Multilineage Hematopoietic Growth Factor Interleukin 3 and Direct Activators of Protein Kinase C Stimulate Phosphorylation of Common Substrates

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In order to investigate early signal transduction events in myeloid cells, the phosphosubstrates of an interleukin 3 (IL 3)-dependent cell line, FDC-P1, have been analyzed. Using synthetic diacylglycerol as a direct activator of the unique calcium-phospholipid-dependent phosphotransferase protein kinase C (PK-C) and genetically engineered homogeneous IL 3, we have demonstrated a common element to signal transduction events associated with these stimuli. One novel substrate, p68 (68,000 kd), was rapidly phosphorylated in either IL 3- or diacylglycerol-stimulated cells. The phosphorylation of p68 was dose-dependent, with both the physiological ligand and diacylglycerol inducing the same maximal level of phosphorylation. Phosphorylation of p68 occurred in a time-dependent manner analogous to previously described kinetics of PK-C subcellular redistribution in the FDC-P1 cell line. The p68 substrate was also phosphorylated in a cell-free system under conditions designed to activate PK-C. Phosphoamino acid analysis demonstrated that the p68 molecule phosphorylated in intact cells as well as in a calcium-phospho-lipid-dependent cell-free system was phosphorylated on threonine residues, not tyrosine. These data support the hypothesis that the activation of PK-C that occurs after IL 3-receptor interaction which leads to the rapid phosphorylation of cellular proteins is an important element of the signal transduction mechanism in FDC-P1 cells. We propose that phosphorylation of the p68 molecule is a physiochemical marker for the activation of PK-C in myeloid cells, in response to the growth-promoting physiological ligand.

Through the interaction between a population of progenitor cells and appropriate polypeptide growth factors, vertebrate organisms are able to regulate the production of heterogeneous blood cells with diverse functional activities. In addition to the biological characterization of distinct peptide signals involved in hematopoiesis, several of these glycoprotein growth factors have been purified to homogeneity and their cDNA nucleotide sequences determined.

Interleukin 3 (IL 3) is one member of the polypeptide hematopoietic growth factors collectively referred to as colony-stimulating factors (CSF). The apparent diversity of biological activities expressed by this molecule in vitro has resulted in several alternative nomenclatures based on the biological assays used, including multi-CSF, P cell-stimulating factor (PCSF), burst-promoting activity (BPA), mast cell growth factor (MCGF), and hematopoietic cell growth factor (HCGF). 

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The true extent of the biological role of IL 3 in vivo remains obscure. Although IL 3 can stimulate erythroid colonies (BPA) in vitro, this growth factor cannot be detected in vivo even in severely anemic animals with greatly elevated levels of erythropoietin, another growth factor closely associated with erythropoiesis. Also, attempts to modulate erythropoiesis or eosinophilopoiesis in vivo using IL 3 have been unsuccessful. Even though the role of IL 3 in vivo is not clear, the fact that it has such a wide range of biological activities in vitro and that its mechanism of action appears to be distinct from those of other growth factors, such as EGF, PDGF, and FGF, suggest that it is important to investigate its mechanisms of action.

The reversible phosphorylation of proteins is now recognized to be one of the major mechanisms by which intracellular events can be controlled by hormonal stimuli. Several distinct phosphorylating enzymes can be distinguished by their cofactor requirements, protein substrate specificity, or the specific amino acid moieties to which they transfer phosphate groups. Among the best characterized phosphotransferases are the cyclic adenosine monophosphate-dependent protein kinases (PK-A) and the calcium-calmodulin phosphotransferase system. Complementing the protein kinases are a group of dephosphorylating enzymes, protein phosphatases (see reference 23 for a review). Two phosphorylating systems have recently been closely associated with the growth and transformation of cells. Tyrosine kinase activity appears to be intrinsic to several retroviral transforming proteins (for review see reference 24) as well as cellular receptors for growth factors, such as EGF and PDGF. The second protein phosphotransferase system recently associated with cell growth and transformation is protein kinase C (PK-C). PK-C is believed to mediate the effects of the tumor-promoting phorbol esters as well as potentially mediate the signal transduction process of oncogenes that have reported tyrosine kinase activity. Phorbol esters physiochemically substitute for diacylglycerol, a hydrolysis product of phosphatidylinositol (PI), in the activa-

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tion of PK-C. Ligand-receptor interaction for a variety of hormones and neurotransmitters is thought to be the physiological signal initiating PI hydrolysis in the plasma membrane (for review see reference 30). Concomitant with PK-C stimulation seems to be its association with the inner surface of the plasma membrane.31-33 The previous observation that IL 3 was able to induce the translocation of PK-C from cytosol to the cell membrane, a phosphotransferase system shared by phorbol esters,34 stimulated our interest in the possible role of this enzyme in the growth and differentiation of hematopoietic cells. The studies presented in this report examine the substrates of protein phosphorylation induced by IL 3 in homogeneous factor-dependent myeloid cells. We report here that IL 3 stimulates phosphorylation of protein substrates identical to those stimulated by direct physicochemical activators of PK-C. These studies provide direct evidence that PK-C has a role in the mechanism of signal transduction that affects the physiological response to IL 3 and characterizes the biochemical substrates of PK-C in myeloid cells.

MATERIALS AND METHODS

Recombinant murine IL 3 and granulocyte-macrophage (GM)-CSF were produced by one of us (D.R.) at DNAX, as previously described.35,36 1-Oleyl-2-acetylglycerol (OAG) was purchased from Avanti Polar Lipids (Birmingham, Ala). 4f3-Phorbol 12f3-myristate was described.9'0 1-Oleyl-2-acetylglycerol (OAG) was purchased from CSF were produced by one of us (DR.) at DNAX, as previously described.

Preparation of particulate membrane and cytosol fractions of FDC-P1 cells and cell-free protein kinase C phosphorylation. Cells were washed free of serum (two washes in 10 mmol/L HEPES saline) and resuspended in 10 mmol/L Tris-HCl pH 7.5, 2 mmol/L PMSF, 5 mmol/L EDTA, 2 mmol/L EGTA, 2 mmol/L NaF, 1% Triton X-100. Cells were then broken in a Dounce homogenizer. Broken cell suspension was layered over a 41% sucrose cushion and centrifuged at 100,000 g for one hour. The cytosol fraction was removed from above the interface while the particulate membrane was removed from the interface. The particulate membrane was washed and pelleted by centrifugation at 100,000 g for one hour and finally resuspended in homogenization buffer. Membrane and cytosol fractions were frozen on dry ice and stored at -70 °C. The basic reaction buffer 20 mmol/L Tris-HCl pH 7.5, 75 mmol/L MgCl2 was used in all reactions. CaCl2 (0.5 mmol/L or 5 mmol/L), phospholipid (12 μg/mL phosphatidylinositol, 0.4 μg/mL [1,2-diolein]), EGTA, and partially purified PK-C were included, as indicated in figure legends. Reactions carried out at 32 °C were started by the addition of adenosine triphosphate (ATP) adjusted from cold ATP and 32P-ATP (>5,000 Ci/mmol) to give a final concentration of 1 mmol/L at 7.5 to 10 × 104 cpm.

Phosphoaminoacid analysis. Slices of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), corresponding to the location of phosphorylated proteins identified by autoradiography, were placed in an extraction buffer, 20 mmol/L ammonium phosphate 1% SDS, 5% 2-mercaptoethanol (2-ME), homogenized and shaken at 37 °C for 48 hours. Extracted proteins were precipitated together with 10 μg of BSA as carrier protein in 20% trichloroacetic acid. The precipitates were washed, twice with ethyl ether (3:1) and once with ether alone, air-dried, and then hydrolyzed with 6 mol/L hydrochloric acid for one hour at 110 °C. HCl was removed by heating under vacuum in a rotor evaporator. Single-dimension thin-layer electrophoresis was carried out at 1,500 V for 40 minutes, using cellulose thin-layer plates and pyridine:glacial acetic acid:water (10:100:1890) buffer pH 3.5. Phosphoaminoacids were identified using unlabeled standards located with ninhydrin.

Sample preparation and electrophoresis. Samples for single-dimension SDS-PAGE were loaded onto 6.6×10 mm, 10% (w/v) acrylamide, 5% SDS, 2% ME, 2% SDS. Samples for nonequilibrating pH gel electrophoresis (NEPHGE) were solubilized in final concentration of 9.5 mmol/L urea, 2% NP-40, 2% α-hydroxyacetone (4:1 pH 5 to 7 : pH 3.5 to 10), 5% 2-DE. For SDS-PAGE 7.5% to 15% gradient, 7.5 mm thick separating gel with a 3% stacking gel were used. Samples were loaded into preformed slots and separation was carried out at 15 mA per gel. For NEPHGE, the sample was loaded at the anode end of a 6% acrylamide, 2% amphotoline (pH 3.5 to 10) tube gel (2 mm ID × 120 mm L). Separation was carried out at 400 V for four hours. Second-dimension SDS-PAGE was essentially the same as single-dimension SDS-PAGE except that after focusing, the NEPHGs were equilibrated in stacking gel buffer 1% 2-DE for 2.5 hours and fixed above the SDS-PAGE with 1% agarose.

Detection of radiolabeled proteins. After electrophoresis, the gels were washed in methanol:water:acetic acid (5:5:1) for at least three one-hour washes. Gels were then rehydrated by soaking in water, supported on filter paper, and dried (Biorad gel drier, Richmond, Calif). Autoradiography was carried out using Kodak XAR 5 film.

RESULTS

The extent of phosphorylation induced by the interaction of IL 3 with its receptor and the role of PK-C in this signal transduction was studied. In order to examine these questions, we selected the murine IL 3-dependent cell line...
FDC-P1 developed by Dexter et al as our homogeneous target cell population. Two nonpeptide chemical compounds, the tumor-promoter PMA and a synthetic analog of diacylglycerol 1-oleyl-2-acetylgllycerol (OAG), are known to activate PK-C in intact cells both directly and physicochemically. The protein phosphorylation patterns induced by these agents were compared with those induced by IL 3 (a growth factor essential for FDC-P1 survival), GM-CSF (a growth factor for which our FDC-P1 cell lines appears to have no biological response and that is unable to replace IL 3), and the tumor-promoter 1,25 dihydroxyvitamin D3, an analog of vitamin D3 that does not appear to activate PK-C. Figure 1 shows the phosphorylated substrates from intact cells after treatment for 15 minutes with different stimulating agents. An obvious increase in phosphorylation of two substrates, a 68,000-dalton molecule (p68) and a 20,000-dalton molecule (p20), was noted in IL 3-, OAG-, and PMA-stimulated cells but not in cells stimulated by GM-CSF or the vitamin D3 analog. We also observed apparent dephosphorylation of two bands (110,000 and 45,000 daltons) in the IL 3-stimulated cells but not in cells treated with any of the other agents, including OAG and PMA. Densitometric scans (Fig 2) of a similar autoradiograph confirm the visual appraisal that the principal changes are (a) increased phosphorylation of the p68 and p20 in IL 3- and OAG-treated cells and (b) dephosphorylation of the p110 and p45 in only IL 3-treated cells. The scans also suggest that other less obvious changes in phosphorylation may be taking place. Comparison of the IL 3-treated cells with mock-stimulated cells (Fig 2A) reveals small changes in phosphorylation of molecules with molecular weights (mol wt) between 100,000 and 60,000 daltons. These changes include increased phosphorylation and dephosphorylation of molecules. OAG treatment (Fig 2B) also increased phosphorylation of several molecules; in fact, the OAG-induced phosphorylation of some of these molecules appeared greater than that induced by IL 3. The only qualitative difference between IL 3 treatment and OAG treatment appears to be dephosphorylation of p110 and p45.

In order to determine better the extent of growth ligand-induced changes in phosphorylation, we compared the phosphoproteins by two-dimensional electrophoresis (Fig 3). The principal phosphorylation and dephosphorylation events associated with ligand stimulation are identified by com-
of a number of additional phosphoproteins. The principal changes observed were highly reproducible and could be identified by both single-dimension SDS-PAGE and two-dimensional NEPHGE x SDS-PAGE. Some of the smaller changes and the dephosphorylation of p45 that were identified by SDS-PAGE were less apparent. These changes were never seen on the two-dimensional phosphorylation patterns, suggesting that the p45 protein may be less abundant.

This study deals with phosphorylation changes in the FDC-P1 cell line. Using other IL 3-dependent cell lines, including FDC-P2, NSF 60, and NSF 60.8, we have observed that IL 3 stimulates the phosphorylation of a substrate with identical charge-mass ratio as p68. The analysis of p68 phosphorylation in these cells will be a subject of a separate paper that focuses on the ability of other growth factors, as well as IL 3, to activate PK-C. The 20,000-dalton protein phosphorylated by IL 3 or OAG stimulation comigrated with myosin light chain, a previously described PK-C substrate. Therefore, we chose to concentrate further studies on the uncharacterized 68,000-dalton molecule. Because the ligand-induced translocation of PK-C from cytosol to membrane is believed to be transient, the temporal kinetic change in p68 phosphorylation associated with IL 3, OAG, and PMA stimulation of the FDC-P1 cells was analyzed. A time course analysis comparing the effects of IL 3, PMA, and OAG on phosphorylation of the 68,000-dalton molecule is represented in Fig 4A. The effect of IL 3 is transient, with peak phosphorylation at around ten minutes. Phosphorylation induced by OAG is also transient, while PMA-induced phosphorylation was more sustained and still significantly greater than background after two hours. Moreover, increases in relative phosphorylation were the result of increased 32P incorporation into protein substrate, since protein levels of the 68,000 substrate did not vary during the experimental time course (data not shown). The phosphorylation pattern from an SDS-PAGE analysis illustrates the same result (Fig 4B). The changes induced by IL 3 appear transient; particularly clear is the change in phosphorylation of the 68,000-dalton molecule, while the effect of PMA was more persistent.

To demonstrate that the phosphorylation observed in intact cells was in fact due to PK-C, we carried out phosphorylation in a cell-free lysate enzyme reaction. In Fig 5A, phosphorylation of cytosolic and membrane fractions of FDC-P1 cells by a calcium–phospholipid-dependent kinase (PK-C) is shown. The reaction conditions described with the figure were designed to detect calcium–phospholipid-dependent PK-C substrates. In the cytosolic fraction, a group of calcium–phospholipid-dependent phosphosubstrates with mol wt of around 68,000 daltons was observed, while in the membrane fraction, a single dominant phosphosubstrate with mol wt of 68,000 daltons was detected. Analysis of the cytosolic (PK-C) phosphosubstrates by two-dimensional electrophoresis (Fig 5B) allows the identification of a molecule with identical charge-mass ratio as the 68,000-dalton substrate phosphorylated by either IL 3 or OAG in intact cells. The 68,000-dalton molecule seen in isolated membranes does not have the same charge-mass ratio (data not shown). It appears that the 68,000-dalton molecule that
Fig 4. (A) Kinetics of p68 phosphorylation after treatment of cells with IL 3, OAG, or PMA. The 68,000-dalton molecule was excised from the gel and homogenized in 30% hydrogen peroxide for 30 minutes. The extent of Cerenkov radiation was then determined in a scintillation spectrometer. IL 3 --- , OAG △△△△, PMA □□□□. (B) Single-dimension analysis of phosphosubstrates at different times after treatment of cells with mock material (M), IL 3, or PMA. Position of 68,000-dalton phosphosubstrate is indicated by arrow. Time since ligand stimulation (and corresponding to end point) is given below each pair of samples.

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constitutes a minor phosphosubstrate for PK-C in cell-free systems is located in the soluble phase in intact cells, although a loose association with the plasma membrane cannot be excluded. Nevertheless, cell-free studies confirmed that the 68,000-dalton protein phosphorylated by IL 3 or OAG in intact cells was an enzymatic substrate of PK-C in vitro.

To confirm that the phosphorylation seen in intact cells was due to a serine-threonine rather than a tyrosine kinase, we carried out phosphoaminoacid analysis of the 68,000-dalton molecule (Fig 6). It is clear that the phosphorylation of a 68,000-dalton protein in intact cells induced by either IL 3 or OAG occurs at threonine residues and not tyrosine.

**DISCUSSION**

Although many of the growth factors associated with the proliferation and differentiation of hematopoietic cells have been characterized and their physiological activities established at least in vitro, little is known about the mechanism of action of these molecules. We chose to begin our investigation of the molecular control of growth and differentiation by identifying the second-message systems affecting signal transduction immediately after ligand-receptor interaction. It has been suggested that phorbol esters potentiate mitogenesis through biochemical pathways that converge with those of hormones and that the PK-C phosphotransferase system may be the one unifying site of this biochemical convergence. With this in mind we set out to establish the role of PK-C as a signal transduction mechanism associated with the biological action of IL 3. By comparing the phosphorylation induced by agents that are known to directly activate PK-C with the phosphorylation seen after IL 3 stimulation of intact cells, we identified two obvious phosphosubstrates with the greatest net change in phosphorylation, p68 and p20, after either treatment with IL 3 or direct PK-C activators. This observation reinforces our previous findings that PK-C translocates to the plasma membrane after the interaction of IL 3 on its receptor. Phorbol esters, such as PMA, have been shown to induce the rapid translocation of PK-C from cytosol to membrane; concomitant with this translocation PK-C is activated. We describe in this paper the phosphorylation events observed in the FDC-P1 cell line. The IL 3- and OAG-stimulated phosphorylation of one major substrate, p68, has been observed in other IL 3-dependent cell lines, FDC-P2 and NSF-60, suggesting that this substrate may be an important element in IL 3 signal transduction. Furthermore, we and others have identified a substrate with identical charge-mass ratio to p68 in cytotoxic T lymphocytes, which is phosphorylated after IL 2 and OAG stimulation of these cells. These findings indicate that p68 may be highly conserved among various lineage cells and is coupled to other growth factor receptors, such as IL 2. Using the FDC-P1 cell line, we have previously shown that IL 3 will induce a rapid but transient translocation of PK-C from the cytosol to the membrane. Interestingly, the transient phosphorylation of p68 we described in this study appears to parallel the kinetics of PK-C subcellular redistribution that we previously reported, thereby providing further evidence that the PK-C phosphotransferase system is physiologically significant for transduction of the signal delivered by IL 3-receptor interaction. Differences primarily in the dephosphorylation pattern between the physiological ligand and OAG were also observed. IL 3 stimulated the dephosphorylation of p110 and p45 substrates, whereas OAG did not. This observation suggests the possibility of an additional receptor-coupled phosphoprotein regulatory system induced by IL 3 that is not stimulated by direct activators of PK-C. Because most physiological growth...
ligands also stimulate calcium mobilization and direct stimulants of PK-C (OAG and PMA) do not, additional enzymatic systems regulated by the physiological ligand may involve calcium.

The inability of PMA and OAG to stimulate proliferation of the FDC-P1 cells is not surprising. First, other phosphorylation events not associated with the activation of PK-C, including the dephosphorylation of p110 as well as the other changes detected by densitometric scanning (Fig 2) may represent biochemical events distinct from PK-C. Second, even if physiological activation of PK-C is responsible for control of proliferation, the use of synthetic agents outside of normal feedback control might be expected to result in discordant biochemical events after long-term stimulation. The value of these pharmacological agents is in the short-term experiment in which the stimulation of rapid biochemical events mimics the activation of IL 3 on its receptor.

There is reasonable evidence that for some growth factors, such as EGF and PDGF, the respective receptors possess tyrosine kinase activity. Accordingly, we examined...
whether the unique phosphosubstrates induced by IL 3 were threonine residues, not tyrosine. These results suggest that treatment and in a cell-free system was phosphorylated on p68 protein phosphorylated in intact cells by IL 3 or OAG, the thin-layer electrophoresis to analyze products of the acid hydrolysis extracted from two-dimensional NEPhGE × SDS-PAGE. Using the phosphorylation of p68 by an enzyme regulated by phospholipid-dependent PK-C but do not exclude the possibility that p68 may be either 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 the Na"+-H" antiporter. However, other candidates for the p68 substrate exist, as suggested by the findings of a similar nuclear lamina phosphoprotein that appears to play a critical role in CHO cell proliferation and has a mol wt of around 68,000 daltons, whereas in neural cells, a 70,000-dalton phosphoprotein has been identified, which is associated with the K⁺ channel thought to be closely associated with depolarization of the cell membrane.

We have demonstrated that PK-C is involved in the transduction of the signal delivered to cells by IL 3, by the identification of common phosphosubstrates. However, IL 3 induced specific changes in the phosphorylation pattern that activators of PK-C did not mimic. Most noticeable and reproducible of these changes was the dephosphorylation event seen in the 110,000-dalton range. Whether this dephosphorylation is due to a protein phosphatase associated with ligand signal transduction or simply reflects changes in molecule solubility after ligand-receptor interaction is under investigation. The finding that additional changes in protein phosphorylation occur with IL 3 stimulation obviously supports the notion that PK-C activation is part of a coordinated signal transduction process that may be evoked by the physiological ligand. Clearly, a role for calcium has been suggested as an additional modulating signal on hormone-receptor interactions.

In conclusion, the data demonstrate that the interaction of IL 3 with its receptor induces the rapid transient translocation and activation of PK-C, which results in the reproducible phosphorylation of unique substrates also phosphorylated by direct activators of PK-C. IL 3 induced additional changes that were not observed when cells were treated with direct activators of PK-C, such as phorbol esters, suggesting that additional phosphoregulatory elements may be involved in the physiological signal transduction system.

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