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Tyrosine protein phosphorylation is required for protein kinase C-mediated proliferation in T cells

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We have studied the role of tyrosine kinase in PMA-stimulated T cells. Protein kinase C (PKC)-mediated D10A cell proliferation is inhibited by the specific inhibitor of tyrosine kinase, tyrphostin. This inhibitor selectively blocks the mRNA expression of the proto-oncogene c-myc in response to the phorbol ester, PMA. On the other hand, the same doses of this inhibitor do not affect the mRNA expression of the proto-oncogene c-fos in PMA-stimulated D10A cells. Phorbol esters induce in this T cell line the tyrosine phosphorylation of a unique protein of 42 kDa and the enzyme PKC is required for this activity.

Protein kinase C; C-myc; Tyrosine kinase; Cell proliferation; T cell

1. INTRODUCTION

Mature T cells are functionally specialized for the antigen recognition in the context of major histocompatibility complex molecules [1]. Such recognition is mediated by the T cell receptor (Tcr) for the antigen that consists of a heterodimer (α and β chains) associated with at least five proteins known as the CD3 complex [2]. Thus, occupancy of the Tcr/CD3 complex by the antigen, monoclonal antibodies or mitogen lectins leads to the hydrolysis of phosphatidyl inositol bisphosphate resulting in the generation of two important second messengers, namely, inositol triphosphate that increases the $[Ca^{2+}]_i$ and diacylglycerol that activates the enzyme protein kinase C (PKC) [3].

The enzyme PKC plays an important role in the mechanisms of T cell activation and phosphorylates specific protein substrates in serine/threonine residues and in so doing, may activate a number of cellular processes such as gene transcription and protein synthesis. PKC is activated physiologically by DAG, but it is also the receptor for phorbol esters which induces a stronger and longer activation of PKC than diacylglycerol [4].

Activation of T and B cells through the antigen receptor triggers phosphorylation of a set of proteins in tyrosine residues [5,6]. At least one of these proteins is phosphorylated through the PKC pathway since it has tyrosine residues that can be phosphorylated by phorbol ester treatment alone. This protein, present also in fibroblasts, has been identified as the 42 kDa Mn^{2+}-dependent kinase that phosphorylates the microtubule-associated protein 2 (MAP-2K) and is involved in cell proliferation, although its function is completely unknown at present [7].

In this report we collect evidence which suggests that this protein is required in D10A cells for the c-myc mRNA expression and cellular proliferation in response to phorbol esters.

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2. MATERALS AND METHODS

2.1. *Media and reagents*

Complete medium was RPMI 1640 (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine (Gibco), 1 mM Hepes, 5×10^{-5} M 2-mercaptoethanol, antibiotics (Gibco), and 10% fetal calf serum (HyClone, Logan, UT). [α-^{32}P]dCTP (800 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate were from Sigma (St Louis, MO). Tyrosine kinase inhibitor tyrphostin was synthesized as previously described [8] and was a gift from Dr C.K. Huang (Univ. of Connecticut Health Center, CT). The mAb, 4G10 (anti-phosphotyrosine) was a gift from Dr T.M. Roberts (Dana Farber Cancer Institute, Boston). ^{125}I-protein A (30 mCi/mg) was from Amersham (Arlington Heights, IL).

2.2. *Proliferation assays*

The generation and maintenance of the D10A cell line has been described elsewhere [9]. Proliferative responses of mycoplasma free D10A cells were assessed after purifying live cells on a Ficoll-Paque gradient (Pharmacia, Piscataway, NJ) at least one week after culture in complete medium without lymphokines. Cells (2.5×10^4/ml) were incubated in 200 μl aliquots in 96 well plates with PMA (10 ng/ml) or IL-2 (10 U/ml) in the presence or absence of tyrphostin. The cultures were incubated for 48 h at 37°C, and [^{3}H]TdR (0.5 μCi/well)

was added for the final 12 h of culture. Radioactivity incorporated into DNA was measured by liquid scintillation counting.

2.3. *RNA preparation and analysis*

Resting D10A cells were stimulated as indicated in the figure legends. Total RNA was extracted as described [10], with some modifications [11]. Twenty μg of total RNA were electrophoresed on a Northern gel (1% agarose, 2% formaldehyde and 1 × Mops), and the gel was blotted onto a nylon membrane (Biotrans Nylon Membrane, ICN, Irvine, CA). The RNA was linked to the membrane by UV irradiation [12]. The blots were prehybridized in 5% SDS, 100 mM NaCl, 50 mM sodium phosphate and 1 mM EDTA for 2 h at 65°C, and then hybridized for 12 h in a new aliquot of the same solution, containing an [α^{32}P]dCTP labelled cDNA probe that was prepared with a random priming kit (Boehringer Mannheim, Germany). The hybridized membranes were washed 3 times for 5 min at 65°C in 5% SDS, 0.5 × SSC and 3 times for 15 min at 65°C in 0.5% SDS, 0.1 × SSC. The blots were exposed to Kodak XAR films at -70°C with enhancer screens.

2.4. *cDNA probes*

The following cDNAs were used for the preparation of random primed probes: c-myc is an *EcoR*I/*Bam*HI fragment isolated from a PGEM plasmid, c-fos is an *Eco*RI fragment from a pBR322 plasmid and β-actin is a *Pst*I fragment from the PGEM plasmid. All plasmids were obtained from ATCC.

2.5. *Immunoanalysis*

The analysis of protein phosphorylated in tyrosine residues was performed as described elsewhere [13]. Resting D10A cells (5×10^6/ml) were stimulated with PMA (10 ng/ml) for the indicated times. In the case of PKC depletion, the cells were treated with PMA (250 nM) for 48 h and then washed 3 times with cold PBS and stimulated again with PMA (10 ng/ml) for 30 min. After stimulation, the cells were spun down and the resulting pellet resuspended in 100 μl of lysis buffer (0.5% Triton X-100, 50 mM Tris, pH 7.6, 300 mM NaCl, 1 mM Na_3VO_4, 5 mM EDTA, 10 μg/ml leupeptin and 2 mM PMSF) and incubated on ice for 5 min. The lysates were centrifuged at 13 000 rpm for 5 min and the supernatants mixed with 100 μl of 2 × Laemmli buffer [14].

Proteins were electrophoresed in a 10% SDS/PAGE gel and then electroblotted onto nitrocellulose paper. Transferred tyrosine phosphorylated proteins were identified using the mAb 4G10 as first antibody. The second Ab used was ^{125}I-protein (0.5 μCi/ml). The blots were exposed to Kodak XAR films at -70°C.

3. RESULTS AND DISCUSSION

To study the role of tyrosine kinase(s) in the proliferation of D10A cells in response to phorbol esters, we have used tyrphostin, a specific inhibitor of tyrosine kinases, at micromolar concentrations; tyrphostin is only a weak inhibitor of PKC and cAMP-dependent kinase when it is used at millimolar concentrations [8]. As shown in Fig. 1, tyrphostin prevents PMA-induced proliferation as well as the proliferation mediated by IL-2. This latter result is consistent with recent reports that demonstrate a role for tyrosine kinase(s) in the IL-2 signal transmission pathways [15,16]. The lack of proliferation in response to IL-2 or PMA in the presence of tyrphostin was not due to toxicity as determined by Trypan blue exclusion (data not shown).

The expression of some proto-oncogenes is closely related to the mechanisms that regulate the processes of cellular proliferation [17,18]. Thus, we were interested

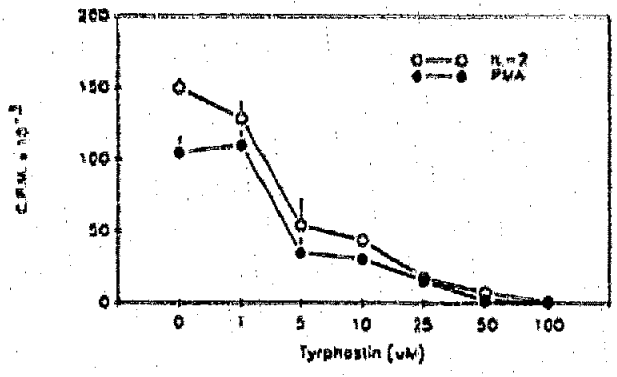

Fig. 1. Tyrphostin inhibition of PMA and IL-2 induced D10A cell proliferation. Resting D10A cells were incubated with PMA (10 ng/ml) or IL-2 (10 U/ml) in the presence of increasing doses of tyrosine kinase inhibitor. Proliferative response was measured at 48 h. Results represent the mean and SD of tripliente wells.

in assessing the role of tyrphostin in the c-fos and c-myc proto-oncogene mRNA expression in D10A cells stimulated with PMA.

To study c-fos mRNA expression, resting D10A cells were stimulated for 30 min at 37°C with PMA in the presence or absence of tyrphostin. Total RNA was extracted and the expression of c-fos was studied by RNA blots. C-fos mRNA expression induced by PMA was not affected by the action of tyrphostin (Fig. 2). This result suggests that c-fos mRNA expression is not essential for D10A cell proliferation in response to PMA. In support of this hypothesis, we have observed that a c-fos oligomer anti-sense could not block the proliferation of D10A cells stimulated with an mAb anti-CD3 (E. Muñoz and B.T. Huber, unpublished

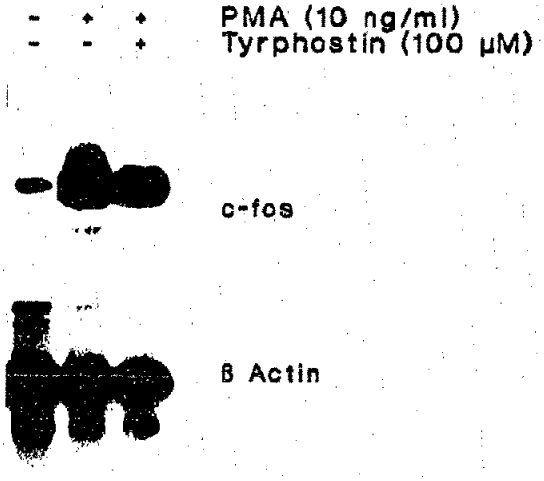

Fig. 2. C-fos mRNA expression in PMA stimulated D10A cells. Cells were stimulated with PMA (10 ng/ml) in the presence or absence of tyrphostin (100 μM) for 30 min. Total RNA was extracted and analyzed on a RNA gel blot. The blot was hybridized with specific cDNA probes for c-fos and β-actin.

results). In contrast, c-fos mRNA expression could be necessary for IL-5 mRNA expression. This gene is PMA inducible and requires the new synthesis of protein to be expressed [9,11]. In addition, tyrphostin did not block the expression of IL-5 and IL-6 mRNA in D10A cells stimulated with PMA (E. Muñoz, A. Zubiaga, C.K. Huang, and B.T. Huber, manuscript submitted for publication).

We have previously shown that c-myc mRNA is accumulated in D10A cells after PKC activation [19]. To address the role of protein tyrosine kinase(s) in this pathway, D10A cells were stimulated with PMA alone or PMA plus tyrphostin for 3 h at 37°C. After this time, total RNA was extracted and c-myc mRNA expression detected by an RNA gel. In Fig. 3 it is shown that c-myc mRNA expression in response to PMA was completely inhibited by tyrphostin, which support c-myc involvement in D10A proliferative response to PMA.

As previously shown, PMA can induce tyrosine protein phosphorylation of different substrates in distinct cell types [5,6-20]. Thus, we were interested in identifying the substrates tyrosine phosphorylated in response to phorbol esters in D10A cells. D10A cells were stimulated with PDB or PMA at different timepoints. Proteins were extracted, blotted and tyrosine phosphorylation identified by using the mAb 4G10. PMA-induced tyrosine phosphorylation of a unique protein of 42 kDa (Fig. 4A, lane 2). This protein was also detected in D10A cells stimulated with another phorbol ester, PDB, and it was rapidly phosphorylated in the first 5 min of stimulation (Fig. 4B, lane 2). To ascertain whether the action of PMA was specifically mediated through PKC, D10A cells were depleted of PKC by pretreating this T cell line with high doses of PMA for 48 h, under which conditions PKC was absent in this cell line [21], and cells were unable to express c-myc mRNA in response to PMA [19]. PMA did not induce the 42 kDa protein tyrosine phosphorylation in PKC-depleted D10A cells (Fig. 4A, lane 3) which demonstrates that PKC is required for PMA-induced tyrosine phosphorylation. Similarly, Einspahr et al. have demonstrated recently that phorbol esters and a synthetic diacylglycerol promote phosphorylation of the 42 kDa protein and also induce the tyrosine phosphorylation of other proteins in natural killer cell lines [20]. We and others have been able to detect phosphorylation only in the 42 kDa substrate either in D10A or in B cells, respectively [6].

This 42 kDa protein is likely to be the MAP-2K which is activated in human T cells in response to Tcr and PMA stimulation [5] and in fibroblasts stimulated with insulin and epidermal growth factor [7]. This enzyme is a serine/threonine kinase that has to be tyrosine phosphorylated by PKC to be active. The results presented in this report strongly suggest that this kinase is required for c-myc mRNA expression and cellular proliferation in D10A cells, since tyrphostin inhibits selectively c-myc proto-oncogene mRNA expression, and the 42 kDa protein is the only substrate which is phosphorylated in tyrosine residues in response to phorbol esters.

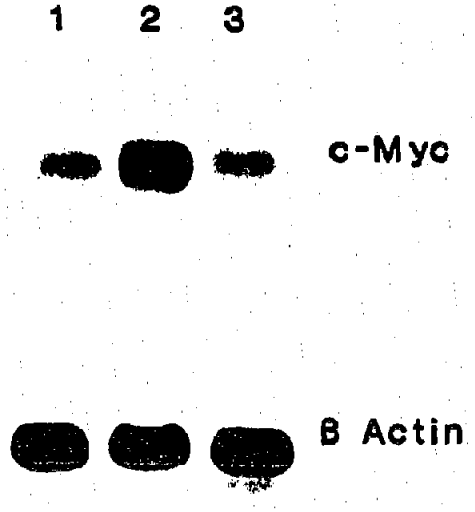

Fig. 3. C-myc mRNA expression in PMA stimulated D10A cells is inhibited by tyrphostin. Resting cells were stimulated as follows: nothing (lane 1), PMA (10 ng/ml) (lane 2), and PMA plus tyrphostin (100 μM) (lane 3). The stimulation was carried out for 3 h and c-myc mRNA expression identified in RNA blot.

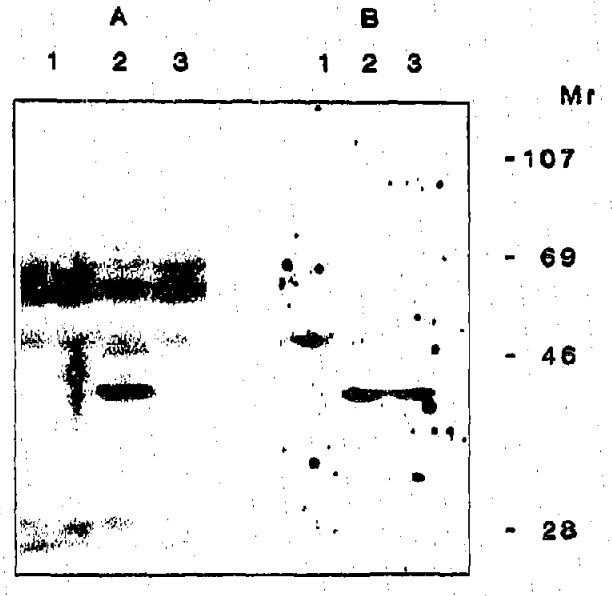

Fig. 4. Phorbol esters induce tyrosine phosphorylation in D10A cells. Resting cells were stimulated as follows. (A) Nothing (lane 1), PMA (10 ng/ml) (lane 2), and PMA (10 ng/ml) in PKC depleted cells (lane 3). The stimulation was carried out for 30 min. (B) Nothing (lane 1), PDB (20 ng/ml for 5 min) (lane 2), and PDB (20 ng/ml for 30 min) (lane 3). Tyrophosphoproteins were identified by Western blot as described in Materials and Methods.

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