Signal transduction of human interleukin 3 and granulocyte–macrophage colony-stimulating factor through serine and tyrosine phosphorylation

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To elucidate the rapid events in signal transduction of human granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL 3), we examined phosphorylation of proteins on both serine and tyrosine residues in a cytokine-stimulated human myeloid cell line. We found increases in tyrosine phosphorylation within 30 s of stimulation with GM-CSF or IL 3, with peak responses occurring within 2 min. IL 3 and GM-CSF also induced serine phosphorylation, though 10 min of stimulation was required for maximum phosphate incorporation. Interestingly, both IL 3 and GM-CSF stimulated phosphate incorporation in identical substrates, a 68 kDa seryl-phosphoprotein (p68) and a 140 kDa tyrosyl-phosphoprotein (p140). Treatment of AML 193 cells with phorbol myristate acetate resulted in serine phosphorylation of p68; however, p140 was not phosphorylated on tyrosine. Depletion of protein kinase C isoenzymes with high concentrations of phorbol myristate acetate resulted in p68 phosphorylation, which was not further increased by IL 3 or GM-CSF. In contrast, cytokine-induced phosphorylation on tyrosine of p140 was observed after protein kinase C depletion. These data demonstrate the co-ordinate yet independent serine and tyrosine phosphorylation in IL 3- and GM-CSF-treated human myeloid cells, and thus suggest a common set of protein kinases stimulated by each separate ligand.

INTRODUCTION

Interleukin 3 (IL 3) and granulocyte–macrophage colony-stimulating factor (GM-CSF) are haematopoietic growth factors which stimulate the differentiation of progenitor cells into each of the major myeloid blood-cell types (Clark & Kamen, 1987). Both of these cytokines produce multi-lineage colonies and activate end-stage cell effector function. GM-CSF supports the development of neutrophils, monocytes, eosinophils, platelets and red blood cells, whereas IL 3 stimulates differentiation into each of these cell types as well as basophils. In addition, GM-CSF has been shown to activate neutrophils and eosinophils, whereas IL 3 enhances eosinophil antibody-dependent cytotoxicity and basophil histamine release (Weisbart *et al.*, 1985; Lopez *et al.*, 1989; Valent *et al.*, 1989). Though much is known about the biology of IL 3 and GM-CSF, there is little information available on signal-transduction pathway activated by these cytokines. Cross-linking studies have indicated that the receptor for human GM-CSF is approx. 85 kDa, whereas mouse IL 3 appears to bind to proteins of 68 and 140 kDa (DiPersio *et al.*, 1988; Sorenson *et al.*, 1989b). Interestingly, Park *et al.*, (1989a,b) have found competition between IL 3 and GM-CSF binding to KG 1 cells as well as in cells from patients with acute myelogenous leukaemia. GM-CSF has also been shown to inhibit binding of IL 3 to normal monocytes and eosinophils (Park *et al.*, 1989a,b; Lopez *et al.*, 1989). These data suggest that one potential mechanism mediating the overlapping biological activities of GM-CSF and IL 3 may be through either a receptor which binds both ligands or a tertiary complex formed by the distinct receptors for each ligand.

Previous work addressing signal transduction by haematopoietic cytokines has focused primarily on colony-stimulating-factor-induced phosphorylation in mouse myeloid cell lines (Evans

et al., 1987; Sorenson *et al.*, 1989a; Ferris *et al.*, 1988, 1989). Though mouse cell lines have served as a useful tool in elucidating the biology of the haematopoietic cytokines, ultimately it is critical to develop human models with which to conduct such studies. To examine the role of phosphorylation in signal transduction of human IL 3 and GM-CSF, we have utilized AML 193 cells, a human leukaemic line which proliferates vigorously in response to either GM-CSF or IL 3. We find that human IL 3 and GM-CSF induce phosphorylation of both serine and tyrosine residues, and that these responses are closely coupled with the biological responses of the cells. Though tyrosine phosphorylation appears independent of protein kinase C (PKC) isoenzymes, the phosphorylation of serine kinase substrates may require PKC isoenzymes. Interestingly, both cytokines also stimulated tyrosine phosphorylation of a 140 kDa protein. A 140 kDa protein has been suggested as an IL 3-binding protein in murine cells (Sorenson *et al.*, 1989b; Isfort *et al.*, 1988). These results may reflect modulation of the human IL 3 receptor by GM-CSF, signal transduction through the putative hybrid GM-CSF/IL 3-binding protein or complex prevalent on eosinophils, monocytes, as well as on cells from patients with acute myelogenous leukaemia (Park *et al.*, 1989a,b; Lopez *et al.*, 1989), or autophosphorylation of a kinase coupling the GM-CSF and IL 3 receptors.

EXPERIMENTAL

Cell lines and growth factors

The human leukaemic line AML 193 was cultured in Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Grand Island, NY, U.S.A.) plus 5% (v/v) fetal-calf serum (FCS; Gibco) and 1% (v/v) conditioned medium from the human bladder car-

Abbreviations used: IL 3, interleukin 3; CSF, colony-stimulating factor; GM-CSF, granulocyte–macrophage CSF; PKC, protein kinase C; IMDM, Iscove's Modified Dulbecco's Medium; FCS, fetal-calf serum; PMA, phorbol myristate acetate; DMSO, dimethyl sulphoxide.

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cinoma cell line 5637 (A.T.C.C., Rockville, MD, U.S.A.). Recombinant human IL 3 and GM-CSF were obtained from the Biological Response Modifiers Program (Frederick Cancer Research Facility, Frederick, MD, U.S.A.) as gifts from Immunex Corp. and Behring respectively.

Proliferation assays

AML 193 cells were washed extensively to remove conditioned media and re-cultured for 3–5 days in IMDM/1% FCS. The cells were then washed twice in serum-free IMDM, and resuspended in IMDM/5% FCS at a concentration of 10^6 cells/ml. Cells (0.1 ml) were added to a 96-well flat-bottomed micro-titre plate (Costart, Cambridge, MA, U.S.A.) and incubated at 37 °C under 5% CO₂ in air for 5 days with 0.1 ml of IL 3, GM-CSF (at concentrations specified in the text) or medium (IMDM/5% FCS). All samples were done in triplicates. Each well was pulsed with 1 μ Ci of [³H]thymidine (NEN, Boston, MA, U.S.A.) for 4 h and then harvested (Scatron Semiautomatic Cell Harvester, Sterling, VA, U.S.A.) on to glass-fibre filter paper (Filtermat; Skatron). Filter stripes were dried and radioactivity was counted in a liquid-scintillation counter (LKB, Bromma, Sweden; model 1216). Data were calculated as the mean radioactivity (c.p.m.) of triplicate cultures and subsequently converted into percentage of maximum response.

Radiolabelling, preparation of cell lysates and immunoprecipitations

At 5–7 days before use, AML 193 cells were washed twice in serum-free IMDM and resuspended in IMDM/1% FCS in the absence of growth factors. Cells to be depleted of protein kinase C isoenzymes were incubated with 500 ng of phorbol myristate acetate (PMA; Sigma, St. Louis, MO, U.S.A.)/ml for 48 h before use. On the test day, factor-depleted cells were washed twice in RPMI 1640 medium without phosphate (ABI; Rockville, MD, U.S.A.), and resuspended at 40×10^6 cells/ml in phosphate-free RPMI 1640 with dialysed 5% FCS and 0.5 mCi of [³²P]P_i (NEN)/ml. This preparation was incubated for 90 min in a shaking water bath at 37 °C. Recombinant human IL 3 or GM-CSF was added to the cells at concentrations described in the text. At the appropriate time after addition of growth factor, samples were rapidly pelleted (400 g, 5 min at 4 °C); the supernatant was discarded and the pellet resuspended in extraction buffer (1% Triton X-100, 50 mM-NaCl, 10 mM-Tris, 5 mM-EDTA, 30 mM-Na₂P₂O₇, 50 mM-NaF, 100 μ M-sodium orthovanadate and 0.1% BSA, pH 7.6). Experiments utilizing total cell lysates did not include BSA in the extraction buffer. Cells (40×10^6) were lysed by addition of 1 ml of the extraction buffer at 0 °C and incubated for a minimum of 45 min. Samples were subjected to a cycle of rapid freeze-thawing to ensure optimum release of proteins and then centrifuged at 5000 g for 20 min at 4 °C. Immunoprecipitations were performed on the clarified supernatant with PY20 (ICN, Costa Mesa, CA, U.S.A.), a monoclonal antibody recognizing phosphotyrosine (Glenney *et al.*, 1988).

Electrophoresis

Two-dimensional electrophoresis was performed as described previously (Evans *et al.*, 1987). Samples for non-equilibrating pH-gel electrophoresis were solubilized in sample buffer [9.5 M-urea, 2.0% Nonidet P-40, 1.6% Ampholines, pH 5–7 (LKB), 0.4% Ampholines, pH 3.5–10, and 5.0% 2-mercaptoethanol] and separated in tube gels (140 mm $\times$ 3 mm) consisting of 4.0% acrylamide, 2.0% Nonidet P-40, 2.0% pH 3.5–10 Ampholines and 3.0% pH 5–7 Ampholines. Determination of pH gradients in isoelectric-focusing gels for pI calculation was performed as described previously (Sorenson *et al.*, 1989a). SDS/PAGE (7.5%

acrylamide in the resolving gel, 4.75% acrylamide in the stacking gel) was utilized for the second dimension. First-dimension tube gels equilibrated for 1–2 h in 2 $\times$ SDS sample buffer (4.6% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.125% Tris and 0.0001% Bromophenol Blue, pH 6.8) were applied to the top of the slab gel and immobilized with 1.0% agarose. Samples subjected to one-dimensional SDS/PAGE were diluted with 1 vol. of 2 $\times$ SDS sample buffer, boiled for 5 min, centrifuged at 5000 g for 1 min, and applied to pre-formed wells in a 5 cm stacking gel (4.75% acrylamide). The resolving gel consisted of 7.5% acrylamide. Gels were fixed in methanol/acetic acid/water (5:1:5, by vol.) and bands were observed by autoradiography (XMAR film, Kodak) with two intensifier screens at –80 °C.

Phosphoamino acid hydrolysis

The proteins of interest were excised from gels, rehydrated for approx. 1 min in distilled water, separated from the plastic backing and placed in 0.4 ml of a buffer containing 20 mM-ammonium phosphate, 1.0% SDS and 5% 2-mercaptoethanol. Samples were then boiled for 5 min and incubated at 37 °C for 48 h. The supernatant was removed and evaporated on a Speed Vac apparatus (Savant, Hicksville, NY, U.S.A.). Trichloroacetic acid (0.5 ml) was then added to each pellet, together with 10 μ g of BSA as a carrier protein. This preparation was incubated for 60 min at 0 °C, centrifuged (5000 g, 20 min at 4 °C), and the resulting pellet was gently washed three times in cold ethanol/ether (3:1, v/v). The pellet was air-dried, resuspended in 0.2 ml of 6 M-HCl and incubated for 90 min at 110 °C. The HCl was removed by evaporation under vacuum in a Speed Vac apparatus. The pellets were then dissolved in 5 μ l of distilled water containing 1 mg each of phosphotyrosine, phosphoserine and phosphothreonine per ml and subjected to single-dimension high-voltage thin-layer electrophoresis. Samples were applied to cellulose (100 μ m thickness)-coated glass plates (E. Merck Nutley, NJ, U.S.A.) and separated at 1.5 kV for 45 min in pyridine/acetic acid/water (1:10:189, by vol.) buffer. Phosphoamino acid standards were identified with ninhydrin and the experimental samples were identified by autoradiography.

Preparation of subcellular fractions and protein kinase C assay

AML 193 cells were harvested, washed three times in RPMI 1640 medium and resuspended at 1×10^7 cells/ml in ice-cold buffer A (20 mM-Tris/HCl, pH 7.5, 2 mM-EDTA, 0.5 mM-EGTA and 2 mM-phenylmethanesulphonyl fluoride). Cell lysis in the hypo-osmotic solution was aided by 20 strokes with a Dounce homogenizer. After removal of nuclei by low-speed centrifugation at 100 000 g for 60 min. The centrifuged membrane pellets were homogenized with a glass rod in 1% Nonidet P-40 in buffer A at 4 °C. Cytosolic fractions also received 1% Nonidet P-40, and both fractions were gently rotated for 30 min at 4 °C, then centrifuged at 6000 g for 15 min. Approx. 500 μ l was passed through a 1 ml column of DEAE-cellulose DE52 (Pharmacia, Piscataway, NJ, U.S.A.) equilibrated with buffer A. Columns were washed first with 15 ml of buffer A, and then with 3 ml of buffer A with 50 mM-NaCl. An additional 1.5 ml of eluate in buffer A with 100 mM-NaCl was collected. All Ca²⁺/phospholipid-dependent protein kinase activity was found in this fraction, as shown previously (Farrar & Anderson, 1985). Samples (50 μ l) were assayed for PKC isoenzyme activity as described by Farrar & Anderson (1985), with 0.5 mM-CaCl₂, 10 mM-MgCl₂, 100 μ M- $[\gamma$ -³²P]ATP (60 c.p.m./pmol; Amersham, Arlington Heights, IL, U.S.A.), 50 μ g of histone H1 (Boehringer Mannheim, Indianapolis, IN, U.S.A.) with or without 5 μ g of phosphatidylserine and 1 μ g of diolein (Sigma). Enzyme reactions were incubated for 3 min at 30 °C and terminated by addition of 20% (v/v) trichloroacetic acid. PKC isoenzyme specific-activity data

are expressed in nmol/min per mg attributed to activity stimulated by phospholipid, with subtraction of the activity stimulated in the absence of lipids.

RESULTS

Regulation of serine phosphorylation

The AML 193 cell line was chosen for studies of human colony-stimulating factor (CSF) signal transduction, since these cells have a vigorous proliferative response to both recombinant human GM-CSF and IL 3 (Santoli *et al.*, 1987). We first

examined the changes in phosphorylation in cell lysates of [32 P] P_i -labelled cells, using two-dimensional electrophoresis. Fig. 1(a) demonstrates that a 10 min treatment with GM-CSF or IL 3 produced an increase in phosphate incorporation by a protein of 68 kDa (p68) and pI 4.8–5.2. Stimulation with phorbol myristate acetate (PMA) also produced increases in phosphorylation of a 68 kDa protein with a pI identical with that of the 68 kDa phosphoprotein observed in GM-CSF- and IL 3-stimulated cells. IL 2, a molecule with no proliferative effects on the AML 193 cell line, produced no changes in p68 phosphorylation.

Fig. 1. Phosphorylation of a 68 kDa protein in response to GM-CSF, IL 3 or PMA in human myeloid cells

(a) Two-dimensional isoelectric-focusing analysis of total cell lysates from AML 193 cells. AML 193 cells (40×10^6 /ml) were washed in phosphate-free media and labelled with [32 P] P_i (0.5 mCi/ml) for 90 min. A 1 ml portion of cells was stimulated with the indicated human cytokine or PMA for 10 min, then pelleted and lysed in extraction buffer. Lysates were subjected to two-dimensional electrophoresis (isoelectric focusing and SDS/PAGE) and made visible by autoradiography. (b) Phosphoamino acid hydrolysis of p68 phosphorylated in AML 193 cells after a 10 min treatment with GM-CSF, IL 3 or PMA. The p68 phosphoprotein was excised from replicate two-dimensional gels, and acid-hydrolysed as described in the Experimental section. Shown is the resolution of phosphoamino acids by thin-layer electrophoresis. (c) Two-dimensional isoelectric focusing of total cell lysates from DMSO-treated HL-60 cells. HL-60 cells were cultured for 4 days in 1.25% DMSO, washed in phosphate-free media, labelled with [32 P] P_i (0.5 mCi/ml), stimulated with recombinant human GM-CSF for 10 min and lysed.

The identity of the amino acid residue phosphorylated in response to cytokine or PMA was examined by acid hydrolysis of p68 and separation of phosphoamino acids by one-dimensional high-voltage thin-layer electrophoresis. Fig. 1(b) demonstrates that only serine residues were phosphorylated in the p68 protein after GM-CSF, IL 3 or PMA treatment.

To assess further the cytokine-induced serine phosphorylation in human myeloid cells, we examined the effects of GM-CSF on the human promyelocytic leukaemic cell line HL-60. Cells were treated for 4 days in the presence of dimethyl sulphoxide (DMSO), a compound which has previously been shown to induce HL-60 cells to differentiate towards a granulocytic lineage as well as to increase expression of GM-CSF receptors (DiPersio *et al.*, 1988). Fig. 1(c) demonstrates that a 68 kDa protein is phosphorylated after GM-CSF treatment in DMSO-treated HL-60 cells.

The dominant phosphoprotein in these studies appears to have the same migration characteristics as a 68 kDa protein identified in IL 3-stimulated murine NSF60.8 cells (Evans *et al.*, 1987). Indeed, isoelectric focusing of p68 from IL 3-stimulated murine NSF60.8 cells and human AML 193 cells showed them to be identical (results not shown). These data indicate that in both human and murine myeloid cells GM-CSF and IL 3 increase serine phosphorylation of a 68 kDa protein. The highly conserved nature of this response suggests that p68 phosphorylation may be a crucial event in signal transduction of haematopoietic factors. Consequently, we chose to focus subsequent studies on characterization of the p68 phosphoprotein. The dose-response of the p68 phosphoprotein was compared with the proliferative responses of the AML 193 cells to human IL 3 (Fig. 2). The half-maximal responses of human IL 3-stimulated p68 phosphorylation and proliferation were nearly identical. This suggests a strong correlation between the regulation of p68 phosphorylation and the biological response of AML 193 cells to human IL 3. Similar responses were obtained with human GM-CSF.

Tyrosine phosphorylation

We next examined induction of tyrosine phosphorylation in the AML 193 cell line by IL 3 and GM-CSF. [32 P]P_i-labelled cells were treated for the indicated times with the appropriate cytokine, lysed, and immunoprecipitated with PY 20, an anti-phosphotyrosine monoclonal antibody (Glenney *et al.*, 1988). Immunoprecipitates of cytokine-stimulated cell lysates were analysed by two-dimensional gel electrophoresis. Fig. 3(a) shows autoradiographs of proteins phosphorylated on tyrosine after IL 3 or GM-CSF treatment. The principal protein phosphorylated in these experiments had an approximate molecular mass of 140 kDa and an estimated pI of 4.9–5.0. Comparison of the migration of the 140 kDa protein tyrosine-phosphorylated in response to either GM-CSF or IL 3 indicated that both proteins migrated identically when subjected to isoelectric focusing in the first dimension. To establish unequivocally that the 140 kDa tyrosyl-phosphoprotein observed after treatment with either cytokine was the same, purified tyrosyl-phosphoproteins from GM-CSF- and IL 3-stimulated cells were mixed and then subjected to two-dimensional electrophoresis. Only one 140 kDa phosphoprotein was observed in the mixed samples, demonstrating p140 to be a conserved tyrosine kinase substrate in GM-CSF- and IL 3-stimulated AML 193 cells (results not shown).

To determine if GM-CSF-induced tyrosine-phosphorylation of p140 was a response unique in AML 193 cells, we examined the effects of GM-CSF stimulation on tyrosine phosphorylation in HL-60 cells (Fig. 3b). Increases in phosphorylation of a protein of approx. 140 kDa were readily apparent in DMSO-treated HL-60 cells stimulated with GM-CSF. In addition,

Fig. 2. Dose-response relationship of IL 3 stimulation of p68 phosphorylation (○) and proliferation of AML 193 cells (●)

Two-dimensional non-equilibrating pH-gel electrophoresis analysis of total cell lysates from AML 193 cells labelled with [32 P]P_i as described in Fig. 1, then treated for 10 min with varied concentrations of IL 3. Shown is a graphic representation of the relationship between the concentration of IL 3 necessary to elicit p68 phosphorylation compared with that necessary to stimulate proliferation of AML 193 cells. Relative phosphate incorporation was quantified by laser densitometry, and data are expressed as percentages of maximum phosphorylation or proliferative response. Concentration of IL 3 is in ng/10⁶ cells.

stimulation of the murine cell line NSF60.8 with either GM-CSF or IL 3 results in tyrosine-phosphorylation of a protein of approx. 140 kDa (D. Linnekin & W. L. Farrar, unpublished work), further supporting our evidence in human myeloid cells that GM-CSF as well as IL 3 stimulates tyrosine-phosphorylation of p140.

In several experiments, a 97 kDa protein (p97) was also phosphorylated in response to IL 3 and GM-CSF (results not shown). Mixing samples from GM-CSF- and IL 3-stimulated cells also showed p97 to be identical in cells stimulated with either cytokine. These data demonstrate that both IL 3 and GM-CSF stimulate phosphorylation of tyrosine residues in common substrates.

The results of phosphoamino acid hydrolysis of the 140 kDa protein phosphorylated in response to GM-CSF are shown in Fig. 3(c). The ratio of phosphate incorporation on tyrosine to that on serine residues was approx. 1:1. Similar results were observed with IL 3.

Ligand-elicited kinase activation is often an extremely rapid event in signal transduction. To characterize the relationship between receptor-ligand interaction and serine and tyrosine phosphorylation, we compared the kinetics of increases in substrate phosphate incorporation stimulated by GM-CSF (Fig. 4). Increases in p140 phosphorylation were observed within 30 s, with a maximum phosphate incorporation in p140 after 2.5 min. In contrast, a 10 min stimulation with GM-CSF was necessary to produce maximum phosphorylation of serine residues on p68. These data indicate that tyrosine phosphorylation is a more proximal event to serine phosphorylation after ligand-receptor interaction. These observations suggest an intimate association of a tyrosine kinase(s) with the GM-CSF receptor. Identical results were obtained with IL 3.

PKC depletion studies

Previous studies using murine IL 3-dependent cells had suggested that phorbol esters could mimic some of the effects of

Fig. 3. Tyrosine phosphorylation in cytokine-treated human myeloid cells

(a) Lysates from AML 193 cells radiolabelled with [32 P]P $_i$ as described in Fig. 1 and treated for 5 min with cytokine were immunoprecipitated with PY-20, a monoclonal antibody specific for phosphotyrosine. Phosphoproteins were resolved by two-dimensional electrophoresis (isoelectric focusing and SDS/PAGE) and made visible by autoradiography. (b) Two-dimensional analysis (non-equilibrating pH-gel electrophoresis and SDS/PAGE) of phosphotyrosyl-proteins immunoprecipitated from the lysates of [32 P]P $_i$ -radiolabelled HL-60 cells treated for 5 min in the absence or presence of recombinant human GM-CSF. (c) Phosphoamino acid hydrolysis of p140 after a 5 min treatment with GM-CSF. The 140 kDa protein was excised from replicate samples and subjected to acid hydrolysis as described in the Experimental section.

Fig. 4. Kinetics of serine and tyrosine kinase activation in AML 193 cells

Lysates from [32 P]P $_i$ -labelled AML 193 cells stimulated with GM-CSF for various times were resolved by two-dimensional electrophoresis (non-equilibrating pH-gel electrophoresis and SDS/PAGE) of total cell lysates or immunoprecipitated with anti-phosphotyrosine antibodies and resolved on a one-dimensional SDS/PAGE gel. Incorporation of [32 P]P $_i$ into either p68 (●; in total cell lysates) or p140 (○; in purified phosphotyrosyl-proteins) was quantified by laser densitometry. Data are expressed as percentages of maximum response.

stimulate PKC translocation (Farrar *et al.*, 1985). To examine the role of PKC in human IL 3- and GM-CSF-induced serine and tyrosine phosphorylation, we performed studies with AML 193 cells functionally depleted of PKC isoenzymes. Previous work has shown that pretreatment of cells for 48 h with high concentrations of PMA accelerates the degradation of PKC (Jaken *et al.*, 1981). To test this effect in our system, AML 193 cells were grown in standard conditioned medium with or without 500 ng of PMA/ml for 48 h (Table 1). The cells were then lysed, and membrane and cytosolic fractions were prepared and analysed for PKC isoenzyme activity by using an assay which detects all the major Ca $^{2+}$ /phospholipid-dependent protein kinase activity (Farrar & Anderson, 1985). Untreated AML 193 cells showed that most of the detectable PKC activity was cytosolic, with approx. 10% found in membrane extracts. Previous experiments with both myeloid and lymphocytic cell lines have shown that initial low-dosage stimulation of PKC with phorbol esters induces the rapid translocation of most of the isoenzymes to the particulate membrane fraction (Farrar & Anderson, 1985; Farrar *et al.*, 1985). On prolonged exposure to PMA, an apparent depletion of Ca $^{2+}$ /phospholipid-dependent activity was observed in the AML 193 cells. Over 20-fold decrease in detectable PKC activity was observed in either fraction of the treated cells, suggesting that this procedure was relatively efficient in decreasing enzyme levels responsive to Ca $^{2+}$ and phospholipid. Although some activity was detected at the limits of the assay, physiological levels of PKC activity which are found in rapidly growing cells dramatically decreased.

IL 3. PMA stimulation has been shown to support the proliferation of murine IL 3-dependent cell lines (Whetton, 1986), to stimulate hexose transport (Whetton *et al.*, 1984), to induce phosphorylation of a 68 kDa protein (Evans *et al.*, 1986) and to

Table 1. PKC activity of AML 193 cells

AML 193 cells were cultured in 500 ng of PMA/ml for 48 h, washed three times in serum-free RPMI 1640, and reconstituted to 1×10^7 cells/ml. The cells were lysed with 1.0 ml of buffer A (20 mM-Tris/HCl, pH 7.5, 0.5 mM-EGTA, 2 mM-EDTA, 2 mM-phenyl-methanesulphonyl fluoride). Lysates were centrifuged for 30 min at 100000 g to obtain cytosolic and membrane fractions. Both fractions were solubilized in buffer A containing 1% Nonidet P40 detergent and fractionated on DEAE-cellulose as described by Farrar & Anderson (1985). Fractions eluted at 100 mM-NaCl contained all detectable Ca^{2+} /phospholipid-dependent activity. PKC activity was determined by subtracting the ^{32}P incorporation into histone in the absence of added phospholipids from the incorporation in the presence of phospholipids. Data are presented as means $\pm$ S.D. of triplicate samples and are expressed as nmol of ^{32}P /min per mg of protein.

	Activity (nmol/min per mg)	
	Cytosol	Membrane
Untreated	17 $\pm$ 0.3	1.2 $\pm$ 0.8
PMA (48 h)	0.6 $\pm$ 0.1	0.8 $\pm$ 0.003

Fig. 5(a) shows two-dimensional gels of total cell lysates from [^{32}P]P-labelled AML 193 cells treated with IL 3, GM-CSF or PMA for 10 min. In control cells, increases in phosphorylation of the p68 phosphoprotein were observed in response to each stimulant. AML 193 cells depleted of PKC before radiolabelling had elevated basal levels of total phosphoproteins, phosphorylation of p68 being particularly prominent. No further increase in phosphate incorporation into p68 was observed in response to treatment with IL 3, GM-CSF or additional PMA in pretreated cells.

Tyrosyl-phosphoproteins from PKC-depleted AML 193 cells were also examined to determine the role of PKC in tyrosine kinase activation (Fig. 5b). IL 3 and GM-CSF, but not PMA, induced p140 phosphorylation in normal and PKC-depleted AML 193 cells. Thus IL 3 and GM-CSF stimulated p140 phosphorylation in PKC-depleted cells, suggesting that receptor coupling to tyrosine kinase(s) occurs independently of PKC.

DISCUSSION

Treatment of a human myeloid cell line with GM-CSF or IL 3 resulted in rapid phosphorylation on serine residues of a protein of apparent molecular mass 68 kDa (Figs. 1a and 1c). A close relationship between concentration of cytokine necessary to induce proliferation of AML 193 cells and p68 phosphorylation was evident for IL 3 (Fig. 2) and GM-CSF (results not shown). The size and pI of p68 suggested a relationship to the 68 kDa phosphoprotein found in IL 3- and G-CSF-stimulated murine NSF60.8 cells. Comparison of phosphoproteins resolved by isoelectric focusing indicated that the human and murine p68 migrated to identical points in two-dimensional gels, strongly suggesting that the two proteins are the same (results not shown). The remarkable conservation of a substrate phosphorylated in response to both IL 3 and GM-CSF (Fig. 1a) as well as in cells of both murine and human origin suggests an important role for this protein in mediation of the biological actions of these molecules. We have also observed phosphorylation of the same protein in human and murine lymphoid cells after IL 2 stimulation (Evans *et al.*, 1987). Phosphorylation of a 68 kDa protein was observed by Sorenson *et al.* (1989a) in B6SutAl cells treated with GM-CSF, IL 3 or PMA.

Although p68 is clearly a major substrate in cells activated by

lymphohaematopoietic cytokines, the identity of p68 has not yet been established. An attractive candidate would be the Na^+/H^+ antiporter, since GM-CSF, IL 3, granulocyte CSF (G-CSF) and macrophage CSF (M-CSF) stimulate cellular alkalization which is amiloride-sensitive (Cook *et al.*, 1989). However, until the structure of the Na^+/H^+ antiporter is better characterized or antisera are available, it will be difficult to determine if p68 is the antiporter or a subunit. Additionally, other proteins of 68 kDa which may be regulated by phosphorylation in haematopoietic cells are lamin B (Hornbeck *et al.*, 1988), one form of S6 kinase (Tabarini *et al.*, 1987), calcimedin (Morse & Moore, 1988) and a Ca^{2+} -binding protein identified in lymphocytes (Owens & Crumpton, 1984).

Cytokine induction of PKC translocation (Farrar *et al.*, 1985), phosphorylation of identical substrates in response to both PMA and cytokines (Evans *et al.*, 1987) and maintenance of cytokine-dependent cell lines on sub-optimal concentrations of PMA (Whetton, 1986) have suggested a role for PKC in signal transduction of cytokines. Depletion of PKC isoenzymes with high concentrations of PMA (Jaken *et al.*, 1981) was performed to delineate if PKC was involved in phosphorylation of p68. By using methods previously demonstrated to detect α and β forms of PKC (Farrar *et al.*, 1985), the PMA-pretreated cells were found to have a greater than 20-fold decrease in PKC activity (Table 1). As shown in Fig. 5(a), cells pretreated with PMA did not produce the increase in p68 phosphorylation observed in control cells. Important to note, however, is the dramatic increase in phosphate incorporation into p68 evident in the PKC-depleted cells not stimulated with IL 3, GM-CSF or PMA. One explanation for these observations is the PMA-induced degradation of PKC to the unregulated catalytic fragment referred to as PK-M (Takai *et al.*, 1977; Ogawa *et al.*, 1981). Although high-dose PMA pretreatment was sufficient to lower PKC isoform activity dramatically compared with physiological levels, it is impossible to determine if all PKC isoenzymes were depleted. Thus additional experimental designs will be necessary to clarify further the role of PKC in the molecular events of cytokine action.

Our studies of human GM-CSF and IL 3 signal transduction also indicate that increases in tyrosine phosphorylation are stimulated by these two colony-stimulating factors (Figs. 3–5). Two-dimensional gel electrophoresis of purified tyrosyl-phosphoproteins from a factor-responsive human myeloid cell line indicated that both GM-CSF and IL 3 stimulate phosphorylation of tyrosine residues in a protein of approx. 140 kDa. An intimate relationship between the receptors for GM-CSF and IL 3 and a tyrosine kinase(s) is suggested by the rapid induction of p140 phosphorylation (Fig. 4). In contrast, maximum increases in phosphate incorporation into p68 were observed 10 min after addition of GM-CSF or IL 3 to AML 193 cells. These data suggest that the activation of a serine kinase in response to stimulation with two human CSFs is an event distal to tyrosine kinase activation. In further support of this hypothesis is the observation that under conditions where PKC isoenzyme activity was minimal, no effect on cytokine-induced phosphorylation of p140 was observed (Fig. 5b).

IL 3-induced phosphorylation of a 140 kDa protein in a murine cell line was originally reported by Koyasu *et al.* (1987) and has been confirmed by the findings of several groups (Morla *et al.*, 1988; Isfort *et al.*, 1988; Sorenson *et al.*, 1989a,b; Schreurs *et al.*, 1989; Ferris *et al.*, 1989). Two recent reports indicate that the 140 kDa tyrosyl-phosphoprotein observed after IL 3 stimulation may be the IL 3 receptor (Sorenson *et al.*, 1989b; Isfort *et al.*, 1988). The results of our studies of cytokine-stimulated tyrosine phosphorylation suggest the convergence of the GM-CSF- and IL 3-stimulated tyrosine kinase(s) on a 140 kDa substrate in a human myeloid cell line. A 140 kDa protein with

Fig. 5. Effects of PKC depletion on kinase activation of AML 193 cells

AML 193 cells cultured for 48 h in the absence or presence of PMA (500 ng/ml) were labelled with [32 P]P_i, stimulated for 5 min with cytokine or PMA, and lysed. (a) Two-dimensional analysis (non-equilibrating pH-gel electrophoresis and SDS/PAGE) of total cell lysates. (b) Two-dimensional analysis (non-equilibrating pH-gel electrophoresis and SDS/PAGE) of phosphotyrosyl-proteins.

pI identical with that observed in AML 193 cells was phosphorylated on tyrosine residues in HL-60 cells stimulated with GM-CSF (Fig. 3b). Additionally, we have observed a tyrosyl-phosphoprotein of approx. 140 kDa in murine NSF60.8 cells treated with both GM-CSF and IL 3 (results not shown). Thus the data that we have obtained in two human and one

murine cell lines suggest that the 140 kDa protein is not a substrate unique to IL 3-stimulated myeloid cells. These data could support several different interpretations. Indeed, IL 3 may be stimulating the tyrosine phosphorylation of a 140 kDa IL 3-binding protein, and GM-CSF-induced phosphorylation of the same protein may represent the regulation of the IL 3 receptor by

GM-CSF. Alternatively, it has been reported that GM-CSF and IL 3 compete for a common binding site on monocytes, eosinophils and on some human myeloid cell lines (Park *et al.*, 1989a,b; Lopez *et al.*, 1989). Three subtypes of GM-CSF/IL 3-binding proteins have been suggested, one specific for GM-CSF, one binding only IL 3, and one which binds both. Interestingly, cells isolated from a number of patients with acute myelogenous leukaemia (AML) exhibit a high degree of cross-competition of GM-CSF and IL 3 for binding. Therefore our studies of AML 193, a cell line derived from a patient with AML, may reflect signal transduction through this putative receptor subtype. It is important to note that the hybrid binding protein present on normal eosinophils and monocytes may mediate the responses of these cells to both GM-CSF and IL 3 and thus be responsible for the closely related biological activities of these two cytokines. The receptors for human GM-CSF and murine IL 3 have been cloned, and neither contains the conserved sequence identified in previously characterized kinases (Gearing *et al.*, 1989; Itoh *et al.*, 1990). The coupling mechanism between the tyrosine kinase(s) and the IL 3 and GM-CSF receptors is as yet undefined. Pertussis-toxin inhibition of GM-CSF-induced tyrosine phosphorylation suggests the coupling of a tyrosine kinase to the GM-CSF receptor through some member of the G-protein family (Gomez-Cabronero *et al.*, 1989). One intriguing speculation is the possibility that the receptors for human IL 3 and GM-CSF may couple to the same tyrosine kinase. Recent evidence has accumulated which suggests that certain components of signal transduction can couple to more than one type of binding protein. The proto-oncogene *lck* has been shown to be associated with both CD4 and CD8 on T lymphocytes (Rudd *et al.*, 1988; Veillette *et al.*, 1988). These types of observations may be germane in consideration of the association of the tyrosine kinase(s) which couple to the human GM-CSF and IL 3 receptors.

Although details of the identity of the tyrosine and serine kinases activated by IL 3 and GM-CSF receptors remain to be elucidated, our findings suggest the intriguing possibility that these receptors may share common kinase systems. Alternately, unique kinases restricted to each receptor may have similar affinity for common substrates with functions important for cellular vitality and commitment to proliferation. These issues will be further addressed as the nature of the receptors and their coupling mechanisms are characterized.

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