adhesion of differentiated HL-60 cells (unpublished data). A different type of topoisomerase II inhibitor, the DNA intercalator *m*-AMSA (34), was also effective in blocking the induction of HL-60 differentiation by PBt_2; however, the inactive isomer *o*-AMSA had no significant effect (Table 1).

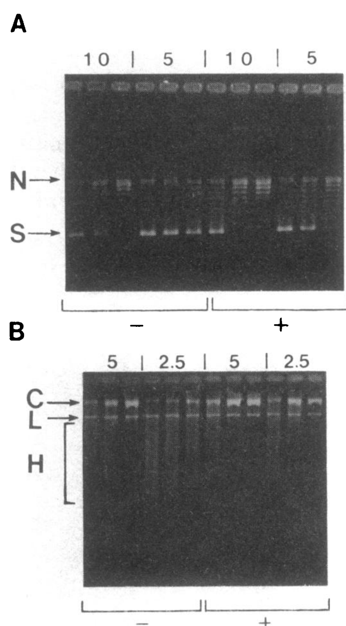

FIG. 5. Activation of topoisomerase II after phosphorylation with protein kinase C. Topoisomerase II (2 μg) was incubated with 60 ng of protein kinase C at 4°C in 40 μl of 50 mM Tris·HCl, pH 7.7/10% glycerol/albumin (0.1 mg/ml)/10 mM $MgCl_2$/100 μM $CaCl_2$/phosphatidylserine (20 μg/ml)/50 μM ATP/1 mM dithiothreitol. After 20 min, 10 volumes of 15 mM sodium phosphate, pH 7.0/50 mM NaCl/0.1 mM EDTA/0.1 mM dithiothreitol/10% glycerol/albumin (1 mg/ml) were added. Then 2.5, 5.0, or 10 ng, as indicated above the lanes, of topoisomerase II were used to assess enzyme activity in the presence of 1.25 mM ATP and 0.3 μg of plasmid pBR322 DNA (*A*) or bacteriophage P4 knotted DNA (*B*). − and + denote control enzyme and enzyme pretreated with protein kinase C, respectively. Each concentration of enzyme was assayed for 3, 7, and 12 min (left to right lanes). N, nicked circular DNA; S, supercoiled circular DNA; C, circular DNA; L, linear DNA; H, heterogeneously knotted DNA. Protein kinase C preparations did not contain detectable topoisomerase activity.

Table 1. Inhibition of phorbol ester-induced differentiation of HL-60 cells by topoisomerase II inhibitors

Inhibitor	Conc., μg/ml	% adherent cells (mean ± SEM)
None	—	95 ± 3
Novobiocin	150	21 ± 1
m-AMSA	5	32 ± 5
	2	40 ± 6
o-AMSA	5	92 ± 3

HL-60 cells (1.1×10^6 per ml) were treated for 11.5 hr with 200 nM PBt_2 in the presence or absence of topoisomerase II inhibitors which were added 30 min prior to the addition of the phorbol ester. Cellular differentiation was measured by assessing the percentage of adherent or suspended cells (44) relative to the total cell number. In all cases, <10% of the cells remaining suspended after 11.5 hr were positive for trypan blue uptake.

DISCUSSION

The mechanism(s) by which tumor promoters modulate gene expression is (are) likely to represent an important step(s) in the regulation of the cell cycle. Recent work on eukaryotic topoisomerases suggests that modification of the topological state of DNA may represent an important component in regulating gene expression. Hence, it was of interest to inquire whether phorbol ester-activated protein kinase C might regulate one or more topoisomerases. Several criteria have to be fulfilled in order to establish topoisomerase II as a mediator of protein kinase C effects on gene transcription. These criteria include (*i*) *in vitro* phosphorylation of topoisomerase II by protein kinase C and modulation of its activity; (*ii*) *in vivo* phosphorylation of topoisomerase II in response to protein kinase C activators, with consequent changes in activity; (*iii*) similar *in vitro* and *in vivo* phosphorylation reactions; and (*iv*) inhibition of the effects of protein kinase C activators by inhibitors of topoisomerase II. Here we present data in support of the first and fourth criteria; additional work is required to investigate the other two. Further, it will be of interest to determine the mechanism by which protein kinase C gains access to topoisomerase II. One such mechanism may involve nuclear translocation of protein kinase C itself. Whether topoisomerase II is a direct target for protein kinase C or whether it merely serves a permissive role for the action of phorbol esters also remains to be determined.

These considerations suggest that activators of protein kinase C may regulate nuclear events by two different mechanisms. Phosphorylation of receptors for growth factors such as insulin (48), somatomedin C (48), epidermal growth factor (49), transferrin (50), and (possibly) platelet-derived growth factor (51) may alter receptor function and/or levels, thereby modulating the efficacy of the corresponding ligand. Alternatively, protein kinase C may act directly on proteins involved in the regulation of DNA transcription or replication: one such protein may be topoisomerase II. Recent evidence indicates that the regulatory subunit (RII) of cyclic AMP-dependent protein kinase acts as a topoisomerase I (52). Thus, the possibility arises that topoisomerase activities may be involved in mediating the nuclear effects of one or more second-messenger systems.

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