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Interleukin 3 and phorbol ester stimulate tyrosine phosphorylation of overlapping substrate proteins

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FDC-P1 is a murine myeloid cell line that requires interleukin 3 (IL3) for survival and proliferation. While the biological effects of IL3 have been well described, the biochemical mechanisms of IL3 actions have only recently been examined. We have investigated whether IL3 or PMA stimulates phosphorylation of proteins on tyrosine as well as on serine/threonine residues as previously described [(1986) Blood 68, 906–913; (1987) Biochem. J. 244, 683–691]. Here we report that both IL3 and PMA stimulate the tyrosine phosphorylation of at least two proteins: pp70 and pp50 in FDC-P1 cells.

Interleukin 3; Phorbol ester; Tyrosine kinase; Phosphorylation

1. INTRODUCTION

IL3 is a member of the family of polypeptide colony-stimulating factors (CSFs) that collectively regulate the proliferation and differentiation of all of the myeloid blood cell lineages [4]. The recent molecular cloning of murine IL3 [5] together with the establishment of IL3-dependent cell lines [6] has allowed investigations into the biochemical mechanisms of IL3 actions. In previous reports we described the IL3-dependent translocation of PK-C as well as the serine/threonine phosphorylation of several proteins that were also phosphorylated in response to pharmacological activators of PK-C [1–3]. We found, however, that while both IL3 and PMA or OAG stimulated the phosphorylation of certain proteins in common, some unique proteins were phosphorylated only in response to IL3. This indicated that while PK-C, or a PK-C-like kinase, was probably involved in IL3 signal transduction, other kinase systems were also activated. These studies were interesting in view of findings that phorbol esters replace IL3 for the regulation of DNA synthesis, ATP regeneration and hexose transport in CSF-dependent cell lines [7].

A variety of oncogenes [8] as well as many growth factor receptors [9] possess protein tyrosine kinase activity. It has been postulated that tyrosine phosphorylation is involved in these growth factor signal transduction pathways and in the abrogation of serum dependence in cell lines transformed by viral oncogenes encoding tyrosine kinases. Moreover, IL3-dependent cell lines have been transformed by tyrosine kinase oncogenic proteins such as v-abl [10,11]. In nontransformed factor-dependent cell lines the importance of tyrosine phosphorylation to signal transduction and proliferation has been difficult to assess because phosphotyrosine typically accounts for less than 1% of the total phosphoamino acids present in cellular extracts. The availability of antiphosphotyrosine antibodies [12], however, has greatly

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simplified analysis of tyrosine phosphorylation and several recent reports have described stimulation of tyrosine kinase activity by IL3 [13,15]. Here we have used antiphosphotyrosine antibodies coupled to Sepharose and two-dimensional gel electrophoresis to purify and characterize proteins phosphorylated on tyrosine residues following either IL3 or PMA treatment of the myeloid, IL3-dependent cell line, FDC-P1.

2. MATERIALS AND METHODS

2.1. *Cells*

FDC-P1 cells were routinely grown in RPMI-1640/10% fetal calf serum/1% glutamine and penicillin-streptomycin supplemented with 10% conditioned medium from the WEHI-3 cell line. For labeling and ligand stimulation, FDC-P1 cells were washed twice in phosphate-free RPMI-1640 and resuspended at 2×10^6 cells/ml for 20 min at 37°C. The cells were then washed and resuspended in phosphate-free RPMI-1640 at 1.5×10^7/ml in a volume of 1 ml in Eppendorf tubes.

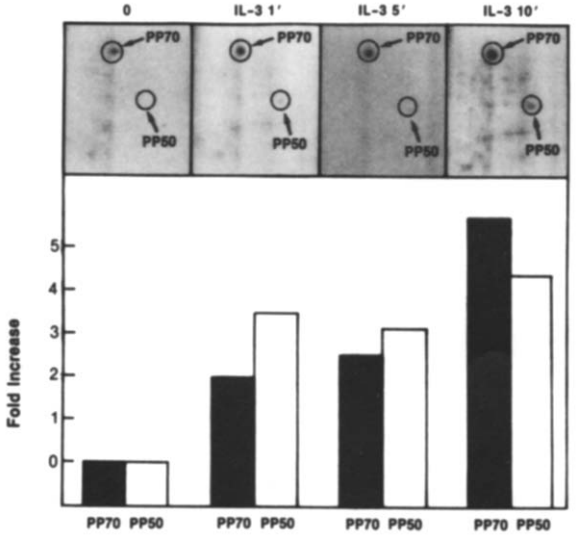

Fig.1. Two-dimensional NEPHGE analysis of antiphosphotyrosine affinity-purified phosphoproteins from IL3-stimulated FDC-P1. The phosphate-labeled, growth-arrested cells were stimulated with IL3 for the indicated times and prepared for NEPHGE analysis as described in section 2. The locations of two phosphoproteins modulated by IL3 are indicated. The histogram shows the -fold increases in radioactivity incorporated into the proteins as determined using a two-dimensional laser densitometer (LKB Ultrascan XL) and LKB scanning software.

2.2. *Phosphate labeling and antiphosphotyrosine affinity adsorption*

[^{32}P]Orthophosphate (Amersham, 8 mCi/ml) was neutralized to pH 7.2 with NaOH and 0.5 mCi (about 0.1 ml) was added to each tube. Following 2–3 h equilibration at 37°C in a shaking water bath, 100 U/10^6 cells of recombinant *E. coli*-derived murine IL3 (Biogen) was added in a volume of 0.05 ml. Cells were resuspended, vortex-mixed for 3 s and replaced in the shaking water bath. At the indicated time points cells were rapidly pelleted (8000 rpm/10 s) in a microfuge and washed once with 1.0 ml cold PBS.

Cell pellets were resuspended in 0.1 ml buffer A (20 mM Hepes, pH 7.2/0.2% SDS/0.15 M NaCl/1 mM EDTA) and boiled in a water bath for 10 min. Upon rapid cooling on ice, extracts were mixed with Triton X-100 to give 1% final concentration. After 30 min on ice, the extracts were clarified by centrifugation at 10000 rpm for 10 min in a microfuge and 0.02 ml antiphosphotyrosine-coupled Sepharose was added. Affinity adsorption proceeded overnight, with rotation, at 4°C. The suspensions were centrifuged at 12000 rpm for 10 min and washed twice with 0.6 ml buffer A. Phosphotyrosyl proteins were specifically eluted from the affinity capture beads by the addition of 0.1 ml of 50 mM phosphotyrosine (Sigma), pH 7.2, in buffer A. The samples were rotated at 4°C for 1 h then centrifuged at 12000 rpm for 10 min.

2.3. *Two-dimensional NEPHGE analysis*

Samples (80 μl) of the supernatants were removed and added to 50 mg urea, 30 μl IEF sample buffer [9.5 M urea, 2% (w/v)

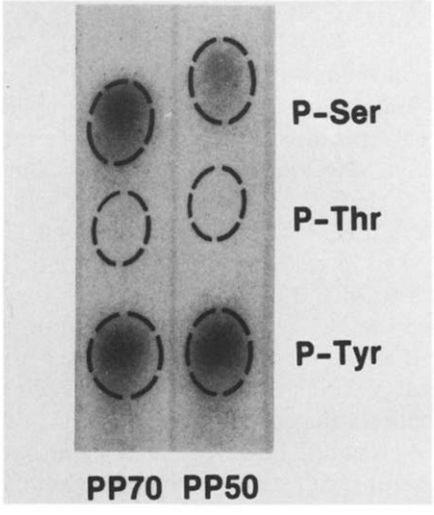

Fig.2. Phosphoamino acid analysis of pp50 and pp70 after IL3 stimulation. The areas on the 2D NEPHGE gels corresponding to pp50 and pp70 on the autoradiographs were sliced out, and the proteins eluted and hydrolyzed in 6 N HCl. The hydrolysates were mixed with phosphoamino acid standard and subjected to high-voltage, single-dimension thin-layer electrophoresis. Positions of the standards were located with ninhydrin (dashed circles) and radioactive phosphoamino acids were located by autoradiography in the presence of intensifying screens.

NP40, pH 5–7 ampholines (1.6%), pH 3.5–10 ampholines (0.4%) 5% 2-mercaptoethanol] and 10 μl of 10% NP40. Standard NEPHGE gels were electrophoresed for 6 h at 500 V, then the tube gels were removed, and equilibrated for 30 min in concentrated SDS sample buffer. Tube gels were attached and electrophoresed on 10% SDS slab gels. Gels were supported on filter paper, dried (Biorad gel drier, Richmond, CA) and autoradiography performed (Kodak X-Omat or SB film) at room temperature.

2.4. *Phosphoamino acid analysis*

The locations of phosphorylated proteins identified by autoradiography were excised from the gels and placed in 0.5 ml extraction buffer (50 mM NH_4CO_3, 0.1% SDS, 5% 2-mercaptoethanol), boiled for 5 min and shaken for 24–36 h at 37°C. Extracted proteins were dried on a rotary evaporator (Savant) and washed once with 0.1 ml of 20% trichloroacetic acid at 4°C and three times with ethanol/ether (3:1). After air-drying the samples were hydrolyzed for 1.5 h at 110°C in 0.4 ml of 6 N HCl and dried on the rotary evaporator.

Single-dimension thin-layer electrophoresis was carried out at 1500 V for 45 min using cellulose acetate thin-layer plates with pyridine/glacial acetic acid/water (10:100:1890) pH 3.5. Phosphoamino acids were identified using unlabeled standards located with ninhydrin and radioactive amino acids were identified by autoradiography.

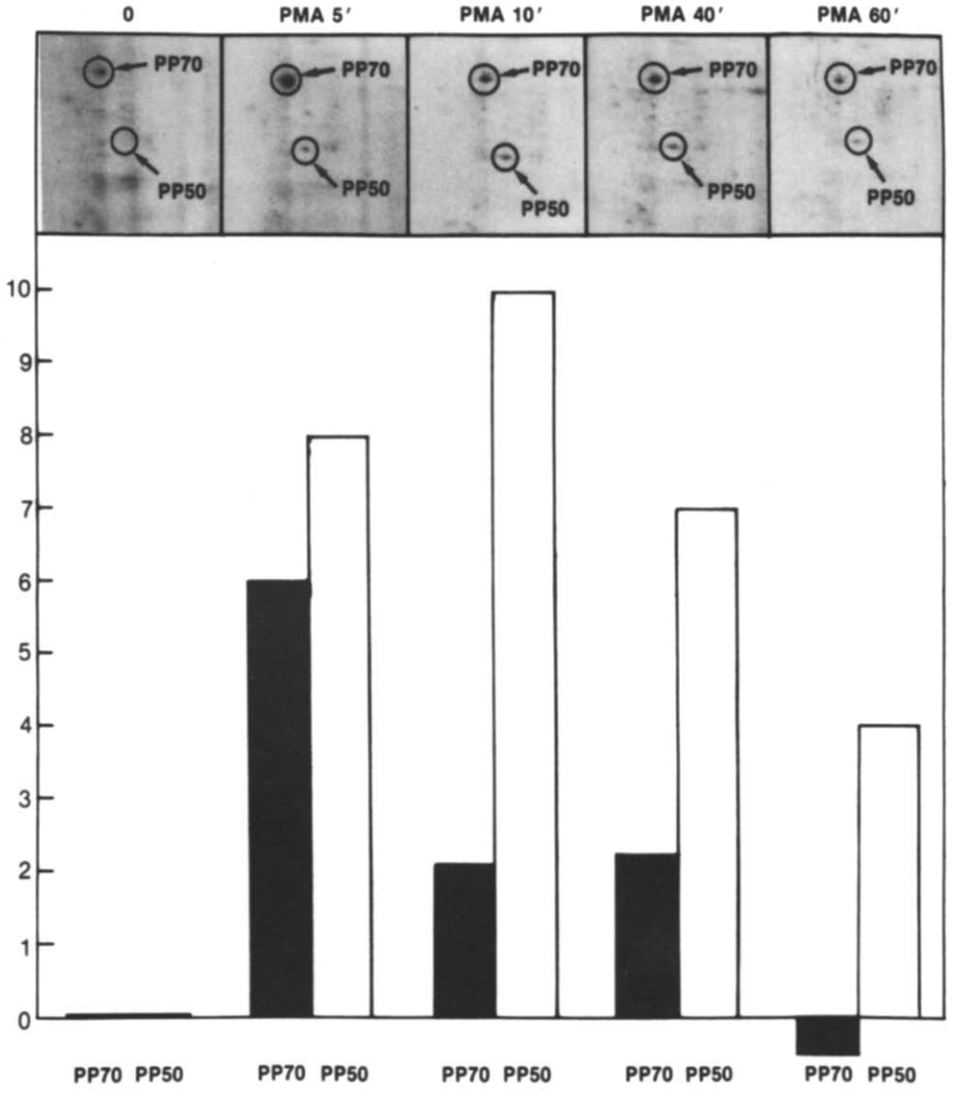

Fig.3. Two-dimensional NEPHGE analysis of antiphosphotyrosine affinity-purified phosphoproteins from PMA stimulated FDC-P1. The phosphate-labeled, growth-arrested cells were stimulated with PMA for the indicated times and prepared and analyzed as described. The histogram shows -fold increases in radioactivity incorporated into the indicated phosphoproteins.

3. RESULTS

3.1. *IL3 stimulated tyrosine phosphorylation*

Actively growing FDC-P1 cells were washed and labeled as described in section 2. After a 2 h equilibration period with $^{32}P_i$ the cells were treated with IL3 for various lengths of time.

We examined the time course of protein phosphorylation induced by IL3 by 2-dimensional nonequilibrating pH gradient gel electrophoresis (NEPHGE) as described. The technique used a low concentration of urea to examine selectively those proteins with a high solubility in urea. This enabled a very high resolution of a few proteins to be obtained. After affinity purification and selective solubilization pp70 and pp50 were the most prominent proteins observed in 2D NEPHGE autoradiographs. In these experiments we found that these two proteins were rapidly phosphorylated in response to IL3 (fig.1). Within 1 min pp70, the most prominent phosphoprotein, underwent a 2-fold increase in phosphorylation and continued to reach 6-fold after 10 min. During the same period, pp50 phosphorylation increased 4.5-fold. Phosphoamino acid analysis (fig.2) confirmed that both of these proteins contained phosphotyrosine as well as phosphoserine. Since the ratios of phosphotyrosine to phosphoserine (40:60 for pp70, and 90:10 for pp50) did not change significantly with time (not shown), we concluded that IL3 simultaneously stimulated phosphorylation by a tyrosine kinase and a serine kinase.

3.2. *Phorbol ester-stimulated tyrosine phosphorylation*

Two-dimensional analysis of these two phosphotyrosyl proteins affinity purified from extracts of PMA-treated cells revealed (fig.3) a more rapid time course of phosphorylation. Increases in phosphorylation of both proteins were apparent within 1 min after PMA application, and the incorporation in pp70 peaked by 5 min, followed by steadily decreasing so that by 60 min the level of pp70 phosphorylation was less than that found initially in the factor-starved cells. In contrast, phosphorylation of pp50 remained significantly elevated even at 60 min.

Phosphoamino acid analysis of pp70 purified from PMA-treated cells (fig.4) revealed a ratio of phosphotyrosine to phosphoserine (45:55) similar to that found in pp70 from extracts of IL3-stimulated cells (40:60). This suggests that the same, or similar, kinase(s) are involved in both cases. We were unable to perform phosphoamino acid analysis on pp50 purified from PMA-treated cells due to low recovery of the excised phosphoprotein.

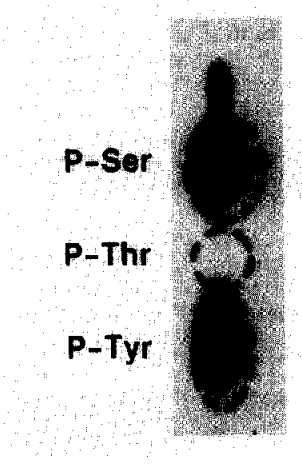

Fig.4. Phosphoamino acid analysis of pp70 after PMA stimulation. The areas on the 2D NEPHGE gels corresponding to pp70 on the autoradiographs were excised, and the proteins eluted and hydrolyzed in 6 N HCl. The hydrolysates were mixed with phosphoamino acid standard and subjected to high-voltage, single-dimension thin-layer electrophoresis. Positions of the standards were located with ninhydrin (dashed circles) and radioactive phosphoamino acids were located by autoradiography in the presence of intensifying screens.

The two proteins examined in extracts from IL3- and PMA-treated cells are identical, as shown by direct comparison of the two-dimensional gel autoradiographs (fig.5).

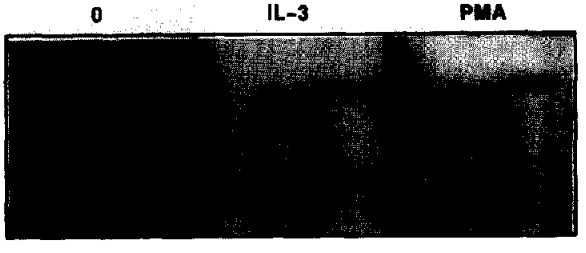

Fig.5. Comparison of PMA- and IL3-stimulated phosphorylation. Autoradiographs from control, 10 min IL3 and PMA NEPHGE are shown.

4. DISCUSSION

Reversible phosphorylation of proteins is recognized as the major mechanism of regulating the functions of proteins. It is important in such diverse cellular processes as intermediary metabolism, differentiation, mitosis and cytokinesis. Early work demonstrated that levels of phosphate incorporated into histones varied with the cell cycle [16,17] and that the phosphorylation state of non-histone DNA-binding proteins affected in vitro transcription rates [18]. The discoveries that certain viral oncogene products [19] and growth factor receptors [20,21] possessed tyrosine kinase activity provided further support for the idea that phosphorylation, and tyrosine phosphorylation in particular, were important to cellular proliferation. Recent molecular studies have shown that the tyrosine kinase activities of a variety of growth factor receptors and oncogene products are required for their biological effects [22–24].

Other kinase systems have also been implicated in growth control. The cyclic adenosine monophosphate-dependent protein kinase (PK-A) has often been associated with negative growth control and even with reversal of transformation [25,26], while the potent effects of the tumor-promoting phorbol esters have been attributed to the activities of the major intracellular phorbol ester-binding protein, PK-C [27]. These studies have provided the conceptual framework to interpret results implicating kinase activity in the biochemical mechanisms of actions of the colony-stimulating factors. Each of the hemopoietic growth factors have now been molecularly cloned, but to date only the receptor for colony-stimulating factor 1 (CSF-1) has been characterized by sequence homology and enzyme activity to be a tyrosine kinase, homologous to the *v-fms* oncogene product [28].

Previously we reported that IL3 as well as G-CSF stimulated the serine/threonine phosphorylation of a 68 kDa protein in a variety of CSF-dependent cell lines. This protein was also similarly phosphorylated in response to pharmacological activators of PK-C. We postulated that PK-C, or an analogous kinase system, was involved in the biological action of IL3 and G-CSF and that p68 phosphorylation might represent a point of convergence for several signal transduction pathways leading to proliferation. Recently, Whetton et al. [29] have shown that IL3 may activate a PK-C-like system in the absence of detectable phosphoinositol 4,5-bisphosphate hydrolysis, suggesting an atypical mechanism activating PK-C.

Abrogation of the IL3 dependence of murine IL3-dependent cell lines has been achieved by infection with the Abelson murine leukemia virus, a virus that encodes the *v-abl* tyrosine kinase oncogene product [10,11]. In addition Pierce et al. [30] have introduced the gene encoding the epidermal growth factor receptor into IL3-dependent cells and established EGF-dependent cell growth. Their work shows that tyrosine kinase activity can substitute for IL3, which suggests that the mechanism of IL3 biological activity involves amplification of tyrosine phosphorylation of a select group of proteins. In support of this idea, Koyasu et al. [13] reported that IL3 induced the tyrosine phosphorylation of a 150 kDa membrane glycoprotein and suggested that the 150 kDa protein might be closely associated with the IL3 receptor. We have shown by two-dimensional analysis of antiphosphotyrosine affinity-purified proteins that IL3 and PMA stimulate phosphorylation on both tyrosine and serine residues of two proteins with similar kinetics. Since we found that the ratios of phosphotyrosine to phosphoserine in pp50 or pp70 did not change after IL3 application we conclude that both a tyrosine and a serine/threonine kinase analogous to PK-C must be co-activated following IL3 treatment. We further suggest that PMA is also able to activate both types of protein kinase activities as demonstrated by increases in serine and tyrosine phosphorylation. This result is in agreement with previous observations of PMA stimulation of tyrosine kinase activity [31,32]. A corollary to this is that the tumor-promoting abilities of phorbol esters may involve the activation of tyrosine kinase systems by PK-C. We believe our findings are consistent with previous reports of interkinase regulation [33,34] and strongly implicate such kinase 'cross-talk' in the IL3 signal transduction pathway.

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