Human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) exert multiple effects on the proliferation, differentiation, and function of myeloid lineage cells through their interaction with specific cell-surface receptors. There is a considerable degree of overlap in the biological effects of these two growth factors, but little is known about the mechanisms of postreceptor signal transduction. We have investigated the effects of GM-CSF and IL-3 on protein tyrosine-kinase activity in a human cell line, MO7E, which proliferates in response to either factor. Tyrosine-kinase activity was detected using immunoblotting with a monoclonal antibody (MoAb) specific for phosphotyrosine. GM-CSF and IL-3 were found to induce a nearly identical pattern of protein tyrosine phosphorylation using both one- and two-dimensional gel electrophoresis. Tyrosine phosphorylation of two cytosolic proteins in particular was increased more than 10-fold, a 93-Kd protein (pp93) and a 70-Kd protein (pp70). Tyrosine phosphorylation of pp93 and pp70 was observed within 1 minute, reached a maximum at 5 to 15 minutes, and gradually decreased thereafter. Other proteins of 150, 126, 63, 56, 42, and 36 Kd were also phosphorylated on tyrosine in response to both GM-CSF and IL-3, although to a lesser degree. Tyrosine phosphorylation was dependent on the concentration of GM-CSF over the range of 0.1 to 10 ng/mL and on IL-3 over the range of 1 to 30 ng/mL. Stimulation of MO7E cells with 12-0-tetradecanoyl-phorbol-13-acetate (TPA) or cytokines such as G-CSF, M-CSF, interleukin-1 (IL-1), interleukin-4 (IL-4), interleukin-6 (IL-6), interferon γ, tumor necrosis factor (TNF), or transforming growth factor-β (TGF-β) did not induce tyrosine phosphorylation of pp93 or pp70, suggesting that these two phosphoproteins are specific for GM-CSF-or IL-3-induced activation. The extent and duration of phosphorylation of all the substrates were increased by pretreatment of cells with vanadate, an inhibitor of protein-tyrosine phosphatases. Importantly, culture of MO7E cells with vanadate (up to 10 μmol/L) resulted in a dose-dependent increase in GM-CSF-or IL-3-induced proliferation of up to 1.8-fold. These results suggest that tyrosine phosphorylation may be important for GM-CSF and IL-3 receptor-mediated signal transduction and that cell proliferation may be, at least partially, regulated by a balance between CSF-induced protein-tyrosine kinase activity and protein-tyrosine phosphatase activity.

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GM-CSF AND IL-3 SIGNAL TRANSDUCTION

receptor, can convert an IL-3-dependent murine cell line to EGF-dependence and also from the finding that factor-dependent cell lines can be converted to factor independence by oncogenes containing tyrosine kinases. cDNAs encoding a low-affinity human GM-CSF and a low-affinity murine IL-3 receptor have recently been cloned. The predicted amino-acid sequences of the receptors suggest that they are not tyrosine kinases, and thus the mechanism leading to activation of cellular tyrosine kinases by these factors is uncertain.

In this study we have investigated GM-CSF- and IL-3-inducible tyrosine-kinase activity in a unique, factor-dependent, human megakaryoblastic leukemia cell line, M07E, which proliferates in response to either human GM-CSF or IL-3 but not to human G-CSF, interleukin-1 (IL-1), or M-CSF. Using an immunoblot technique with a monoclonal antibody (MoAb) specific for phosphoryrosine, it is demonstrated that there is rapid, dose-dependent tyrosine phosphorylation of similar sets of substrate proteins in response to physiologic concentrations of either GM-CSF or IL-3 but not in response to a variety of other hematopoietins or to activation of protein-kinase C by 12-O-tetradecanoylphorbol-13-acetate (TPA). Furthermore, the degree of tyrosine phosphorylation can be increased by pretreatment of M07E cells with the protein-tyrosine phosphatase (PT-Pase) inhibitor vanadate. This pretreatment does not induce proliferation by itself but does result in a dose-dependent increase in CSF-dependent proliferation. These results suggest that both GM-CSF and IL-3 induce tyrosine phosphorylation of an identical or overlapping set of cellular proteins and further suggest that this may be functionally important in factor-dependent proliferation.

MATERIALS AND METHODS

Reagents. Recombinant human (rh) GM-CSF, IL-3, G-CSF, interleukin-1 (IL-1), interleukin-6 (IL-6), and M-CSF were gifts from Drs Steven Clark and Gordon Wong, Genetics Institute, Cambridge, MA. GM-CSF, IL-3, and M-CSF were highly purified, while other factors were in the form of media from transfected CHO cells. Recombinant IL-1β was purchased from Genzyme Corporation (Boston, MA), and recombinant TNF was a gift from Asahi Chemical Co., (Shiznuka, Japan). Recombinant human interferon-γ was a gift from Biogen. TGFβ and platelet-derived growth factor (PDGF) were purchased from Collaborative Research (Waltham, MA). Phorbol myristate acetate (PMA), bovine serum albumin (BSA; fraction V), o-phosphotyrosine, and sodium orthovanadate were purchased from Sigma (St. Louis, MO).

The antiphosphotyrosine antibody is a murine MoAb generated using phosphotyrosine as the immunogen (Drucker et al., unpublished data, February 1989). This antibody is specific for tyrosine-phosphorylated proteins and does not cross-react with phosphoryserine, phosphothreonine, phosphohistidine, or tyrosine sulfate. When used for immunoblotting, the addition of 1 mmol/L phosphotyrosine completely eliminates all immunoreactive bands, while phosphothreonine or phosphoserine have no effect. Anti-GM-CSF antibody (3092) is a murine MoAb that neutralizes human natural and recombinant GM-CSF (Y. Kanakura and J.D. Griffin, manuscript in preparation).

Normal and leukemic cells. Normal peripheral blood and bone marrow cells were obtained from healthy adult volunteers, and mononuclear cells were prepared by ficoll-hypaque density gradient centrifugation. Monocytes were prepared from blood mononuclear cells by removing E rosetting cells and then collecting plastic adherent cells as previously described. Granulocytes were prepared from Ficoll-Hypaque pellets by dextran sedimentation as previously described and were greater than 99% granulocytes by morphological analysis and immunofluorescent staining with a MoAb reactive with a granulocyte-specific epitope of FeRIII (CD16; J. Griffin, unpublished data, July 1988).

Blood or marrow aspirate samples were obtained at diagnosis from adult patients with acute myelogenous leukemia (AML). The diagnosis of these leukemias was established by morphology, cytochemical staining, and surface-marker analysis using a panel of MoAbs. Leukemic cells were recovered by Ficoll-Hypaque density-gradient sedimentation, and mononuclear cells were cryopreserved in 10% dimethylsulfoxide in the vapor phase of liquid nitrogen until use. After thawing, samples selected for study were required to have a viability of leukemic cells of greater than 90%. All tissue samples were obtained after informed consent of donors and under institutional review board approved protocols.

Cell lines. The human GM-CSF and IL-3-dependent cell line, M07E, was obtained from Dr Steve Clark, Genetics Institute, and was originally derived by Avanzini et al from the peripheral blood of an infant with acute megakaryocytic leukemia. The cell line was cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), rh GM-CSF, 10 ng/mL, or rh IL-3, 10 ng/mL. Human myeloid leukemia cell lines HL60 and THP-1 were obtained from the American-Type Culture Collection (Rockville, MD). Characterization of the human myeloid leukemia cell line, JOSK-M, was reported previously. HL-60, THP-1, and JOSK-M were cultured in RPMI 1640 containing 10% FBS. MTT [3-(4,5-dimethylthiazolyl-2)-5-(3,4-diphenyl-tetrazolium bromide; Sigma] incorporation was used to quantitate factor-induced proliferation of M07E cells as previously described. Briefly, triplicate aliquots of M07E cells were cultured in flat-bottom microtiter plates (100 μL/well) for 72 hours at 37°C. MTT was added for the final 4-hour of culture (10 μL of a 5 mg/mL solution of MTT in phosphate-buffered saline [PBS]). At 72 hours, 100 μL of acid isopropanol (0.04 N HCl in isopropanol) was added to all wells, mixed, and the optical density measured on a microelisa plate reader at 540 nm. This assay has been more reproducible and reliable than 1H-thymidine incorporation or cell enumeration, although equivalent results are obtained with all three assays.

Stimulation with factors and cell lysis. Exponentially growing M07E cells were washed free of serum and growth factors and incubated in serum-free RPMI 1640 containing 0.5% BSA for 6 to 18 hours at 37°C to factor deprive the cells. In some experiments sodium orthovanadate (0.8 to 20 μmol/L) was added for the last 2 to 3 hours of culture. The cells were then exposed to various factors at 37°C for 30 seconds to 120 minutes. Other leukemic cell lines and normal and primary leukemic cells were washed twice with serum-free medium and exposed to growth factors. After stimulation, cells were washed with cold PBS and lysed in Lysis Buffer (20 mmol/L tris-HCl, pH 8.0, 137 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40) containing 1 mmol/L phenyl methylsulfonyl fluoride (PMSF, Sigma), 0.15 U/mL aprotinin (Sigma), 10 mmol/L EDTA, 10 μg/mL leupeptin (Sigma), 100 mmol/L sodium fluoride, and 2 mmol/L sodium orthovanadate at 4°C for 20 minutes. Insoluble material was removed by centrifugation at 4°C for 15 minutes at 10,000g.

Cytosol and membrane fractions were prepared according to the method of Koyasu et al. Briefly, the cells stimulated with growth factors were pelleted by centrifugation, snap frozen in a dry ice-acetone bath, and thawed in lysis buffer containing protease and phosphatase inhibitors as described above but without detergent. The cells were then homogenized, subjected to three cycles of...
The supernatant cytosol fraction was mixed with applied horizontally to the top of a second gel. which was then some experiments sections from nonreducing gels were excised and with 2 NP-40. The pellets were suspended in lysis buffer containing NP-40.

**Gel electrophoresis and immunoblotting.** Lysates (~150 µg for 13 × 12-cm gels and 40 µg for 8 × 7.5-cm minigels) were mixed 2 × sodium dodecyl sulfate (SDS) sample buffer with (reducing) or without (nonreducing) 2-mercaptoethanol and heated at 100°C for 5 minutes prior to one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% polyacrylamide. In some experiments sections from nonreducing gels were excised and applied horizontally to the top of a second gel, which was then run under reducing conditions. After electrophoresis, proteins were electrophoretically transferred from the gel onto a 0.2-mm nitrocellulose filter (Schleicher & Schuell) in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol at 0.4 amp for 4 hours at 4°C. Residual binding sites on the filter were blocked by incubating the nitrocellulose in TBS (10 mmol/L Tris, pH 8.0, 150 mmol/L NaCl) containing 1% gelatin (Bio-Rad Laboratories) for 1 hour at 25°C. The blots were then washed in TBS (TBS with 0.05% Tween 20) and incubated overnight with anti-P-Tyr MoAb (1.5 µg/mL in TBST). The primary antibody was removed, and the blots were washed four times in TBST. To detect antibody reactions, the blots were incubated 2 hours with alkaline phosphatase-conjugated antimouse IgG (Promega Biotec) diluted 1:2000 in TBS (1.5 mmol/L Tris-HCl, pH 9.5). The blots were washed four times in TBS, and then placed in TBS containing 1% gelatin (Bio-Rad Laboratories) for 15 minutes, and then placed in a buffer containing 100 mmol/L Tris-HCl, pH 9.5, 100 mmol/L NaCl, 5 mmol/L MgCl2, 330 µg of Nitro blue tetrazolium (NBT) per milliliter, and 150 µg of 5-bromo-4-chloro-3-indolyl phosphate (BICP) per milliliter for 10 to 30 minutes. Enzymatic color development was stopped by rinsing the blots in deionized water.

**Metabolic labeling and peptide mapping.** Peptide mapping was undertaken in two ways. First, M07E cells were treated with GM-CSF or IL-3, lysed, and subjected to SDS-PAGE. Specific bands were excised and inserted into contiguous slots of a 12-cm gel. The gels were then immunoblotted using a MoAb specific for phosphotyrosine (Fig 1). Increased phosphotyrosine was observed in proteins particularly at mol wt 93,000 and 70,000 (pp93 and pp70, respectively) after both GM-CSF or IL-3 but not after exposure to G-CSF, M-CSF, or other cytokines for 10 minutes. Changes in tyrosine phosphorylation were detected by Western blotting with a MoAb specific for phosphotyrosine (Fig 1). Increased phosphotyrosine was observed in proteins particularly at mol wt 93,000 and 70,000 (pp93 and pp70, respectively) after both GM-CSF or IL-3 but not after exposure to G-CSF, M-CSF, IL-1, IL-4, IL-6, IFN-γ, TNF, TGF-β, or PMA. A MoAb that neutralizes GM-CSF completely abolished the tyrosine phosphorylation of the pp93 and pp70 (Fig 1). Immunoblotting in the presence of an excess of phosphotyrosine completely abolished detection of all bands, while phosphoserine and phosphothreonine did not (data not shown). The high number of bands observed in the M07E cell line, both stimulated and factor deprived, is attributable to the high level of sensitivity that this MoAb has for phosphotyrosine.
containing proteins compared to many other antiphosphotyrosine antibodies (Druker and Roberts, unpublished data, 1989). Other proteins of 150, 125, 63, 55, 42, and 36 Kd were also phosphorylated in response to both GM-CSF and IL-3 but were better visualized after inhibition of tyrosine phosphatases (see below). Tyrosine phosphorylation of a mol wt 110,000 protein was observed after treatment with IL-4, a mol wt 125,000 protein after IFN-γ, and a mol wt 42,000 protein after PMA. The PMA-inducible 42 Kd protein is likely to be the previously described microtubule-associated protein-2 kinase. 

The phosphorylation of both pp93 and pp70 was dose dependent, with readily detectable activity at 1.0 ng/mL GM-CSF and maximal activity at 10 ng/mL (Fig 2A). IL-3 activity was also detectable at 1.0 ng/mL but maximal at 30 ng/mL (Fig 2B and data not shown). MO7E cells were exposed to GM-CSF for 10 minutes and then crudely fractionated into membrane-enriched and cytoplasm-enriched fractions (Fig 3). Both pp93 and pp70 were enriched in the cytoplasm fraction and depleted in the membrane fraction.

GM-CSF and IL-3 induce tyrosine phosphorylation of an overlapping set of substrates. Simultaneous exposure of MO7E cells to optimum concentrations of both GM-CSF and IL-3 did not increase the amount of pp93 visualized on the phosphotyrosine immunoblot compared to either factor alone, suggesting that phosphorylation of two proteins of identical apparent molecular mass was unlikely (data not shown). Isoelectric focusing was technically unsatisfactory because several proteins, notably pp93, failed to enter the focusing gel. Therefore a two-dimensional gel system, in which the first dimension is run under nonreducing conditions and the second dimension is run under reducing conditions, was used to further show the overall similarity of the pattern of tyrosine phosphorylation induced by GM-CSF or IL-3 (Fig 4). This gel system resolved pp93 into three distinct spots. An identical pattern was observed after either GM-CSF or IL-3 stimulation. One of the spots was approximately 200 Kd under nonreducing conditions and 93 Kd under reducing conditions, suggesting the possibility that it was either a homodimer or complexed to another protein through a reducible bond. The other two spots were 90 to 95 Kd under both reducing and nonreducing conditions. The relationship of these three spots to each other is currently under investigation.

In an effort to further compare the tyrosine phosphorylation substrates of GM-CSF and IL-3, additional studies of pp93 were undertaken because of the high abundance of this particular phosphoprotein in CSF-treated MO7E cells. The pp93 band was excised from a one-dimensional gel, and the eluted proteins were digested with staphylococcal V8 protease. The digestion products were separated on a second reducing gel and immunoblotted (Fig 5). The phosphotyrosine peptide maps of the 93-Kd region after both GM-CSF and IL-3 treatment were identical. Also, MO7E cells were labeled in vivo with 32P orthophosphate, stimulated with
GM-CSF or IL-3, and subjected to SDS-PAGE. The 93-Kd band was excised, treated with V8 protease as above, and phosphopeptides visualized by autoradiography after SDS-PAGE. Again, the peptide maps of the 93-Kd band were identical after either GM-CSF or IL-3 treatment (data not shown). Although this analysis was not performed for all the new bands visualized in Fig 3, the results shown in Fig 5 confirm the apparent identity of the pp93 protein(s) phosphorylated in response to both GM-CSF and IL-3.

**Time course of CSF-induced protein-tyrosine phosphorylation.** The kinetics of protein-tyrosine phosphorylation induced by GM-CSF and IL-3 were similar (Fig 6A and B). Peak levels of phosphorylation were observed after GM-CSF at 5 minutes and at 5 to 15 minutes after IL-3 in multiple experiments. Phosphorylation of pp93 was detectable as early as 30 seconds after GM-CSF and 1 minute after IL-3 (data not shown). Tyrosine phosphorylation patterns returned to baseline at 90 to 120 minutes (data not shown).

**Effects of sodium orthovanadate on CSF-induced tyrosine phosphorylation.** Vanadate is an inhibitor of many PTPase's. The effects of vanadate on GM-CSF- and IL-3–induced protein-tyrosine phosphorylation were evaluated by culturing factor-deprived MO7E cells with 10 μmol/L vanadate for 2 hours. Factor was then added and immunoblot analysis performed over the next 0 to 60 minutes (Fig 6). Treatment of cells with vanadate by itself resulted in very little change in the phosphorylation of most phosphoproteins over the total 3-hour period of this study. Figure 6 shows the effects of vanadate alone at 2 hours (the “0” time after factor addition). Increased tyrosine phosphorylation of pp70, pp63, and pp55 were seen in some experiments following treatment with vanadate alone (compare “0” time points in control- and vanadate-treated cells). No increase in tyrosine phosphorylation of pp150, pp125, or pp93 was detected with this method (Fig 6). However, pretreatment of MO7E cells with vanadate significantly enhanced GM-CSF– and IL-3–induced tyrosine phosphorylation of several proteins, including proteins at mol wt 225,000, 150,000, 125,000, 93,000, 70,000, 63,000, 55,000, 42,000, and 36,000. There was a fivefold to 10-fold increased tyrosine phosphorylation of pp93 and pp70 with vanadate pretreatment when compared to tyrosine phosphorylation of the same proteins in cells pretreated with media alone. Furthermore, vanadate...
also prolonged the kinetics of CSF-induced tyrosine phosphorylation (Fig 6). For example, tyrosine phosphorylation of pp70 in the absence of vanadate peaked at 5 minutes and declined rapidly thereafter in the absence of vanadate. However, in the presence of vanadate, pp70 tyrosine phosphorylation of pp70 peaked at 30 minutes and declined at 60 minutes in multiple experiments (Fig 6). For other proteins, however, vanadate had a greater effect on degree of phosphorylation than on duration of phosphorylation (compare pp93 phosphorylation in Fig 6A and 6B). These results are consistent with the known activity of vanadate as an inhibitor of tyrosine phosphatases. Additionally the increased degree of tyrosine phosphorylation observed in Fig 6 facilitates comparison of the effects of GM-CSF and IL-3, again demonstrating the near identity of the effects of these two factors on protein-tyrosine phosphorylation.

Effects of vanadate on M07E cell proliferation. Since vanadate treatment increases the degree of GM-CSF–and IL-3–induced protein-tyrosine phosphorylation, the effects of vanadate treatment on GM-CSF and IL-3–induced cellular proliferation were investigated. M07E cells were cultured in the presence of 0 to 20 μmol/L sodium orthovanadate and 0 to 31.6 ng/mL GM-CSF or 0 to 316 ng/mL IL-3 for 72 hours, followed by measurement of cell proliferation (Fig 7). Vanadate by itself had a minimal effect on the proliferation of M07E cells but significantly increased GM-CSF- and IL-3–dependent proliferation in each of three separate experiments (P < .01). At 1 ng/mL of GM-CSF, for example, 4 μmol/L vanadate increased M07E proliferation by 1.9 ± 0.2-fold; and at 10 ng/mL of GM-CSF, there was an increase of 1.57 ± 0.1-fold increase. Similarly, at 3.16 ng/mL of IL-3, 4.0 μmol/L vanadate increased M07E proliferation by 1.84 ± 0.1-fold; and at 31.6 ng/mL of IL-3, vanadate resulted in an 1.32 ± 0.6-fold increase in proliferation. In control
Tyrosine phosphorylation in normal myeloid cells and factor-independent leukemic cell lines. Using immunoblot analysis, readily detectable increased phosphorylation of pp93 in response to GM-CSF was observed in normal monocytes, neutrophils, and the leukemic cell lines THP-1 and JOSK-M but not in HL-60 cells (data not shown). Phosphorylation of pp93 was increased in 10/10 cases of primary AML treated with GM-CSF (10 ng/mL) for 10 minutes, and in 5/6 cases treated with IL-3 (10 ng/mL; data not shown). Normal bone marrow mononuclear cells did not have detectable phosphotyrosine in pp93, but treatment of cells with GM-CSF resulted in a detectable level. These results show that at least some of the common substrates for GM-CSF–and IL-3–induced tyrosine phosphorylation identified in the M07E cell line are observed in response to CSF stimulation of other types of myeloid cells.

DISCUSSION

The diverse effects of GM-CSF and IL-3 on proliferation, differentiation, and activation of both mature and immature hematopoietic cells are mediated through binding of these factors to high-affinity surface receptors. The mechanisms of signal transduction of the GM-CSF and IL-3 receptors are largely unknown. In this study we have investigated the effects of these two factors on tyrosine-kinase activity in a unique human megakaryoblast cell line, M07E, which expresses functional receptors for both GM-CSF and IL-3 and requires physiologic concentrations of either factor for viability and growth.25 The results show that exposure of factor-deprived cells to either GM-CSF or IL-3 induces rapid (less than 1 minute) tyrosine phosphorylation of a closely related, or identical, set of proteins. Tyrosine phosphorylation of two proteins was particularly prominent, pp70 and pp93. Tyrosine phosphorylation of pp93 and pp70 was unique to GM-CSF and IL-3 and was not observed after treatment of these cells with G-CSF, M-CSF, TNF, interferon-γ, IL-1, IL-4, IL-6, TGF-β, or PMA. The apparent similarity of the proteins that are phosphorylated on tyrosine residues in response to either GM-CSF or IL-3 was observed in one-dimensional and two-dimensional immunoblots and both before and after pretreating the cells with the phosphatase inhibitor vanadate to enhance the immunoblot signals. Furthermore, peptide mapping of the 93-Kd band with Staphylococcus V8 protease was carried out, again demonstrating identity of the effects of GM-CSF and IL-3 in this cell line. These results show that both GM-CSF and IL-3 induce protein tyrosine phosphorylation of an apparently overlapping set of substrates.

We also present data that suggest that GM-CSF–and IL-3–induced tyrosine kinase activity may be important for the proliferation of these cells. This suggestion is based on several observations. First, the tyrosine kinase activity is rapidly induced (1 minute or less after factor addition to the medium), which is typical for other growth factor receptors where signal transduction is mediated by tyrosine kinase activity, such as the PDGF and CSF-1 receptors.74 Second, the dose-response curves for proliferation and for tyrosine kinase activity are similar (compare Figs 2 and 7). Finally, experiments not shown, the MTT assay27 was shown to accurately measure cell number in the presence or absence of vanadate; furthermore, the results of the MTT assay shown in Fig 7 were confirmed by counting cells and by measuring 3H-thymidine incorporation. These results are consistent with the hypothesis that the induction of tyrosine-kinase activity by these factors is associated with proliferation. The effects of vanadate were dose dependent with maximum activity at 10 µmol/L, a concentration that was shown to have significant effects on protein-tyrosine phosphorylation (Fig 6). Higher concentrations of vanadate were inhibitory to cell growth (Fig 7).
pretreatment of MO7E cells with the tyrosine-phosphatase inhibitor vanadate results in enhanced factor-dependent tyrosine phosphorylation of multiple substrates and also reproducibly results in enhanced factor-dependent cell growth. Vanadate has been widely used to investigate the effects of protein-tyrosine phosphatases in signal transduction and has been used to demonstrate the role of PTPase's in dephosphorylation of receptors for insulin, and insulin-like growth factor, and epidermal growth factor. Our results with vanadate pretreatment of MO7E cells are consistent with inhibition of one or more PT-Pase. The results in Fig 6 show that vanadate treatment has a profound effect on the tyrosine-kinase activity induced by either GM-CSF or IL-3 but little effect on tyrosine-kinase activity in the absence of these factors. Furthermore, vanadate by itself did not induce cell proliferation of MO7E cells. Thus vanadate is not directly inducing a tyrosine-kinase activity in MO7E cells, and the increased protein tyrosine phosphorylation observed in Fig 6 is most likely due to inhibition of PT-Pase activity. However, direct studies of the effects of vanadate on specific PT-Pase's in hematopoietic cells are necessary. It is also possible that vanadate is stimulating some cellular process unrelated to tyrosine phosphorylation that is responsible for cell proliferation, and studies with other phosphatase inhibitors are warranted. MO7E cells have been shown to express high levels of at least one isoform of a known PT-Pase, CD45. In lymphoid cells, CD45 has been shown to dephosphorylate the tyrosine-kinase pp56ck. Furthermore, CD45 is known to be inhibitable by vanadate. Thus our data suggest that direct studies of the role of CD45 and other PT-Pase's in MO7E cells may be important in understanding growth regulation of hematopoietic cells.

Our data suggest that induction of tyrosine-kinase activity is important in the signal-transduction pathway for CSF-induced proliferation, and one or more of the substrate proteins may serve as intermediate messengers in linking the surface receptors to control of proliferation in the nucleus. As noted above, two proteins were prominently phosphorylated, and at least six other proteins were variably phosphorylated on tyrosine within 5 minutes of CSF addition. It is likely that some substrate proteins are not related to mitogenesis but stimulate other cellular processes. For example, GM-CSF and IL-3 induce similar tyrosine phosphorylation of pp93, but the mitogenic response to both factors is different (Fig 7). Thus phosphorylation of pp93 may not directly correlate with the mitogenic signal. In comparing our observations in human cells to those of others in murine cells, some potentially common substrate proteins can be identified. For example, IL-3 was reported to induce tyrosine phosphorylation of both a 90- and 95-Kd protein in the IC-2.9 cell line, as well as phosphorylation of proteins of 160, 70, and 55 Kd. In this line, however, GM-CSF had a minimal effect on tyrosine phosphorylation. Isfort et al have shown that murine IL-3 binds to a 140-kd phosphotyrosine-containing cell surface protein in FDC-P1 cells and that proteins of 140, 70, 56, and 38 Kd are rapidly phosphorylated on tyrosine in response to IL-3 binding. In B6SU1A, cells, Sorenson et al showed that IL-3 induced tyrosine phosphorylation of proteins at 140, 90, 68, 55, and 40 Kd. Interestingly, GM-CSF, although equivalent as a growth factor when compared to IL-3, induced tyrosine phosphorylation only of a 90-Kd protein. Very few studies of tyrosine phosphorylation have been done with human cells. Gomez-Cambronero et al has shown that treatment of human neutrophils with GM-CSF results in increased tyrosine phosphorylation of at least five proteins with molecular masses of 118 Kd, 92 Kd, 78 Kd, and 40 Kd. Tyrosine phosphorylation of a 75-Kd protein has also been demonstrated in factor-treated HL-60 cells. Thus in various cells of two different species, increased tyrosine phosphorylation of proteins at apparent mol wt of approximately 90 Kd and 70 Kd are commonly observed in response to GM-CSF and/or IL-3. However, it is possible that multiple tyrosine-kinase substrates are found in these molecular weight ranges, and direct comparison of these proteins will need to be done by peptide mapping or immunoprecipitation when appropriate antibodies are available.

Despite the fact that an increasing number of growth-factor receptors have been shown to be tyrosine kinases or to be associated with rapid induction of tyrosine kinase activity, very few substrates for these receptor-associated kinases have been identified. Platelet-derived growth factor stimulates the tyrosine phosphorylation of an 85-Kd protein that may be a phosphatidylinositol (PI) kinase. Transformation of 3T3 cells with v-fms also results in enhanced tyrosine phosphorylation of 85-Kd and increased PI kinase activity. Expression of v-fms, v-src, or v-sis in 3T3 cells or treatment of 3T3 cells with PDGF or EGF induces phosphorylation and activation of Raf-1 (c-raf), a serine/threonine protein kinase of mol wt 74,000. It is possible that the pp70 described here is c-raf, and experiments to test that possibility are currently underway. Preliminary data from others suggest that other c-raf may be phosphorylated in response to IL-3 in some murine cell lines. Activation of the EGF receptor has been shown to lead to tyrosine phosphorylation and activation of phospholipase C. Finally, the GTPase-activating protein, GAP (mol wt = 125,000), is phosphorylated on tyrosine in cells transformed by v-src or v-fps or in fibroblasts in response to activation of the epidermal growth factor receptor. The potential identity of these known tyrosine-kinase substrates with the proteins observed in this study is currently being investigated.

A cDNA encoding a low-affinity GM-CSF binding protein has recently been cloned from a human placental library. This receptor does not bind IL-3 but does have some homology to a family of factor receptors including the erythropoietin, IL-6, and IL-2 receptors. Similarly, a cDNA encoding a low-affinity murine IL-3 receptor has been cloned. Neither of these proteins has the structure typical of a tyrosine kinase. It is not known at this time if these are the only GM-CSF and IL-3 receptors. If these receptors are representative of the functional GM-CSF and IL-3 receptors on MO7E cells, our results would suggest that they induce rapid activation of one or more intracellular tyrosine kinases such as c-fes, c-abl, c-src, c-hek, etc, and further that the GM-CSF and IL-3 receptors may activate the same tyrosine kinases. It is possible, for example, that the receptors exist in
a complex with a tyrosine kinase or are coupled to a kinase through an intermediate step of signal transduction such as activation of a G protein. Further studies will be necessary to define the nature of the receptors for these factors and how they induce tyrosine kinase activity. We also suggest, based on preliminary studies with vanadate, that the function of this receptor/kinase complex may be modulated by one or more PTPases and that events that regulate PTPase activity could be important in regulating some types of hematopoietic cells.

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Signal transduction of the human granulocyte-macrophage colony-stimulating factor and interleukin-3 receptors involves tyrosine phosphorylation of a common set of cytoplasmic proteins

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