Identification of a signal-transduction pathway shared by haematopoietic growth factors with diverse biological specificity

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The haematopoietic growth factors multi-colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, granulocyte colony-stimulating factor and interleukin 2 specifically control the production and proliferation of distinct leucocyte series. Each growth factor acts on a unique surface receptor associated with an appropriate signal-transduction apparatus. In this report we identify a 68 kDa substrate which is phosphorylated after stimulation of different cell types with multi-colony-stimulating factor, granulocyte colony-stimulating factor and interleukin 2. The 68 kDa substrate is also phosphorylated in each cell line stimulated with synthetic diacylglycerol, a direct activator of protein kinase C. Interestingly, granulocyte/macrophage colony-stimulating factor does not induce phosphorylation of the 68 kDa molecule. The 68 kDa molecule that is phosphorylated after stimulation with each ligand yielded similar peptide maps after chymotryptic digestion; furthermore, the substrate was always phosphorylated on threonine residues. Phosphorylation of the same residues in the 68 kDa substrate suggests that activation of protein kinase C is one common signal-transduction event associated with the action of multi-colony-stimulating factor, granulocyte colony-stimulating factor and interleukin 2.

INTRODUCTION

The expansion and differentiation of blood cells (haematopoiesis) and maintenance of the mature immune system are exquisitely regulated by a variety of exogenous regulatory cytokines acting on specific receptors expressed by progenitor cells [1–6]. Within the haematopoietic system, two principal cell series can be distinguished. Cells of the myeloid series (monocytes, granulocytes, erythrocytes and megakaryocytes) are produced in the bone marrow, whereas cells of the lymphoid series (T and B lymphocytes) are produced in the spleen and other lymphoid organs, and to a lesser extent in the bone marrow [7]. It appears that both the myeloid and lymphoid series originate from a common stem-cell population and that many of the mature haematopoietic cells can be generated *in vitro* from bone-marrow cells stimulated with the appropriate growth factors [1–6]. Many of the haematopoietic growth factors have been genetically engineered or otherwise purified to homogeneity. Although each of these growth factors expresses a distinct spectrum of activities on heterogeneous bone-marrow-cell populations, the appearance of distinct receptors for several colony-stimulating factors has been observed on cloned differentiated haematopoietic cells.

Multi-CSF (or IL 3), granulocyte/macrophage-CSF (GM-CSF) and granulocyte CSF (G-CSF) are all polypeptide haematopoietic growth factors, and are collectively referred to as colony-stimulating factors

(CSFs). Multi-CSF is synthesized by mitogen- or antigen-activated lymphocytes, and appears to control the growth and differentiation of pluripotent stem cells, leading to the production of all of the myeloid blood-cell types [3,5,8–11]. GM-CSF is more restricted than multi-CSF in terms of its biological activity, stimulating the production of mature granulocytes, macrophages and eosinophils as well as initiating proliferation of some multi-potential erythroid and megakaryocyte precursors [2–14]. G-CSF has an even more limited specificity *in vitro*, stimulating only the production of granulocytes [15,16]. Interleukin 2 (IL 2) is a polypeptide growth factor that acts on the lymphoid lineage, which is released from antigen- or lectin-stimulated T lymphocytes [17,18]. Although IL 2 was originally suggested to be specific for T lymphocytes, subsequent studies have shown that IL 2 also induces the activation of large granular lymphocytes to express natural killer activity and the proliferation of B lymphocytes [19,20]. It is clear that biologically the haematopoietic growth factors act on distinct populations of cells which are ontologically related. It is also becoming clear that these growth factors are structurally distinct and effect their response through different receptors [21,22]. An important question is how such a diverse group of growth factors stimulate the proliferation of distinct but overlapping populations of cells.

Protein phosphorylation has been associated with the regulation of many systems critical to cell function, including intermediary metabolism, protein synthesis

Abbreviations used: CSF, colony-stimulating factor; GM-CSF, granulocyte/macrophage CSF; G-CSF, granulocyte CSF; IL 2, interleukin 2; OAG, 1-oleoyl-2-acetyl-glycerol; CFU, colony-forming units; PMA, phorbol myristate acetate; p68, 65 kDa phosphoprotein; PAGE, polyacrylamide-gel electrophoresis.

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and growth-factor-receptor function [23–25]. The initial response of a cell to growth-factor stimulation might therefore be reflected by a change in the phosphorylation state of key regulatory molecules. It has previously been established that stimulation of CT6 or FDC-P1 cells with IL 2 or multi-CSF, respectively, leads to the rapid phosphorylation of numerous substrates. In particular, within each cell line a group of substrates is phosphorylated after stimulation of the cells with either appropriate growth factor or a synthetic diacylglycerol [26,27]. Diacylglycerol is the endogenous second-messenger molecule associated with activation of protein kinase C [28]. In the present study a comparative analysis of protein phosphorylation stimulated by four unique cytokines with a direct activator of protein kinase C has been performed. We conclude that both synthetic diacylglycerol and physiological ligand stimulate the threonine-specific phosphorylation of a protein, p68, which is highly conserved within a variety of myeloid and lymphoid lineage cells. The phosphorylation of p68 by haematopoietic growth factors correlates well with the stimulation of proliferation of distinct lineages. Except for GM-CSF, which did not induce phosphorylation of p68 under the conditions tested, the stimulation of p68 phosphorylation by two other distinct CSFs was correlated with cellular growth.

MATERIALS AND METHODS

Materials

Recombinant murine multi-CSF and GM-CSF were produced by D.R. at DNAX as previously described [29,30]. Recombinant IL 2 was provided by Dr. Tom Sayer, Sandoz (Vienna, Austria). Purified natural G-CSF was obtained from Dr. Jonathan Keller (Biological Response Modifier Program, Frederick, MD, U.S.A.). Purified natural human IL 2 (CR1-IL 2) was purchased from Collaborative Research (Lexington, MA, U.S.A.). 1-Oleoyl-2-acetyl-glycerol (OAG) was purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). All other chemicals were from standard sources.

Maintenance of cell line

All cell lines were maintained in RPMI 1640 (Advanced Biotechnology, Silver Spring, MD, U.S.A.), containing glutamine (0.1 mg/ml), 10% (v/v) fetal-calf serum (Hyclone, Logan, UT, U.S.A.), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Appropriate growth factors were added at final concentrations of 10% Wehi-3 growth-conditioned media for FDC-P1 [31] and NSF-60 [32] cell lines, and 0.01% partially purified recombinant GM-CSF for NSF-60.8 and 2% CR1-IL 2 for CT6 cell lines [33]. Proliferation and DNA synthesis by cells was measured by [³H]thymidine incorporation. Briefly, growth-factor-depleted cells (10⁴/well) were reseeded in micro-titration culture plates under appropriate conditions, cultured overnight and then pulsed for 2 h with 1 μ Ci of [³H]thymidine.

Radioisotopic labelling and ligand stimulation of cells

Before use, cells were washed three times, twice in normal media but without growth factor, and once with serum-free growth-factor-free media. The cells were then rested by culture in medium with 1% fetal-calf serum and no growth factor. The length of time for which each cell

line could be cultured in the absence of growth factor (rested) without loss of proliferation potential was determined empirically. The optimum period of time for resting the cell lines was 3 h for FDC-P1, 6 h for NSF-60.8 and 16 h for CT6 and NSF-60. Rested cells were washed twice in phosphate-free RPMI 1640 + glutamine (0.1 mg/ml), and 30 min later 0.5 mCi of [³²P]P_i was added to each 1 ml suspension. The P_i pools were allowed to equilibrate for 60 min. Cells were stimulated for the times indicated in the Figure legends (0–30 min) with either a mock material (prepared exactly as the genetically engineered growth factors, but without growth factor), OAG (1 μ g/ml) multi-CSF (10⁵ CFU/ml), GM-CSF (10⁵ CFU/ml), G-CSF (10⁵ CFU/ml) or IL 2 (1000 units/ml) (50 CFU stimulate half-maximal colony formation in a 1 ml culture seeded with 75000 bone-marrow cells; 1 unit of IL 2 stimulates half-maximal proliferation in a 1 ml culture seeded with 100000 cells).

Phosphoprotein analysis by SDS/PAGE

After stimulation, 10 vol. of ice-cold RPMI 1640 was added to the cells. The cell pellet collected by centrifugation (1500 rev./min for 5 min) was lysed with 1% Triton X-100/10 mM-Tris/NaCl (pH 7.5)/10 mM-NaF/10 mM-EDTA and kept on ice for 30 min. Detergent-insoluble material was removed by centrifugation at 100000 *g* for 10 min. Detergent-soluble material was prepared for immediate analysis by SDS/PAGE or non-equilibrated pH-gel electrophoresis in the appropriate sample buffer [26] or else was rapidly frozen and stored at –70 °C. Both types of electrophoresis were carried out as previously described [26]. Autoradiography of dried gels was carried out with Kodak XAR 5 film, except for Fig. 2(a) FDC-P1 gels, when Kodak MR-1 film was used.

Phosphopeptide and phosphoamino acid analysis

Regions of two-dimensional gels corresponding to protein p68 were removed from the backing paper and placed in an extraction buffer (20 mM-ammonium phosphate/1% SDS/5% 2-mercaptoethanol), homogenized and shaken at 37 °C for 48 h. Extracted proteins were precipitated together with a carrier protein (10 μ g of bovine serum albumin) by 10% trichloroacetic acid. Precipitates were washed twice with ethanol/ether (3:1, v/v) and once with ether alone, air-dried and then resuspended in digestion buffer [1.25 mM-Tris/HCl (pH 6.8)/0.5% SDS/10% glycerol/chymotrypsin (10 μ g/ml)] or hydrolysed with 6 M-HCl (1 h at 100 °C).

Chymotryptic digests were analysed by SDS/PAGE in a 30% -acrylamide separating gel, and acid-hydrolysed amino acids were separated by thin-layer electrophoresis (1500 V for 40 min, on cellulose thin-layer plates with pyrimidine/acetic acid/water, 1:10:189, by vol.).

RESULTS

Properties of cell lines used for growth-factor-stimulated proliferation

In order to investigate phosphorylation of substrates associated with the signal-transduction pathway stimulated by haematopoietic growth factors, it is necessary to use homogeneous populations of cells which exhibit ligand-specific receptors. Four cell lines which exhibited selective growth-factor dose-response patterns were

Fig. 1. Proliferation assays of growth-factor responsiveness

(a) CT6, (b) FDC-P1, (c) NSF-60 and (d) NSF-60.8 cells were tested for proliferation in a thymidine incorporation assay. Each cell line was tested for dose-response to multi-CSF (Δ), GM-CSF (∇), G-CSF ($\square$) and IL 2 ($\circ$).

selected for study (Fig. 1). The ability of a cell line to proliferate in response to the appropriate growth factor was our criterion for the expression of a functional receptor. Two previously described cloned cell lines, CT6 [33] and FDC-P1 [31], proliferate in response to IL 2 and multi-CSF respectively (Figs. 1a and 1b). The lymphoid cell line (CT6) did not respond to any CSFs, and conversely the myeloid cell lines did not respond to IL 2. The myeloid cell line NSF-60 and a subclone of this line, NSF-60.8, proliferated in response to multi-CSF and G-CSF (Figs. 1c and 1d); NSF-60.8 cells, however, also respond to GM-CSF (Fig. 1d). The NSF-60 and NSF-60.8 cell lines are Thy 1^+ , express the granulocyte myeloid-specific markers 8C5 and M1, but are FcE $^-$ and histamine $^-$. The effect of phorbol esters on proliferation of the cell lines used in this study was investigated (Table 1). Phorbol 12-myristate 13-acetate (PMA) was used in place of OAG because, unlike OAG, PMA is not rapidly metabolized and is therefore able to maintain a transmembrane signal. PMA alone was not able to support proliferation of any of the cell lines used.

However, PMA was able to potentiate the proliferation of CT6, NSF-60 and NSF-60.8 cell lines cultured in suboptimal concentrations of growth factor (Table 1). The extent of potentiation was dose-dependent and variable. Typically 1–10 ng of PMA/ml was optimum, and maximum potentiation was approx. 50% above growth-factor-supported proliferation (Table 1).

Protein phosphorylation

Protein kinase C phospho-substrate, of approx. 68 kDa, has been previously identified and associated with growth-factor signal transduction in IL 2 stimulation of T lymphocytes [27] and multi-CSF (IL 3) stimulation of the myeloid cell line FDC-P1 [26]. To determine if this might be the same molecule, and whether other haematopoietic growth factors such as GM-CSF and G-CSF can stimulate phosphorylation of the molecule, two-dimensional (non-equilibrated pH-gel electrophoresis and SDS/PAGE) phosphorylation patterns of growth-factor-stimulated cells were compared. We confirmed that the principal substrate phosphoryl-

Table 1. PMA-potentiated proliferation

Proliferation was determined by measuring incorporation of [3 H]thymidine: 10^4 cells in 200 μ l of RPMI 1640/10% fetal-calf serum were stimulated with IL 2, multi-CSF or PMA. After overnight culture, the cells were pulsed for 2 h with 1 μ Ci of [3 H]thymidine/well. Results are means $\pm$ S.E.M. for three experiments.

Cell line	Treatment	[3 H]Thymidine incorporation (c.p.m.)
CT6	–	7060 $\pm$ 1220
	IL 2 (1 unit/ml)	10320 $\pm$ 2030
	IL 2 (10 units/ml)	37640 $\pm$ 1765
	IL 2 (1 unit/ml)+PMA (10 ng/ml)	15446 $\pm$ 1027
	PMA (10 ng/ml)	5428 $\pm$ 786
NSF-60	–	2604 $\pm$ 674
	Multi-CSF (1 CFU/ml)	8752 $\pm$ 423
	Multi-CSF (10 CFU/ml)	29867 $\pm$ 1437
	Multi-CS (1 CFU/ml)+PMA (10 ng/ml)	14320 $\pm$ 881
	PMA (10 ng/ml)	3016 $\pm$ 1224
NSF-60.8	–	1876 $\pm$ 106
	Multi-CSF (1 CFU/ml)	8464 $\pm$ 1036
	Multi-CSF (10 CFU/ml)	17995 $\pm$ 3642
	Multi-CSF (1 CFU/ml)+PMA (10 ng/ml)	14024 $\pm$ 895
	PMA (0 ng/ml)	2164 $\pm$ 151
FDC-P1	–	256 $\pm$ 108
	Multi-CSF (1 CFU/ml)	10285 $\pm$ 1260
	Multi-CSF (10 CFU/ml)	40765 $\pm$ 2468
	Multi-CSF (1 CFU/ml)+PMA (10 ng/ml)	21242 $\pm$ 1576
	PMA (10 ng/ml)	198 $\pm$ 50

ated by both growth factor and a direct activator of protein kinase C (OAG) in CT6 and FDC-P1 cells was a 68 kDa molecule (p68) (Fig. 2a). Analysis of the phosphorylation patterns of NF-60 and NF-60.8 showed that multi-CSF, G-CSF and synthetic diacylglycerol stimulated phosphorylation of protein p68 in both cell lines (Fig. 2b). GM-CSF, which stimulates proliferation of NSF-60.8 cells, did not stimulate the phosphorylation of protein p68 in this cell line. Except for GM-CSF, each of the growth factors stimulated phosphorylation of a 68 kDa molecule with identical charge/mass ratio and which co-migrate in two-dimensional analysis. Phosphorylation of protein p68 was detected in those cell lines which respond to the growth factor by proliferation. IL 2 stimulated protein-p68 phosphorylation only in the lymphoid cell line, which physiologically responds to IL 2. We have previously reported that the phosphorylation of protein p68 in FDC-P1 cells is IL 3- or OAG-dose-dependent [26] and that the concentration of IL 3 required for maximum proliferation is sufficient to induce maximum phosphorylation of p68. Similar concentration effects have been observed for each of the growth factors investigated (results not shown).

Kinetics of phosphorylation of p68

If a phosphorylation event is associated with an early signal transduction initiated by ligand–receptor interaction, then the kinetics of phosphorylation should be relatively rapid. In Fig. 3, the phosphorylation of p68 after growth-factor stimulation is shown. Fig. 3(a) shows the phosphorylation of p68 in CT6 cells stimulated with IL 2. A slight increase in phosphorylation of p68 was detected within 3 min of stimulation, and maximum phosphorylation was achieved within 10 min and maintained for at least 20 min. Phosphorylation of p68

subsequently decreased to a steady state intermediate between the maximum stimulated value and the resting (IL 2-deprived) cell value (results not shown). Similarly, Fig. 3(b) shows the percentage of maximum incorporation of 32 P into p68 for each of the growth factors. The data show that IL 2, multi-CSF and G-CSF stimulate the rapid phosphorylation of p68. GM-CSF fails to stimulate p68 phosphorylation within 2 h of stimulation of NSF-60.8 cells, even though multi-CSF and OAG both stimulated rapid phosphorylation of p68 in this cell line.

Biochemical analysis of p68: peptide mapping and phosphoamino acid analysis

In order to confirm that the p68 phosphorylation stimulated by each growth factor represented a similar biochemical event, partial biochemical characterization of phosphorylated p68 was carried out. Maximally phosphorylated p68 was excised from two-dimensional gels and subjected to chymotryptic digestion. The resultant phosphopeptides were analysed by SDS/PAGE. In Fig. 4, densitometric scans of the phosphopeptides detected by autoradiography are shown; the phosphopeptide patterns are similar for each cell line investigated, suggesting that the p68 substrate was phosphorylated in the same manner after stimulation with the physiological ligand (IL-2, multi-CSF, G-CSF) or diacylglycerol. Four principal phosphopeptides were generally observed on proteolytic digestion. The major peptide had an apparent molecular mass of 27 kDa, and the other peptides were of 33, 38 and 43 kDa. The relative abundance and molecular masses of the peptides generated by chymotryptic digestion suggest two possible digestion patterns. First, the 27 kDa substrate may represent the most stable digestion product, with each of the other less-abundant peptides representing less-stable

Fig. 2. Phosphorylation of p68 in growth-factor-stimulated cells

Cells were deprived of growth factor and pre-equilibrated with [32 P]P_i. P_i-loaded cells were stimulated for 10 min with 1000 units of IL 2/ml, 10⁶ CFU of multi-CSF, GM-CSF or G-CSF/ml, or 1 μ g of OAG/ml, and then phosphoproteins were separated by two-dimensional electrophoresis (non-equilibrated pH-gel electrophoresis and SDS/PAGE). The resolved phosphoproteins were revealed by autoradiography. Only the portion of gel in which p68 was resolved is shown. (a) CT6 cells stimulated with IL 2 and OAG; (b) FDC-P1 cells stimulated with multi-CSF and OAG; (c) NSF-60 cells stimulated with multi-CSF, OAG, GM-CSF, G-CSF and IL 2; (d) NSF-60.8 cells stimulated with multi-CSF, OAG, GM-CSF, G-CSF and IL 2.

intermediates. Second, the 27 kDa and 43 kDa substrates might represent then the products of a single cleavage site. The 33 kDa and 38 kDa substrates would represent further digestion of the 43 kDa molecule. In order to determine the residues phosphorylated, maximally

phosphorylated p68 was excised from two-dimensional gels and subjected to acid hydrolysis and thin-layer electrophoresis. The results from this analysis are given in Table 2. In all cases the principal phosphoamino acid identified was threonine.

Fig. 3. Time course of p68 phosphorylation

Phosphorylation was performed as described in the Materials and methods section. In the upper panel, phosphorylation of p68 in CT6 cells is shown as revealed by two-dimensional electrophoresis [non-equilibrated pH-gel electrophoresis (NEPHGE) and SDS/PAGE]. Inserts show phosphorylation after (A) 0 min, (B) 3 min, (C) 10 min and (D) 30 min of IL 2 stimulation (1000 units/ml). The lower panel shows data from similar experiments with CT6, FDC-P1 and NSF 60.8 cells. The extent of phosphorylation was determined after stimulating the cells with each of the different growth factors (1000 units of IL 2/ml, or 10^5 CFU of multi-CSF, GM-CSF and G-CSF). The extent of 32 P incorporation into p68 was determined by extracting the piece of gel corresponding to p68 and liquid-scintillation counting of radioactivity.

Fig. 4. Chymotryptic peptides generated from phosphorylated p68

The phosphorylated p68 molecule isolated from cells treated with appropriate growth factor (1000 units of IL 2/ml, or 10⁵ CFU of multi-CSF, GM-CSF and G-CSF/ml) or OAG (1 µg/ml) was digested with chymotrypsin and separated by SDS/PAGE, in a 20%-acrylamide separating gel. Separated phosphoproteins were revealed by autoradiography, and a quantitative analysis of the phosphopeptides was performed by densitometric scanning of the autoradiograph. A–D indicate the peptide fragments obtained.

Table 2. Phosphoamino acid analysis of p68

Results are shown of phosphoamino acid analysis of the 68 kDa molecule extracted from two-dimensional electrophoresis. Thin-layer electrophoresis was used to analyse the p68 after acid hydrolysis. The analysis was performed on p68 phosphorylation in intact cells after growth-factor or OAG treatment. Regions of the cellulose plate corresponding to the position of serine, threonine and tyrosine were removed and counted for radioactivity by liquid scintillation. Total radioactivity (c.p.m.) recovered is set at 100%.

Cell line	Growth factor or OAG	Relative contribution of phosphoamino acids (% of total)		
		Serine	Threonine	Tyrosine
CT6	IL 2	6	91	3
	OAG	5	93	2
FDC-P1	Multi-CS	4	93	3
	G-CSF	7	91	2
	OAG	7	88	5
NSF-60.8	Multi-CSF	3	95	2
	G-CSF	5	92	3
	OAG	3	94	3

DISCUSSION

Although many of the growth factors associated with the proliferation and differentiation of haematopoietic cells have been characterized and their biological activities established, at least *in vitro*, little is known about the mechanism of action of these molecules. Many ligands have been shown to activate protein kinase C by stimulating the hydrolysis of phosphatidylinositol [28,34]. It has also been demonstrated that IL 2 stimulates the generation of diacylglycerol via inositol lipid hydrolysis [27,35]. However, other evidence suggests that diacylglycerol can be generated more directly from phosphatidylcholine [36], although such a pathway had not yet been identified in the IL-2-stimulated generation of diacylglycerol. The mechanism by which diacylglycerol is generated in myeloid cell lines has not been elucidated, although initial experiments do suggest that multi-CSF and G-CSF stimulate the production of diacylglycerol (E. Bonvini, unpublished work).

It has been suggested that phorbol esters potentiate mitogenesis through biochemical pathways that converge with those of hormones and that the protein kinase C phosphotransferase system may be a unifying site of this biochemical convergence [37,38]. PMA has been shown to support or potentiate the proliferation of several

lymphoid cell lines [39]. In lymphoid cells, which do not proliferate in the presence of PMA alone, the Ca^{2+} ionophore A23187 is often able to complement PMA and allow proliferation [39]. The data in Table 1 support the idea that PMA is able to potentiate proliferation of lymphoid cells and is also able to potentiate the proliferation of some myeloid cells. With this in mind we set out to establish the role of protein kinase C as a signal-transduction mechanism associated with the biological action of haematopoietic growth factors. By comparing the phosphorylation induced by agents which are known to activate protein kinase C directly with the phosphorylation after growth-factor stimulation of intact cells, we identified one obvious substrate (p68) phosphorylated in each haematopoietic cell line investigated. The p68 substrate is phosphorylated after treatment with either growth factor or direct protein kinase C activators. Further, the phosphorylation of p68 by growth factor or OAG is dose-dependent [26,27]. Using the CT6 and FDC-P1 cell lines, we have previously shown that IL 2 and multi-CSF respectively will induce a rapid but transient translocation of protein kinase C from the cytosol to the membrane [40,41]. Interestingly, the rapid transient phosphorylation of p68 that we describe here appears to parallel the kinetics of protein kinase C subcellular redistribution previously reported, thereby providing further evidence that the protein kinase C phosphotransferase system is physiologically significant for transduction of the signal delivered by certain haematopoietic-growth-factor-receptor interactions.

Peptide mapping of p68 from each of the cell lines investigated provides further evidence that the p68 proteins identified in each cell line (having the same charge-to-mass ratio) by two-dimensional gel electrophoresis are the same molecule.

There is evidence that the respective receptors for some growth factors, such as epidermal growth factor and platelet-derived growth factor, possess tyrosine kinase activity [42,43]. Accordingly, we examined whether phosphorylation of the p68 substrate induced by haematopoietic growth factors was at tyrosine or serine/threonine residues. After stimulation of intact cells with multi-CSF, IL 2 or OAG, the p68 protein was phosphorylated on threonine residues and not on tyrosine. We have previously shown that protein kinase C will phosphorylate p68 on threonine in a cell-free system and that phosphorylation of p68 is highly dependent on the presence of Ca^{2+} and phospholipid *in vitro* [26,27]. Together, these results suggest that the phosphorylation of p68 is effected by the Ca^{2+} - and phospholipid-dependent protein kinase C. However, the results do not exclude the possibility that a more complicated enzyme cascade has been effected to achieve phosphorylation of p68 by a protein kinase C-regulated enzyme. It is clear, however, that p68 is not being phosphorylated by a tyrosine kinase.

There is no direct evidence for a functional role for the p68 molecule; however, a molecule of approximately the same size is known to be associated with the Na^+/H^+ anion exchanger in red-cell membranes [44]. Since activation of the Na^+/H^+ antiporter is one of the earliest events detected in IL 2-stimulated lymphocytes [45], as well as in multi-CSF- and G-CSF-stimulated myeloid cells (S. W. Evans & W. L. Farrar, unpublished work), we suggest the possibility that p68 may be a regulatory

subunit associated with the cytosolic domain of an anion exchanger, or alternatively a component of a phosphorylation cascade associated with the regulation of Na^+/H^+ antiporter. The phosphorylation of ribosomal protein S6 and increased initiation of protein synthesis, which occurs after IL 2 stimulation of CT6 cells [46] and 3T3 fibroblasts [47], may be dependent on the intracellular alkalization established by Na^+/H^+ antiport activity [48]. These observations suggest a common cascade following growth-factor-receptor interaction in which activated protein kinase C phosphorylates a Na^+/H^+ -antiport regulatory unit (possibly p68), leading to intracellular alkalization, which permits phosphorylation of ribosomal S6 protein by the protein kinase C-activated S6 kinase.

In this paper we have demonstrated by the identification of a common phospho-substrate, p68, that protein kinase C is involved in the transduction of the signal delivered to cells by several haematopoietic growth factors. One important exception was that GM-CSF fails to stimulate phosphorylation of p68, suggesting the possibility of an alternative mechanism which may affect the GM-CSF-receptor signal-transduction mechanism. Since only the IL 2 receptor has been structurally studied [49], and shown to have no intrinsic kinase domain, the possibility exists that the GM-CSF receptor might contain a kinase domain which affects signal transduction in a manner distinct from the IL-2, multi-CSF and G-CSF receptors.

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