

# Selective phosphorylation of human DNA methyltransferase by protein kinase C

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Received 25 November 1985; revised version received 18 December 1985

Human DNA methyltransferase, the enzyme thought to be responsible for the somatic inheritance of patterns of DNA methylation, is an effective substrate for phosphorylation by protein kinase C. This provides a plausible mechanistic link between the action of tumor promoting phorbol esters, which stimulate protein kinase C, and abnormal patterns of DNA methylation often observed in transformed cells.

*DNA methylation*      (*Human placenta*)      *DNA methyltransferase*      *Phorbol ester*      *Protein kinase C*  
*Protein phosphorylation*

## 1. INTRODUCTION

The strong preference of the DNA methyltransferase for hemimethylated DNA in vitro [1,2] suggests that the enzyme may function in the 'maintenance methylation' mechanism proposed by Holiday and Pugh [3] and Riggs [4] for the inheritance of patterns of DNA methylation. These authors suggested that a fully methylated CG doublet would give rise to two hemimethylated doublets in daughter strands and that the maintenance methylase would recognize and methylate all half-methylated sites in nascent DNA to regenerate the parental pattern in both daughter strands. These processes in fact occur in living cells. Transfection experiments have shown that hemimethylated DNA prepared in vitro is converted to the fully methylated form [5] and that methylation patterns applied in vitro are somatically inherited [6,7].

Abnormal methylation patterns associated with transformed cells have been reported [8–10]. This suggests that a disruption of the normal maintenance methylation process may occur dur-

ing the generation of the transformed phenotype. Here we demonstrate that the human placental DNA methyltransferase is selectively phosphorylated by protein kinase C in vitro, and that this phosphorylation is stimulated by the tumor promoter TPA (12-tetradecanoylphorbol-13-acetate). This result suggests a plausible link between the action of tumor promoters that activate protein kinase C and altered DNA methyltransferase activity.

## 2. MATERIALS AND METHODS

The methods used for placental DNA methyltransferase purification, assay, and DNA substrate preparation are described in [11]. Protein phosphorylation conditions have been described [12,13]. After incubation at 30°C, reactions were terminated by boiling with SDS buffer for 5 min and the <sup>32</sup>P incorporation from [ $\gamma$ -<sup>32</sup>P]ATP into protein analyzed by polyacrylamide gel electrophoresis, [14]. The preparation of protein kinases is described in [12,13]. Casein kinase II, cyclic AMP-dependent protein kinase catalytic subunit, phosphorylase kinase, and calmodulin-dependent protein kinase were essentially

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homogeneous preparations. Casein kinase I was approx. 50% pure, and  $F_A$ /GSK-3 was extensively purified but not homogeneous. Protein kinase C was purified to a stage of 25-fold stimulation by  $Ca^{2+}$  and phospholipid [13] by the method of Kikkawa et al. [15]. Approximately equivalent amounts of protein kinase activity toward glycogen synthase (0.05–0.1 unit/ml) were used in each incubation, where 1 unit is the amount of protein kinase that incorporates 1 nmol phosphate/min into glycogen synthase at 0.2 mg/ml [12,13]. DNA methyltransferase protein concentration was estimated by the method of Bradford [16]. To determine amino acid species phosphorylated, the region of the polyacrylamide gel corresponding to the methyltransferase was excised and hydrolyzed in 6 N HCl for 4 h at 110°C. The hydrolysate was analyzed by thin-layer electrophoresis at pH 1.9 [17], and  $^{32}P$  was detected by autoradiography.

### 3. RESULTS

The capacities of 7 different protein kinases were compared with respect to their ability to phosphorylate the human DNA methyltransferase. When the protein kinases were present at similar levels of enzyme activity, only protein kinase C was effective in phosphorylating methyltransferase. Cyclic AMP-dependent protein kinase, phosphorylase kinase, calmodulin-dependent protein kinase, casein kinases I and II, and  $F_A$ /GSK-3 (glycogen synthase kinase-3) introduced less than 0.026 phosphates per subunit into methyltransferase (not shown). Fig.1 shows an experiment of this type in which the activity of protein kinase C present during phosphorylation was reduced to 0.01 U/ml. Protein kinase C (track 5) catalyzed a more extensive phosphorylation of the 126 kDa polypeptide, even when present at 5–10-fold lower levels compared to the other protein kinases. The lower molecular mass phosphorylated species (tracks 2, 5 and 6) were detected at equivalent intensities in controls lacking DNA methyltransferase and represent autophosphorylation of constituents of the respective protein kinase incubations. The DNA methyltransferase preparation did not phosphorylate itself (track 1). The phosphorylated species corresponded to the methyl-

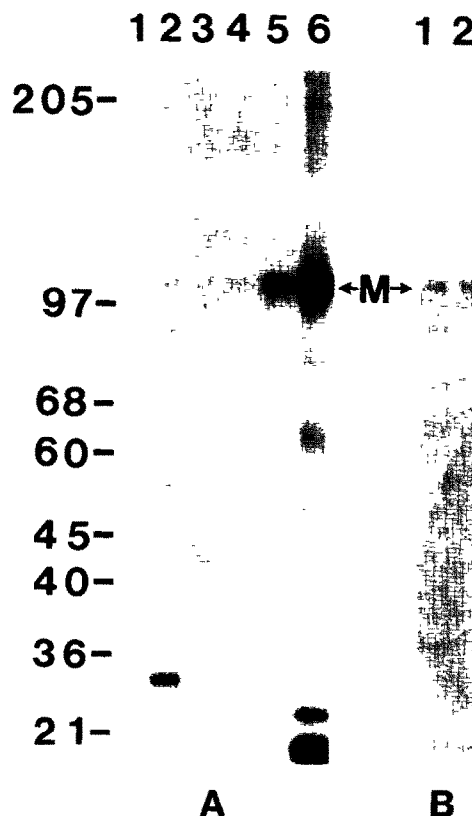


Fig.1. Protein kinase specificity of DNA methyltransferase phosphorylation. Purified DNA methyltransferase (7  $\mu$ g/ml) was incubated with [ $\gamma$ - $^{32}P$ ]ATP (~20000 cpm/pmol) and different protein kinases for 60 min as described in section 2. Each lane contained 0.14  $\mu$ g methyltransferase. As autoradiogram (3 h exposure at  $-70^{\circ}C$  with image intensifying screens) prepared from a dried 7.5% polyacrylamide gel is shown in A. Calmodulin-dependent kinase, casein kinase I, and phosphorylase kinase did not significantly phosphorylate the methyltransferase and are not included. The DNA methyltransferase incubated under phosphorylation conditions in the absence of added kinases is shown in track 1. Casein kinase II (track 2), cyclic AMP-dependent protein kinase catalytic subunit (track 3),  $F_A$ /GSK-3 (track 4) and protein kinase C (tracks 5 and 6) gave the autoradiographic signals shown. In the experiment shown protein kinase C was tested at 0.01 U/ml in the absence of added  $Ca^{2+}$  and phospholipid (track 5) or presence (track 6) of 0.5 mM  $Ca^{2+}$  in excess over EGTA plus phospholipid (0.2 mg/ml phosphatidylserine and 40  $\mu$ g/ml diolein). Note that the amount of protein kinase C activity used in this experiment is 5-fold less than that of the other kinases tested. A gel stained with Coomassie blue prior to autoradiography was used to show the correspondence of  $^{32}P$  incorporation (autoradiography, A) and protein staining (Coomassie blue staining, B).

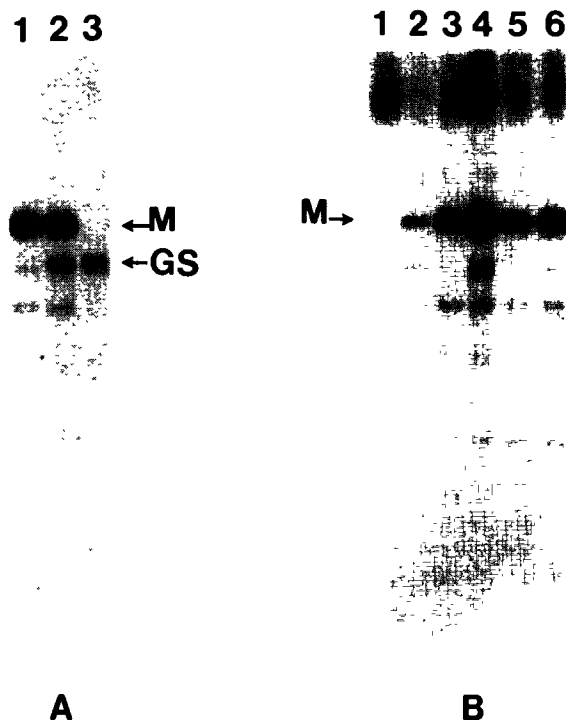


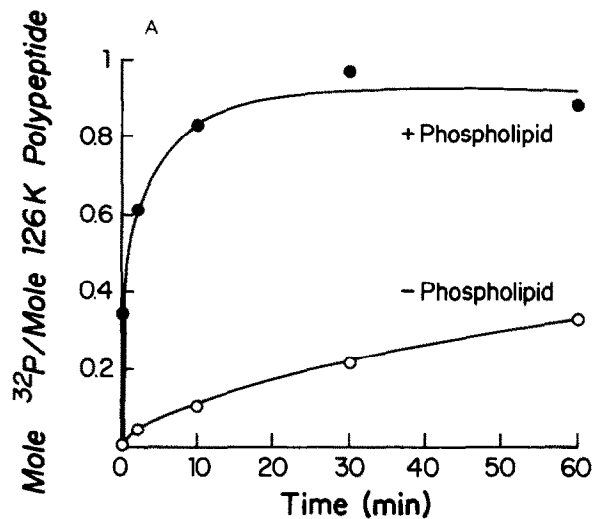
Fig.2. Characterization of DNA methyltransferase phosphorylation. (A) Comparison of the DNA methyltransferase and glycogen synthase as substrates for protein kinase C (0.012 U/ml) analyzed as described in the legend to fig.1 except that a 6–20% gradient polyacrylamide gel was used. An autoradiogram is shown. DNA methyltransferase (track 1), glycogen synthase (track 3) or a mixture of both (track 2) were phosphorylated for 5 min by protein kinase C in the presence of  $\text{Ca}^{2+}$  (0.5 mM) and phospholipid (0.2 mg/ml phosphatidylserine and 40  $\mu\text{g}/\text{ml}$  diolein). Each substrate protein was present at 54 nM with respect to subunits. Results of a similar experiment to test various effectors of protein kinase C action on methyltransferase phosphorylation. An autoradiogram is shown. Stimulation of phosphorylation by added  $\text{Ca}^{2+}$  (track 2), added phospholipid (track 3), or added  $\text{Ca}^{2+}$  plus phospholipid (track 4) is seen by comparison with a control in which neither compound was added (track 1). Activation of protein kinase C by TPA is seen by comparing track 6 (15 ng/ml TPA) with 5 (no TPA). For TPA stimulation, phosphatidylserine (0.2 mg/ml) but not diolein was included in the assay. Protein kinase C was present at 0.005 U/ml (of glycogen synthase kinase activity) and the reaction mixture was incubated for 5 min.

transferase protein (fig.1B) and the phosphorylation was markedly stimulated by  $\text{Ca}^{2+}$  plus phospholipid (track 6).

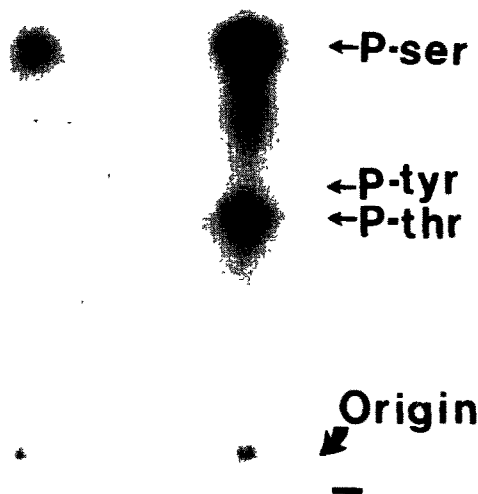
It was also of interest to determine whether DNA methyltransferase was a preferred substrate for phosphorylation. Since unequivocal physiological substrates for protein kinase C are not readily available, glycogen synthase, known to be a good substrate [13] *in vitro*, was used for the comparison. An equimolar mixture of glycogen synthase and DNA methyltransferase was incubated with protein kinase C. The DNA methyltransferase was preferentially phosphorylated at a 7-fold higher rate than the competing substrate (fig.2A). Similar results were obtained with histone H1 as competitor (not shown). The effects of  $\text{Ca}^{2+}$ , phospholipid, and TPA on the phosphorylation of the methyltransferase are shown in fig.2B. The reaction stimulations observed with  $\text{Ca}^{2+}$  or phospholipid alone are approximately additive when both are present. TPA stimulated the reaction of low  $\text{Ca}^{2+}$  concentration.

The time dependence of phosphorylation of the enzyme by protein kinase C is depicted in fig.3A. In the absence of phospholipid, the reaction was relatively slow, yielding less than 0.5 mol  $^{32}\text{P}$  per mol 126 kDa polypeptide. In the presence of phospholipid, the initial rate was stimulated about 6-fold in the experiment shown, yielding about 0.5 mol  $^{32}\text{P}$  per mol 126 kDa polypeptide in less than 1 min. Phosphorylation proceeded to a stoichiometry of approx. 1 phosphate/subunit. An analysis of the phosphoamino acids produced by protein kinase C action is shown in fig.3B. In the absence of  $\text{Ca}^{2+}$  and phospholipid, most of the phosphate introduced by protein kinase C was recovered in phosphoserine. Stimulation by  $\text{Ca}^{2+}$  plus phospholipid resulted in an increased level of phosphoserine but now a significant proportion of the phosphate was associated with phosphothreonine. The methyltransferase therefore contains at least two phosphorylation sites.

The effect of phosphorylation on enzyme activity was studied in the experiments shown in table 1. Under the conditions employed here, phosphorylation produced a consistent stimulation of activity on each substrate tested. Physiologically, the enzyme is thought to operate on newly replicated DNA that contains 5-methylcytosine only on the parental strand, and is thus hemimethylated. The enzyme prefers this substrate *in vitro* [1,2]. The ratio of the activity on hemimethylated double-stranded M13 DNA to that on unmethylated M13



1 2 +  
B



DNA observed here was about 10:1. This ratio did not change as a function of phosphorylation. The activity on either of these substrates was stimulated about 30%, while that on single-stranded DNA was stimulated about 50%.

Fig.3. Time dependence of DNA methyltransferase phosphorylation by protein kinase C. With an experimental protocol similar to that described in the legend to fig.1, DNA methyltransferase was incubated with protein kinase C (0.05 U/ml) for the indicated times in either the absence (○) or presence (●) of 0.5 mM  $\text{Ca}^{2+}$  plus 0.5 mg/ml phosphatidylserine and 40  $\mu\text{g}/\text{ml}$  dioleoin (A). After gel electrophoresis the region of the gel corresponding to the migration of methyltransferase was excised and the  $^{32}\text{P}$  quantified. (B) Analysis of the amino acids modified by protein kinase action. Methyltransferase phosphorylated by protein kinase C in the absence (0.5 phosphorylated/subunit) or presence (1.2 phosphates/subunit) of  $\text{Ca}^{2+}$  plus phospholipid was separated by gel electrophoresis (as in fig.1) and, after excising the appropriate region of the gel, hydrolyzed in 6 N HCl for 4 h at  $110^\circ\text{C}$ . The hydrolysate was analyzed by thin-layer electrophoresis at pH 1.9. A representative autoradiogram is shown as well as the migration of phosphoamino acid standards.

Table 1

Effect of phosphorylation of the DNA methyltransferase on enzyme activity in vitro

DNA substrate	Activity		Stimulation (%)
	- ATP	+ ATP	
Single-stranded			
<i>M. lysodeikticus</i>	3.82	5.69	49
Hemimethylated			
double-stranded			
M13	2.29	3.12	36
Unmethylated			
double-stranded			
M13	0.24	0.31	29

Single-stranded template was heat-denatured *Micrococcus lysodeikticus* DNA. Unmethylated and hemimethylated DNA substrates were prepared from M13 DNA. 4 U purified enzyme (fraction VII) were used in each assay. Phosphorylation by protein kinase C (0.05 U/ml) was carried out in a complete reaction mixture containing all reaction components required for maximal phosphorylation as described in fig.3. Reactions in which ATP was omitted served as controls in which phosphorylation was prevented. Reaction rates are given in pmol [ $^3\text{H}$ ]methyl groups incorporated into trichloroacetic acid-precipitable DNA per h

#### 4. DISCUSSION

Evaluation of the relevance of *in vitro* protein phosphorylation reaction is not simple but the striking features in this study were: (i) that of 7 different protein kinases tested, only protein kinase C interacted significantly with DNA methyltransferase and (ii) that DNA methyltransferase was a much more effective substrate of protein kinase C than glycogen synthase or histone H1. Whether protein kinase C, and by inference agents that stimulate diacylglycerol production, are physiologically important regulators of DNA methyltransferase cannot be judged but is an interesting prospect.

Perhaps the more interesting link suggested by this work is between the tumor promoter TPA and the phosphorylation of DNA methyltransferase. Experiments with TPA and telocidin show that tumor promoters of this type induce alterations in DNA methylation patterns that are associated with the induction of oncogenic herpesviruses [18-20]. These findings suggest a correlation between the action of this class of tumor promoter and alterations in DNA methylation patterns. The phorbol ester tumor promoters are thought to exert their effect via interaction with protein kinase C [21]. The DNA methyltransferase could be a key control point in this process. TPA by usurping normal receptor-mediated control of protein kinase C can lead to an unnatural and persistent activation of the protein kinase [21,22]. From our experiments, a plausible result could be the uncontrolled phosphorylation of DNA methyltransferase, the generation of aberrant methylation patterns in DNA, and corresponding aberrations in gene expression in the effected cells and their progeny. Transient activation of protein kinase C by inositol phospholipid breakdown might not produce the same effect.

The physiological role of the observed phosphorylation of DNA methyltransferase remains to be established. However, our results suggest a possible mechanistic link between DNA methylation and the tumor promoting activity of the phorbol esters.

#### ACKNOWLEDGEMENTS

This work was supported by grants GM32863

(S.S.S), AM27221 (P.J.R.), AM27240 (P.J.R) and AM01089 (P.J.R.) from the National Institutes of Health, and by grant 1571R1 (S.S.S) from the Council for Tobacco Research USA, Inc. We are grateful to Zafeer Ahmad for supplying the protein kinase C.

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