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Inhibition of protein kinase C is associated with a decrease in *c-myc* expression in human myeloid leukemia cells

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Treatment of human myeloid leukemic cells with phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is associated with activation and then partial down-regulation of protein kinase C activity. Previous work has suggested that the activation of protein kinase C by TPA contributes to the decrease in *c-myc* expression during differentiation of these cells. The present studies demonstrate that the decline in *c-myc* mRNA levels following exposure of HL-60 cells to TPA is preceded by an increase in expression of this gene. In contrast, exposure of HL-60 cells to inhibitors of protein kinase C activity is associated with down-modulation of *c-myc* expression. Similar findings have been obtained in U-937 myeloid leukemia cells. Taken together, these findings suggest that phorbol esters have a biphasic effect on *c-myc* expression. Whereas the activation of protein kinase C by phorbol esters may be associated with an increase in *c-myc* gene expression, the subsequent partial down-regulation of kinase activity may initiate a cascade of events resulting in the down-modulation of *c-myc* expression.

Protein kinase C; *c-myc*

1. INTRODUCTION

Phorbol esters induce the terminal differentiation of several human myeloid leukemia cell lines [1]. The identification of protein kinase C as the phorbol ester receptor has indicated that the pleiotropic effects of TPA are related to the activation of this enzyme [2]. Translocation of protein kinase C to the cell membrane is followed by proteolytic degradation of the enzyme, presumably through the activity of a calcium-activated neutral protease [3]. This degradation is associated with a decrease in enzyme activity [4], a decrease in protein level as assessed by immunoblotting [4], and a decrease in the number of phorbol ester binding sites [5]. In this regard, binding of [20-^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDB) to HL-60 cells reaches maximal levels at 15–20 min and then decreases by 60–70% at 1 h [5]. A variant HL-60 subclone which exhibits no down-regulation of [^{3}H]PDB binding was, unlike the parent HL-60 cell line, refractory to differentiation induction by phorbol esters [5]. These findings suggest that the down-regulation of protein kinase C by phorbol esters may be an important event in cellular differentiation.

TPA induces both HL-60 and U-937 cells to differentiate along the monocytic lineage [6,7]. This differentiation is associated with a decrease in *c-myc* expression [6,7]. Previous studies have indicated that a decrease in *c-myc* mRNA levels is necessary and perhaps sufficient for induction of hematopoietic cell differentiation [8,9]. Since activation of protein kinase C is associated with induction of *c-myc* expression in certain resting cells [10], we undertook studies to investigate the hypothesis that the dual effects of phorbol esters on protein kinase C activity may correlate with differential expression of the *c-myc* gene in myeloid leukemia cells. The results demonstrate that translocation of protein kinase C by phorbol ester is initially associated with an increase in *c-myc* transcripts and that down-regulation of protein kinase C is associated with a decrease in expression of this gene.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

HL-60 and U-937 cells (American Type Culture Collection, Rockville, MD) were grown as described [11]. TPA was obtained from Sigma Chemical Co., St. Louis, MO. 1-(5-isoquinolinylsulfonyl)-2-Methylpiperazine (H-7) and *N*-(2-guanidinoethyl)-5-isoquinolinsulfonamide (HA1004) (Seikagaku America, Inc.) were dissolved in phosphate-buffered saline (PBS). Staurosporine (Sigma) was dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in all experiments was 0.1%. Sangivamycin (provided by the Natural Products Branch, National Cancer Institute) was used at a final concentration of 10 μM.

2.2. *Northern-blot analysis*

Total cellular RNA was isolated by the guanidine isothiocyanate/cesium chloride method, separated in a 1% agarose/2.2 M formaldehyde gel, transferred to a nitrocellulose filter and hybridized to the following ^{32}P-labeled DNA probes: (1) the 1.8-kb *ClaI/Eco*RI fragment containing the human *c-myc* 3′ exon purified from the pM C41-3

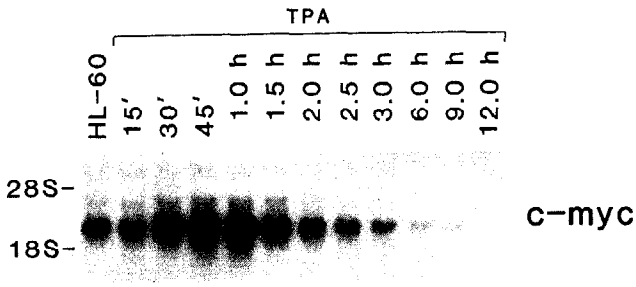

Fig. 1. Effects of TPA on c-myc expression in HL-60 cells. HL-60 cells were treated with 32 nM TPA. Total cellular RNA (20 μg/lane) was isolated at the indicated times for Northern analysis with the ^{32}P-labeled *c-myc* probe.

RC plasmid [12]; and (2) the 2.0-kb *Pst*I cDNA of the chicken β-actin gene purified from the pA1 plasmid [13].

2.3. *Protein kinase C assays*

HL-60 cells (2×10^7) were suspended in 0.4 ml ice-cold TEM buffer (20 μM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 10 mM β-mercaptoethanol) containing 25 μg/ml leupeptin and 25 μg/ml aprotinin. The cells were disrupted using a syringe and 25-gauge needle, incubated on ice for 30 min and then spun in a microcentrifuge for 10 min. The supernatant (cytosol) was kept for partial purification on DEAE cellulose columns. The pellet (membrane fraction) was suspended in 0.4 ml ice-cold TEM buffer containing 0.5% Triton X-100, 25 μg/ml leupeptin and 25 μg/ml aprotinin. The suspension was sonicated in glass tubes for 15 s, incubated on ice for 30 min and then spun in a microcentrifuge for 10 min. The supernatant (detergent-solubilized membrane) was partially purified on DEAE cellulose columns. Both cytosol and membrane fractions were eluted with 1.5 ml of column buffer containing 200 mM NaCl as previously described [14]. The DEAE eluate was diluted in TEM containing 200 μM NaCl to obtain a range of three different enzyme concentrations. Protein kinase C activity was determined as described [15, and Protein Kinase C Assay System, Gibco BRL, Grand Island, NY]. The partially purified protein extract in TEM/NaCl was incubated for 5 min at 30°C in phospholipid (phosphatidyl serine and phorbol ester in Triton X-100 mixed micelles: Gibco BRL), [γ-^{32}P]ATP and protein kinase C synthetic peptide [Ser^{25}] PKC(19–31) (Gibco BRL). All assays were also performed in the presence of a protein kinase C inhibitor peptide (Gibco BRL) to ensure specificity of the phosphorylation reaction. The samples were dried on phosphocellulose, washed in 1% H_3PO_4 and assayed by scintillation counting. Protein kinase C activity was determined as described in the Protein Kinase C Assay System (Gibco BRL).

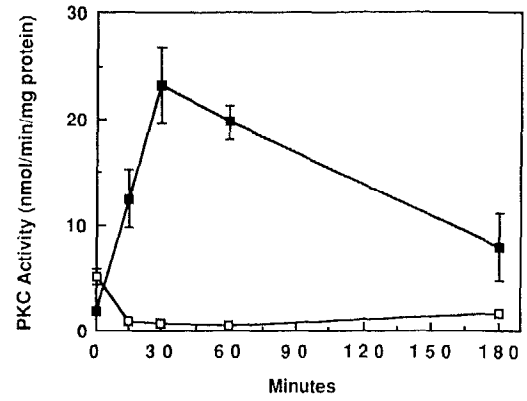

Fig. 2. Activation and translocation of protein kinase C in TPA-treated HL-60 cells. HL-60 cells were treated with 32 nM TPA. At the indicated times, cells were harvested, lysed, and cytosol and membrane-bound fractions prepared. Protein kinase C activity was assayed using a synthetic peptide substrate as described in section 2. Protein kinase C (PKC) activity of the membrane (■) and cytosolic (□) fractions are expressed as nmol/min/mg protein.

3. RESULTS

Fig. 1 demonstrates the biphasic effect of TPA on *c-myc* mRNA levels. The *c-myc* gene is constitutively expressed in HL-60 cells and exposure to 32 nM TPA was associated with an increase in *c-myc* transcripts that was maximal at 60 min (Fig. 1). In contrast, longer TPA exposures resulted in a progressive decline in *c-myc* expression to nearly undetectable levels by 6 h (Fig. 1). These findings were compared to the effects of TPA on protein kinase C activation. Treatment of HL-60 cells with 32 nM TPA was associated with a translocation of protein kinase C activity from the cytosol to the membrane fraction (Fig. 2). This activation of protein kinase C was maximal at 30 min and corresponded to the maximal increases in *c-myc* expression. In contrast, exposure to TPA for 1–3 h was associated with a partial down-regulation in membrane-associated protein kinase C activity (Fig. 2). These findings suggested that down-regulation, rather than activation, of protein ki-

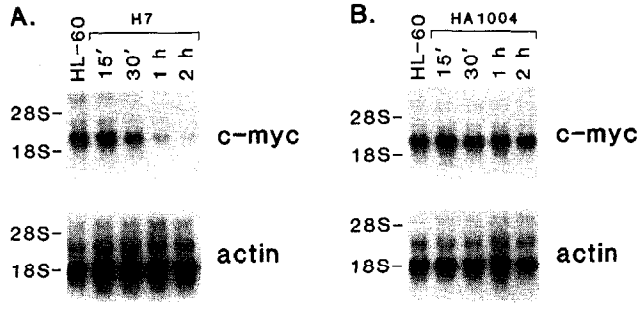

Fig. 3. Effects of the protein kinase inhibitors H7 and HA1004 on *c-myc* expression in HL-60 cells. HL-60 cells were treated with 10 μM H7 (A) or 10 μM HA1004 (B). Total cellular RNA (20 μg/lane) was isolated at the indicated times for hybridization with the labeled *c-myc* and actin probes.

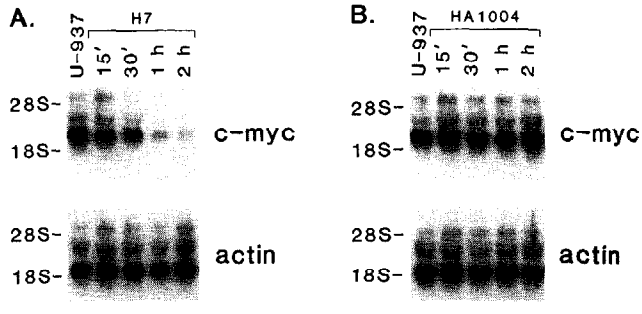

Fig. 4. Effects of the protein kinase inhibitors H7 and HA1004 on c-myc expression in U-937 cells. U-937 cells were treated with 10 μM H7 (A) or 10 μM HA1004 (B). Total cellular RNA (20 μg/lane) was isolated at the indicated times for Northern analysis with the labeled *c-myc* and actin probes.

nase C might contribute to decreases in *c-myc* expression.

In order to further address the effects of protein kinase C down-regulation on *c-myc* mRNA levels, we used H7, an isoquinolinsulfonamide inhibitor of this enzyme [16]. Exposure of HL-60 cells to H7 resulted in a decrease in *c-myc* transcripts to nearly undetectable levels by 60 min (Fig. 3A). In contrast, H7 had no detectable effect on actin mRNA levels (Fig. 3A). Since H7 also inhibits cyclic nucleotide dependent protein kinases, we performed similar experiments with HA1004, an agent related to H7 with more selective effects against cAMP and cGMP dependent protein kinases [17]. Exposure of HL-60 cells to HA1004 had no effect on either *c-myc* or actin expression (Fig. 3B). In order to determine whether these effects were restricted to HL-60 cells, we performed additional studies with the U-937 cell line. Exposure of U-937 cells to H7 similarly resulted in a rapid decrease in *c-myc* transcripts, while there was no change in actin expression (Fig. 4A). In contrast, exposure of U-937 cells to HA1004 had little effect on either *c-myc* or actin mRNA levels (Fig. 4B). Taken together, these findings indicated that the effects of H7 on *c-myc* expression were related to inhibition of protein kinase C and not cyclic nucleotide-dependent protein kinases.

Other studies were performed with staurosporine, a structurally distinct inhibitor of protein kinase C [18]. Exposure of HL-60 cells to this agent resulted in down-regulation of *c-myc* expression to nearly undetectable levels by 1 h (Fig. 5A). *c-myc* mRNA levels were also decreased following staurosporine treatment of U-937 cells (Fig. 5B). These changes in *c-myc* expression were associated with little if any effect on actin mRNA levels. Recent studies have demonstrated that sangivamycin, a member of the pyrrolo[2,3-d]pyrimidine class of nucleoside analogues, is a potent inhibitor of protein kinase C [19,20]. Exposure of U-937 cells to sangivamycin was also associated with a decrease in *c-myc* mRNA levels (Fig. 6). Taken together, these data suggest that multiple agents which inhibit protein kinase C activity are associated with down-regulation of *c-myc* expression.

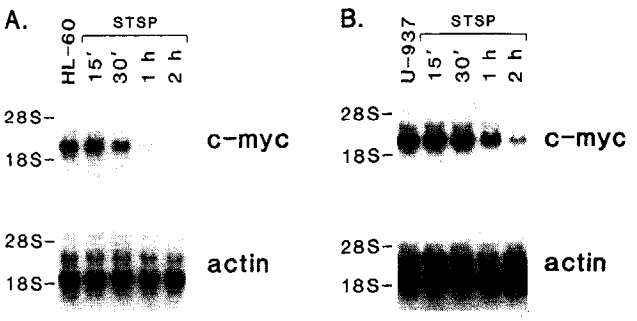

Fig. 5. Effects of the protein kinase inhibitor staurosporine (STSP) on *c-myc* expression in HL-60 and U-937 cells. HL-60 cells (A) and U-937 cells (B) were treated with 50 μM staurosporine. Total cellular RNA (20 μg/lane) was isolated at the indicated times for hybridization to the labeled *c-myc* and actin probes.

4. DISCUSSION

A decrease in *c-myc* gene expression is a necessary step for hematopoietic cell differentiation. For example, Friend murine erythroleukemia (MEL) cells transfected with constitutively expressed *c-myc* sequences are resistant to induction of differentiation [21]. In addition, *c-myc* antisense oligonucleotides inhibit HL-60 cell growth [9] and accelerate MEL cell differentiation in response to DMSO [22]. The precise mechanism whereby the *c-myc* protein regulates growth and differentiation is unclear, however there is recent data to suggest that *c-myc* may function in part as a transcriptional repressor, perhaps through its effect on other transcription factors such as CTF/NF-1 [23].

Although much is known about the regulation of *c-myc* gene expression, little is known about the second messenger pathways involved in controlling the decrease in its expression during hematopoietic differentiation. Treatment of MEL cells with DMSO results in an early decrease in inositol-triphosphate and diacylglycerol levels which precedes the decrease in both *c-myc* gene and protein expression [24]. As diacylglycerol is the physiological activator of protein kinase C [25], these findings suggested a possible relationship between a decrease in kinase activity and a decrease in *c-myc* expression. Taken together with the results presented in this study, the decrease in *c-myc* expression seen during hematopoietic differentiation may be due in part to the down-regulation of protein kinase C. Further studies will be needed to define the role of the catalytically active fragment PKM [3], as well as that of the individual protein kinase C isoenzymes [26–28] in *c-myc* regulation.

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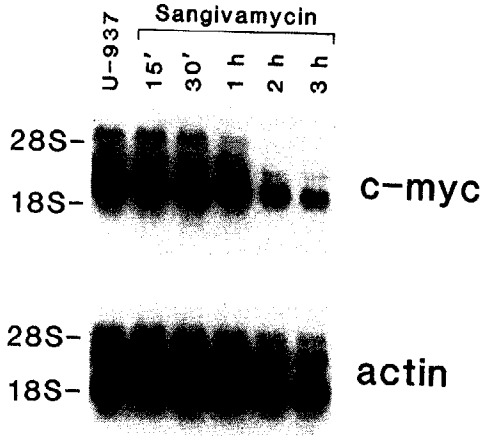

Fig. 6. Effects of the protein kinase inhibitor sangivamycin on *c-myc* expression in U-937 cells. U-937 cells were treated with 10 μM sangivamycin. Total cellular RNA (20 μg/lane) was isolated at the indicated times for hybridization to the labeled *c-myc* and actin probes.

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