Phosphorylation of human topoisomerase I by protein kinase C *in vitro* and in phorbol 12-myristate 13-acetate-activated HL-60 promyelocytic leukaemia cells

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Topoisomerase I was phosphorylated *in vitro* by protein kinase C (PKC) purified from rat brain with high affinity (K_m about 0.1 μ M). Tryptic phosphopeptide mapping indicated that two major topoisomerase I peptides phosphorylated *in vivo* were co-migrating with minor peptides phosphorylated by PKC *in vitro*.

Topoisomerase I phosphorylation was stimulated 3-fold in HL-60 cells exposed to the tumour promoter phorbol 12-myristate 13-acetate. The results suggest that topoisomerase I phosphorylation in HL-60 cells is indirectly controlled by PKC.

INTRODUCTION

Protein kinase C (PKC) is a serine/threonine kinase important in signal transduction; activation of PKC is dependent on Ca^{2+} and phospholipids (Nishizuka, 1989). Tumour-promoting phorbol esters can activate PKC *in vitro* and *in vivo* (Castagna et al., 1982; MacFarlane, 1986; Hornbeck et al., 1988). Among protein substrates phosphorylated by PKC are nuclear proteins (McFarlane, 1986) suggestive of PKC activity in the nucleus. Additional evidence for the presence of PKC in the nucleus was gained from studies on subcellular distribution of PKC by immunofluorescence, Western blots and enzymic activity; importantly, the amount of PKC in the nuclear membrane increased several-fold in response to treatment with phorbol 12-myristate 13-acetate (PMA), indicative of a nuclear translocation of PKC (Halsey et al., 1987; Leach et al., 1989).

Topoisomerase I is a nuclear enzyme that catalyses DNA topological changes essential in DNA transcription and recombination (Wang, 1985; Liu, 1989). In earlier studies we and others have reported that topoisomerase I can be regulated by phosphorylation with nuclear NII kinase (a type II casein kinase): dephosphorylation decreased or abolished topoisomerase I activity, and phosphorylation with NII kinase re-activated topoisomerase I (Durban et al., 1983; 1985; Kaiserman et al., 1988).

More recent studies have demonstrated a potential second regulatory phosphorylation pathway for topoisomerase I mediated by PKC. Topoisomerase I activity was increased by phosphorylation with PKC *in vitro* (Pommier et al., 1990; Samuels et al., 1989).

The present study shows that human topoisomerase I is phosphorylated *in vitro* by PKC with high affinity. Phosphorylation of topoisomerase I in HL-60 cells *in vivo* occurs rapidly within 20 min of PMA administration, suggesting that PKC controls topoisomerase I phosphorylation *in vivo*.

MATERIALS AND METHODS

Purification of topoisomerase I

Topoisomerase I was purified as described by Durban et al. (1981). Briefly, HeLa cells were washed with RSB buffer (10 mM

Tris/HCl, pH 7.2, 10 mM NaCl, 1.5 mM $MgCl_2$), resuspended in RSB buffer containing 0.5% Nonidet P-40, homogenized in a Dounce homogenizer and centrifuged. Nuclei were lysed in 10 mM Tris/HCl, pH 8.0, and chromatin was extracted with 0.35 M NaCl. Each buffer contained 1 mM phenylmethanesulphonyl fluoride (PMSF) (Sigma) and 0.1 ml/l aprotinin (Sigma) as protease inhibitors. The extracted material was centrifuged overnight at 100 000 *g* and the supernatant was precipitated with $(NH_4)_2SO_4$. The 50–85%-satd. fraction was dissolved in 1 mM dithiothreitol (DTT)/1 mM PMSF/50 mM potassium phosphate buffer, pH 7.3, and dialysed against the same buffer. Calcium phosphate gel (17 mg/ml of water) was added to the dialysed fraction. The calcium phosphate gel was washed with the above buffer and then eluted consecutively with 0.15 M, 0.35 M and 0.8 M phosphate buffer, pH 7.3, containing 1 mM DTT and 1 mM PMSF. The 0.8 M-phosphate-buffer eluate was dialysed against 20 mM Tris/HCl (pH 8.0)/1 mM DTT/10% glycerol/0.2 M KCl/1 mM PMSF/0.1 ml/l aprotinin and applied on a phosphocellulose column pre-equilibrated with dialysis buffer. The column was eluted consecutively with the same buffer containing 0.5 M KCl, 0.8 M KCl and 1 M KCl. Fractions eluted in 1 M KCl were verified for topoisomerase I purity by SDS/PAGE (single band at 100 kDa) and specific activity.

Purification of PKC

PKC was purified from rat brain by a modification of the method of Kikkawa et al. (1982) and Huang et al. (1986). Briefly, seven rat brains were homogenized, centrifuged at 100 000 *g* for 1 h, and the supernatant was applied to a DEAE-cellulose column as described by Kikkawa et al. (1982). PKC activity was eluted at about 0.1 M NaCl when a gradient of 0–0.3 M NaCl was applied to the column. The active fractions were pooled and allowed to absorb to calcium phosphate gel [pooled volume: calcium phosphate gel slurry (17 mg/ml water) = 1:1]. The gel was washed with 20 mM potassium phosphate (pH 7.5)/0.5 mM EDTA/0.5 mM EGTA/1 mM DTT/10% glycerol/1 mM PMSF, and the active enzyme was eluted with 0.2 M phosphate buffer. Buffer composition was that used for elution of PKC

Abbreviations used: PKC, protein kinase C; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; PMA, phorbol 12-myristate 13-acetate ('TPA').

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from hydroxyapatite as described by Huang et al. (1986). Specific activity was 37 nmol/min per mg of protein, which is comparable with that of PKC purified to a similar extent on hydroxyapatite (Huang et al., 1986).

Assay for PKC

The assay mixture contained 20 mM Tris/HCl, pH 7.6, 5 mM $MgCl_2$, 40 μg of phosphatidylserine + 8 μg of diolein/ml of 0.5 mM $CaCl_2$, 2 μCi of $[\gamma\text{-}^{32}P]ATP$ (30 Ci/mmol) 25 μl of enzyme (0.1 unit), and 25 μg of histone H1 as substrate. Reactions were allowed to proceed for 3 min at 30 °C, stopped by addition of 1 ml of trichloroacetic acid (10%), and the precipitate was collected on Millipore HA filters and counted by Čerenkov radiation in a scintillation counter.

Topoisomerase I phosphorylation *in vitro* and tryptic cleavage

Topoisomerase I eluted from phosphocellulose in 1 M KCl was directly used as substrate for PKC. The phosphorylation assay contained 5 μl of topoisomerase I (equivalent to 1 μg), 5 mM $MgCl_2$, phosphatidylserine (40 μg /ml), diolein (8 μg /ml), 12.5 μCi of $[\gamma\text{-}^{32}P]ATP$ (sp. radioactivity 30 Ci/mmol; 4.2 μM final concn.), 0.5 mM $CaCl_2$ and 10 μl (0.04 unit) of PKC in 100 μl total volume. The reaction was stopped by addition of 100 μl of Laemmli (1970) sample buffer, and the phosphorylation mixture was run on SDS/7.5%-polyacrylamide gels. In order to obtain tryptic digests, gel slices containing ^{32}P -labelled topoisomerase I were soaked in 10% methanol for 4 h, freeze-dried and then incubated in 1 ml of 50 mM NH_4HCO_3 buffer, pH 8.0, containing 50 μg of trypsin [treated with tosylphenylalanylchloromethane ('TPCK'); Sigma] for 24 h at 37 °C. The supernatant was removed from the gel slices and freeze-dried as described by Durban et al. (1985).

Topoisomerase I phosphorylation *in vivo*

Human HL-60 promyelocytic leukaemia cells were grown in suspension in RPMI 1640 medium/0.5% fetal-calf serum (Gibco). For ^{32}P labelling, cells were harvested, washed once with RPMI 1640 phosphate-free medium/0.5% fetal-calf serum and resuspended in the same medium at 10^6 cells/ml. $[^{32}P]P_i$ was added to the cell culture at a final concentration of 0.4 mCi/ml. After incubation for 1 h at 37 °C, PMA, dissolved in dimethyl sulphoxide, was added at final concentration of 0.5 μM (to control cells, the same amount of dimethyl sulphoxide was added without PMA); incubation was continued for the times indicated. Cells were then harvested, washed once with PBS buffer (85 mM Na_2HPO_4 /1.6 mM NaH_2PO_4 /0.145 M NaCl, pH 7.2) and nuclei were isolated by using the same procedure used for HeLa cells. Nuclei were extracted in the triple-detergent buffer of Sambrook et al. (1989): 50 mM Tris/HCl, pH 8.0, 50 mM NaCl, 0.02% NaN_3 , 0.1% SDS, 1.0% Nonidet P-40, 0.5% sodium deoxycholate. Each buffer used in this procedure contained 10 mM NaF and 10 mM $NaHSO_3$ as phosphatase inhibitors, and 1 mM PMSF.

Two-dimensional gel electrophoresis

The HL-60 cell nuclear extract, diluted 1:5 with 0.5 M Tris/HCl (pH 8.0)/1 mM PMSF, was concentrated on Amicon micro-concentrators (30000- M_r cut-off), washed first with 0.5 ml of distilled water containing 1 mM PMSF, and then with 1 ml of 6 M urea/0.1% Ampholine (pH 3.5–10)/2 mM DTT/1 mM PMSF and loaded on the acidic side of isoelectric-focusing

SDS/4%-polyacrylamide gels, to be subjected to two-dimensional gel electrophoresis as described by Durban et al. (1981).

Tryptic cleavage of topoisomerase I phosphorylated *in vivo*

Gel slices, containing phosphorylated topoisomerase I, were excised from two-dimensional gels and processed as described above for topoisomerase I phosphorylated *in vitro*.

Two-dimensional tryptic-peptide maps

Freeze-dried ^{32}P -labelled tryptic peptides were dissolved in water, spotted on thin-layer cellulose plates (E. Merck), separated by electrophoresis in 1% $(NH_4)_2CO_3$, pH 8.9, at 500 V for 20 min and 200 V for 20 min (first dimension) and ascending chromatography (second dimension) in isobutyric acid/butan-1-ol/pyridine/acetic acid/water (1250:38:96:58:558, by vol.) (Hunter and Sefton, 1980; Boyle et al., 1991).

RESULTS

Topoisomerase I phosphorylation *in vitro* by PKC

Human topoisomerase I was phosphorylated efficiently with PKC in a phospholipid/ Ca^{2+} -dependent manner (Figure 1). Maximal uptake was 1.6 mol of phosphate/mol of topoisomerase I in 25 min. The apparent V_{max} of topoisomerase I was approx. 7-fold lower than that for histone H1 (31 pmol and 208 pmol of phosphate incorporated respectively, by using 1 unit of PKC). The apparent K_m for topoisomerase I as a substrate was 1.9 μg /250 μl assay volume, or about 0.1 μM (Figure 2), indicating high affinity of PKC for topoisomerase I. Affinity of PKC for other protein substrates appears to be somewhat lower; the K_m for good substrate peptides S6 peptide and epidermal-growth-factor-receptor peptide was 0.5 μM and 1.4 μM respectively (House et al., 1987). The K_m for histone H1 under our assay conditions was 2 μM , confirming the value reported previously (Kikkawa et al., 1983). Phosphorylation of topoisomerase I was associated with a 2.5–3-fold increase in topoisomerase I activity

Figure 1 Phosphorylation of HeLa-cell topoisomerase I *in vitro* by PKC

Purified topoisomerase I was phosphorylated with PKC at 30 °C and electrophoresed on an SDS/polyacrylamide 7.5% gel. Lane 1: phosphorylation assay contained 5 μl (1 μg) of topoisomerase I in 1 M KCl/5 mM $MgCl_2$ containing phosphatidylserine (40 μg /ml), diolein (8 μg /ml), 12.5 μCi of $[\gamma\text{-}^{32}P]ATP$ (4.2 μM final concn.), 0.5 mM $CaCl_2$ and 10 μl (0.04 unit) of PKC in 100 μl total volume. Reaction was stopped by addition of 100 μl of Laemmli (1970) sample buffer. Lane 2: as lane 1, but with addition of 1 mM EGTA instead of phosphatidylserine, diolein and $CaCl_2$. Lanes 3 and 4: as lanes 1 and 2 respectively, with addition of Tris/HCl, pH 7.6, to a final concentration of 20 mM, instead of topoisomerase I. The gel was dried and exposed to an X-ray film (Kodak XAR-5) for 3 h. Arrow points to topoisomerase I.

Figure 2 Apparent K_m of PKC for topoisomerase I

Topoisomerase I at different concentrations was phosphorylated with 12.5 μ Ci of [γ -³²P]ATP (1.6 μ M final concn.) as described in Figure 1, except for an assay volume of 250 μ l. The reaction was stopped by addition of 1 ml of 5% trichloroacetic acid/1.5% pyrophosphate, and the precipitate was collected on Millipore HA filters and counted by Čerenkov radiation. K_m was evaluated from a Lineweaver-Burk plot.

(results not shown), in agreement with previous results (Pommier et al., 1990).

Topoisomerase I in HL-60 cells: phosphorylation *in vivo*

Exponentially growing HL-60 cells were treated with PMA for 20 min in the presence of ³²P in the incubation medium. Nuclear extracts of these cells were evaluated by two-dimensional isoelectric focusing-SDS/PAGE, and phosphoproteins were detected by autoradiography (Figure 3). Topoisomerase I was identified by co-migration with authentic purified topoisomerase I stained by Coomassie Blue as described previously (Durban et al., 1981). Topoisomerase I from cells treated with PMA for

Figure 3 Autoradiogram of two-dimensional gels of HL-60-cell nuclear extract

HL-60 cells were incubated, in the presence of [³²P]P_i, with PMA for 20 min (b) or without PMA (a) (as described in the Materials and methods section). Gels were dried and exposed to an XAR-5 film overnight at room temperature. Relative densities of topoisomerase I spots were evaluated on an LKB densitometer (Ultrascan-XL). Isoelectric focusing was in the horizontal dimension (acidic pH at left, basic pH at right). SDS/PAGE was in the vertical dimension. Arrows point to position of topoisomerase I.

Table 1 Time-dependent effect of PMA on topoisomerase I phosphorylation in HL-60 cells

HL-60 cells, incubated in the presence of ³²P, with (+) and without (−) PMA at different times (5, 20 or 60 min) were harvested and nuclear extracts run on two-dimensional gels (as described in the Materials and methods section). Gels were dried and exposed to X-ray film (Kodak XAR-5). Degree of phosphorylation was quantified by densitometric analysis on a LKB densitometer (Ultrascan XL). Abbreviation: AU, relative absorption units, computed by integration of area under absorption peak.

Time (min)	Ratio + PMA (AU)/ − PMA (AU)
5	0.76
20	2.76
60	0.73

Figure 4 One-dimensional tryptic phosphopeptides or topoisomerase I labelled by PKC *in vitro* and of topoisomerase I from HL-60 cells labelled *in vivo*

³²P-labelled topoisomerase I isolated from gel slices was digested with trypsin, and peptides were separated on t.l.c. plates by high-voltage electrophoresis at pH 8.9 as described in detail in the Materials and methods section. (a) Peptides labelled *in vitro*; (b) mixture of material labelled *in vitro* and *in vivo*; (c) phosphopeptides labelled *in vivo*. +, Origin; electrophoresis was from negative to positive poles.

20 min was phosphorylated to an approx. 3-fold higher degree than topoisomerase I from untreated cells (Figures 3b and 3a respectively).

To evaluate the kinetics of PMA treatment, cells were exposed to PMA for different lengths of time. A semi-quantitative densitometric evaluation of the degree of phosphorylation showed 2.5–3-fold higher phosphorylation of topoisomerase I after 20 min exposure to PMA. This time of treatment resulted in the highest response, whereas no difference in degree of phosphorylation was observed 5 min or 60 min after PMA treatment (Table 1). In addition to topoisomerase I, several other

Figure 5 Comparison of phosphopeptides labelled in uninduced HL-60 cells (a) and in cells treated with PMA (b)

Procedures were described in Figure 4.

nuclear proteins were phosphorylated to a higher degree after PMA treatment (Figure 3).

PKC phosphorylation sites of topoisomerase I

The one-dimensional (electrophoretic) phosphopeptide map of human topoisomerase I labelled *in vitro* and in HL-60 cells *in vivo* is shown in Figure 4. Five phosphopeptides were resolved from topoisomerase I labelled by PKC *in vitro*, one of which (spot 3) was much more phosphorylated than the others (lane a). Phosphopeptides from topoisomerase I labelled *in vivo* are shown in lane c; six spots were detected. Three of these (spots 2, 3 and 4) were identical with minor spots 2, 3 and 4 labelled *in vitro* by PKC. Spots 6, 7 and 8 were not seen *in vitro*. Some variability in

intensity of spots 3, 6 and 7 was noted from experiment to experiment; however, the spots identified in the Figures were reproducible. No qualitative change in phosphopeptide pattern was observed after treatment of HL-60 cells with PMA (Figure 5), i.e. no additional spots were found, nor were any spots lost, after PMA treatment. Further evidence for identity of phosphopeptides labelled *in vitro* and *in vivo* was gained from two-dimensional t.l.c. tryptic maps (Figure 6). Some of the spots labelled *in vitro* were resolved into two spots in the two-dimensional map, i.e. 1a and 1b, 2c and 2d, 3e and 3f, and 4g and 4h. Spots 2c and 4g co-migrated *in vitro* and *in vivo*. Spot 3 labelled *in vitro* apparently co-migrated with a minor spot labelled *in vivo* in the one-dimensional maps (Figure 4). However, it is not clear if spot 3 labelled *in vivo*, which did not migrate in the second dimension, is present in the '*in vitro*' map, due to trailing of the strongly labelled spot 3e (Figure 6).

DISCUSSION

Phosphorylation of topoisomerase I increased upon treatment of HL-60 cells with PMA for 20 min. The major cellular response to PMA is activation of PKC (Castagna et al., 1982; MacFarlane, 1986; Hornbeck et al., 1988) and an increase in nuclear localization of PKC (Halsey et al., 1987; Leach et al., 1989), which suggests that topoisomerase I phosphorylation is controlled by PKC. Comparison of phosphopeptide maps of topoisomerase I phosphorylated in HL-60 cells *in vivo* and by PKC *in vitro* does not provide unequivocal evidence for direct phosphorylation of topoisomerase I by PKC, since the major phosphopeptide found *in vivo* is not the major phosphopeptide *in vitro*. Thus increased phosphorylation of topoisomerase I in HL-60 cells *in vivo* may be mediated through an intermediate kinase which is under direct control of PKC. An alternative explanation may be that the conformation of topoisomerase I is different *in vivo* and *in vitro*. *In vivo*, topoisomerase I is loosely bound to DNA/chromatin, on the basis of its extraction properties (Durban et al., 1981), whereas the purified protein *in vitro* is in the free form, and perhaps in a different conformation which may permit altered efficiency of phosphorylation at a particular site. A major site *in vivo* may no longer be the most accessible site for PKC phosphorylation *in vitro*.

An increase in phosphorylation of several other nuclear proteins was observed after PMA treatment of HL-60 cells

Figure 6 Two-dimensional map of tryptic phosphopeptides

Sample preparation, electrophoresis and chromatography conditions are described in the Materials and methods section. After air drying, plates were exposed to Kodak XAR-5 films. Two-dimensional maps are shown of (a) phosphopeptides labelled *in vitro*, (c) phosphopeptides labelled *in vivo*, and (b) mixture of material labelled *in vivo* and *in vitro*. O, origin; E, electrophoresis, from negative to positive poles; C, ascending chromatography.

(Figure 3), in agreement with observations by MacFarlane (1986). Thus PKC may act on several nuclear proteins in a concerted fashion as part of a general regulatory pathway. In this context, it is noteworthy that DNA polymerase α is phosphorylated by PKC *in vitro* and stimulated 3-fold in its enzymatic activity (Krauss et al., 1987).

Recently, Samuels and Shimizu (1992) presented evidence that mouse topoisomerase I is phosphorylated by PKC *in vivo*. Interestingly, fundamental differences seem to exist in phosphorylation of topoisomerase I in human and mouse cell lines. Whereas our '*in vitro*' map of HeLa-cell topoisomerase I is essentially in agreement with that of Samuels and Shimizu (1992), our homologous map of human topoisomerase I labelled *in vivo* appears different from that of mouse topoisomerase I. Since the mouse sequence is not yet available, even minor changes in amino acid sequence could account for differences in phosphorylation sites (Durban et al., 1988). An important physiological difference in mouse and human cell systems was the PMA response time to reach maximal topoisomerase I phosphorylation: optimal phosphorylation was observed 20 min after PMA treatment in human HL-60 cells (Table 1), whereas 60 min or more of PMA treatment was required in the mouse fibroblasts (Samuels and Shimizu, 1992). This difference may relate to intrinsic physiological differences: PMA induces terminal differentiation of the human promyelocytic leukaemia cell line HL-60 (Gallagher et al., 1979; Huberman and Callahan, 1979), whereas cultured fibroblasts undergo general mitogenic metabolic and biochemical changes in response to PMA (Rozenfurt, 1986; Shimizu et al., 1986).

In earlier studies we have shown that topoisomerase I is phosphorylated by NII kinase (casein type II kinase) *in vivo* and *in vitro*. This phosphorylation *in vitro* is associated with enzyme activation (Mills et al., 1982; Durban et al., 1983, 1985). Topoisomerase I activity is also increased by phosphorylation with PKC *in vitro* (Pommier et al., 1990). However, the actual phosphorylation sites are different: NII kinase prefers serine/threonine in an acidic environment (Chan et al., 1986; Edelman et al., 1987), whereas PKC prefers serine/threonine in a basic environment (House et al., 1987). The presence of unique phosphopeptides labelled *in vivo* (spots 6–8) not labelled by PKC *in vitro* (Figure 4) suggests that topoisomerase I has multiple phosphorylation sites whose function is related to stimulation of enzymic activity and may be part of at least two different regulatory phosphorylation cascades. Whether these sites are inter-related in their regulatory function (Roach, 1991) needs to be addressed in future studies.

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