Internalization of the Granulocyte-Macrophage Colony-Stimulating Factor Receptor Is Not Required for Induction of Protein Tyrosine Phosphorylation in Human Myeloid Cells

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) exerts its biologic activities through binding to specific high-affinity cell surface receptors. After binding, the ligand/receptor complex is rapidly internalized in most hematopoietic cells. Using a human factor-dependent cell line, M07, and normal human neutrophils, we found that the internalization is exquisitely temperature-dependent, such that ligand/receptor internalization does not detectably occur at 4°C. Activation of the GM-CSF receptor has previously been shown to stimulate a number of postreceptor signal transduction pathways, including activation of a tyrosine kinase and activation of the serine/threonine kinase, Raf-1. The GM-CSF-stimulated increase in tyrosine kinase activity occurs rapidly at both 4°C and 37°C, and therefore is likely to be independent of receptor internalization. At 37°C, the protein tyrosine phosphorylation was transient in M07 cells, with maximum phosphorylation observed after 5 to 15 minutes, followed by a rapid decline. At 4°C, the protein tyrosine phosphorylation of the same substrates was greater than at 37°C, and no decline in substrate phosphorylation was observed for at least 90 minutes. In contrast to tyrosine phosphorylation, the activation and hyperphosphorylation of Raf-1 observed at 37°C in both M07 cells and neutrophils was markedly diminished at 4°C. These results indicate that at least one postreceptor signal transduction mechanism, activation of a tyrosine kinase, does not require ligand/receptor internalization, and indicate that receptor internalization may be a consequence, rather than the initiator, of signal transduction.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a member of a family of hematopoietic growth factors that stimulate the proliferation, differentiation, and activation of blood cells and their precursors. These biologic effects are mediated through binding of the factors to specific, high-affinity cell surface receptors. However, postreceptor signaling events remain largely unknown. In both human and murine myeloid cell lines, GM-CSF has been shown to stimulate protein tyrosine kinase activity resulting in rapid phosphorylation of several membrane and cytoplasmic proteins. For example, we have previously shown that both GM-CSF and interleukin-3 (IL-3) activate one or more tyrosine kinases in the human factor-dependent megakaryoblastic cell line, M07, resulting in phosphorylation of substrates of apparent Mr = 150,000, 125,000, 93,000, 70,000, 55,000, 42,000, and 36,000. Evidence that tyrosine phosphorylation is important for proliferation comes from observations that introduction of a known tyrosine kinase receptor, such as the epidermal growth factor (EGF) 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. In addition to activating tyrosine kinases, both GM-CSF and IL-3 rapidly induce activation of a serine/threonine kinase, Raf-1, in M07 cells and in normal myeloid cells. A cDNA encoding a low-affinity receptor for GM-CSF has been cloned and a potential second subunit of the receptor has also been identified. The predicted amino acid sequences indicate that neither subunits of the receptor are likely to be kinases, suggesting that the receptor activates tyrosine and serine/threonine kinases indirectly.

In addition to activating cellular kinases and other biochemical events, the binding of GM-CSF to the GM-CSF receptor causes the receptor-ligand complex to be rapidly internalized. Receptor internalization is also observed in response to certain activating agents, such as phorbol myristate acetate (PMA) or the calcium ionophore A23187. GM-CSF receptor internalization is more rapid and complete in neutrophils than in myeloid leukemic cell lines, and this may reflect a difference in receptor function that is related in some way to stage of differentiation. The susceptibility of the GM-CSF receptor to internalization appears to be inversely correlated with whether IL-3 can cross-compete with GM-CSF for binding to the GM-CSF receptor. It is not known if receptor internalization is required for, or associated with, signal transduction.

Receptor internalization appears to be a general property of the hematopoietin receptors, either in response to binding of specific ligand or in response to activation of a receptor for another CSF (receptor transmodulation). It has previously been shown that there is a hierarchical transmodulation of hematopoietic growth factor receptors in murine granulocyte-macrophage cells. Such cells simultaneously express receptors for IL-3, GM-CSF, granulocyte-CSF (G-CSF), and macrophage-CSF (M-CSF). Binding of IL-3 to its receptor induces downregulation of the receptors for the other three factors, while GM-CSF induces downregulation of the receptors only for G-CSF and M-CSF. It has been suggested that receptor transmodulation is associated with receptor activation and that this may be important in mediating some of the pleiotropic effects of factors such as GM-CSF and IL-3.

In contrast, it is also possible that receptor internaliza-
tion is a regulatory mechanism to limit the response of a cell to a specific CSF, and as such serves primarily to remove functional receptors from the cell surface. If so, it is likely that receptor internalization is a consequence of, rather than the initial step in, signal transduction. To better understand the significance of receptor internalization, we have investigated receptor signaling under conditions in which receptor internalization is blocked. The results indicate that receptor internalization can be separated from some aspects of receptor activation.

MATERIALS AND METHODS

Reagents. Highly purified recombinant human (rh) GM-CSF and IL-3 were gifts from Drs Steven Clark and Gordon Wong (Genetics Institute, Cambridge, MA). Bovine serum albumin (BSA, Fraction V), FHA, sodium azide, and sodium orthovanadate were purchased from Sigma (St Louis, MO). Antiphosphotyrosine monoclonal antibody (MoAb) was generated using phosphotyramine as the immunogen. This antibody is specific for tyrosine-phosphorylated proteins and does not cross-react with phosphoserine, phosphothreonine, phosphohistidine, or tyrosine sulfate. When used for immunoblotting, the addition of 1 mmol/L phosphotyrosine completely eliminated all immunoreactive bands, while phosphothreonine or phosphoserine had no effect. Anti-GM-CSF antibody (3092) is a murine MoAb that neutralizes human natural and recombinant GM-CSF.19 In control experiments, 3092 was shown to block the increase in protein tyrosine phosphorylation induced by GM-CSF. Rabbit anti-Raf-1 antiserum was generated against a synthetic peptide (CTLTSPRLPVF) corresponding to amino acid residues 315-326 of Raf-1, and was affinity-purified as described.20 The antibody was used for immunoblotting at a dilution of 1:4,000.

Cells. The human GM-CSF- and IL-3-dependent cell line, MO7, was obtained from Dr Steven Clark (Genetics Institute) and was originally derived by Avanzi et al from the peripheral blood (PB) cells of an infant with acute megakaryocytic leukemia.21 The cell line was cultured in RPMI1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), rhGM-CSF 10 ng/mL, or rhIL-3 10 ng/mL. Normal PB was obtained from healthy adult volunteers and mononuclear cells were removed by Ficoll-Hypaque density gradient centrifugation. Granulocytes were prepared from Ficoll-Hypaque pellets by dextran sedimentation as previously described,22 and were greater than 99% granulocytes by morphologic analysis and immunofluorescent staining with an MoAb reactive with a granulocyte-specific epitope of Fc receptor III (FcRIII) (CD16; J. Griffin, unpublished).

Stimulation with factors and cell lysis. Exponentially growing MO7 cells were washed free of serum and growth factors and incubated in serum-free RPMI1640 containing 0.5% BSA for 6 to 18 hours at 37°C to factor-deprive the cells. The cells were then equilibrated at 37°C or on wet ice in a 4°C cold room, and then further exposed to GM-CSF or IL-3 (10 ng/mL unless otherwise indicated) for 0 to 120 minutes. After stimulation, cells were lysed on ice without centrifugation or washing by adding 1 volume of boiling 2X sample buffer (80 mmol/L Tris-HCl, pH 6.7, 8% sodium dodecyl sulfate, 20% glycerol, 10 mmol/L 2-mercaptoethanol, 6 mmol/L EGTA, 3 mol/L EDTA, 1 mol/L phenylmethylsulfonyl fluoride [PMSF], 0.15 U/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L sodium orthovanadate [reagents from Sigma]). Insoluble material was removed by centrifugation at 4°C for 15 minutes at 10,000g. Neutrophils were pretreated with 1 mmol/L diisopropylfluorophosphate (Sigma) for 30 minutes at 4°C to inhibit proteases before stimulation with GM-CSF (10 ng/mL) at either 37°C or 4°C. After stimulation, the neutrophils were washed with cold phosphate-buffered saline (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 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. Centrifugations were performed in a refrigerated microfuge (Eppendorf 5402, Hamburg, Germany) to eliminate any possible sample warming during centrifugation.

Gel electrophoresis and immunoblotting. Lysates (~150 μg for 13 × 12 cm gels) were electrophoresed on one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% to 12% polyacrylamide. After electrophoresis, proteins were electrophoretically transferred from the gel onto a 0.2-mm nitrocellulose filter (Schleicher & Schuell, Keene, NH) in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol at 0.4 A 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 for 1 hour at 22°C. The blots were then washed in TBS (10 mmol/L Tris, pH 8.0, Tween 20) and incubated overnight with either anti-P-Tyr MoAb (1.5 μg/mL in TBS) or anti-Raf-1 polyclonal antibody (1:4,000 in TBS). The primary antibody was removed and the blots washed four times in TBS. To detect antibody reactions, the blots were incubated for 2 hours with alkaline phosphatase-conjugated antimouse or antirabbit IgG (Promega Biotech, Madison, WI) diluted 1:2,000 in TBS, washed three times in TBS, 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 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) per milliliter for 10 to 30 minutes. Enzymatic color development was stopped by rinsing the filters in deionized water. Prestained protein molecular weight standards were run on each gel and were obtained from BRL (Gaithersburg, MD).

Binding of 125I-labeled GM-CSF to myeloid cells. MO7 cells or neutrophils were washed and briefly treated with mild acid conditions to remove prebound GM-CSF (1.5 × 105 cells in 5 mL of cold PBS titrated to pH 3.0 with HCl for 2 minutes at 4°C), followed by immediate washing in 50 mL of cold PBS.23 We have previously shown that this treatment removes greater than 80% of receptor-bound GM-CSF without damaging the GM-CSF receptor.23 Aliquots of 5 × 105 cells were then cultured at either 4°C or 37°C in 300 μL of binding buffer (BB) consisting of RPMI 1640 (GIBCO) with BSA (2 mg/mL) and 25 mmol/L HEPEs, pH 7.5, with 0.028 to 6.9 ng/mL (455 pmol/L) 125I-GM-CSF (New England Nuclear Dupont, Boston, MA; specific activity, 70 to 100 mCi/mg) in the presence or absence of an approximately 100-fold excess of cold ligand to assess specifically bound counts, with a final incubation volume of 500 μL in 1.5-mL polypropylene tubes (Sarstedt Inc, Princeton, NJ). Binding was performed for 0 to 90 minutes. Under these conditions, nonspecific binding was typically in the range of 5% to 20%. After incubation, the cells were resuspended, layered over a cushion of 0.75 mL of 75% FBS, and spun for 90 seconds in a microfuge. After aspirating the supernatant, the cell pellets were cut off and counted with an efficiency of 95% (LKB Gamma master 1277; Turku, Finland). Specific binding (SB) of GM-CSF was determined from the mean of duplicate samples and was defined as follows: SB = (binding observed in the absence of cold competitor) – (nonspecific binding observed in the presence of a 100-fold excess of GM-CSF [NSB]). To measure the internalization of ligand, aliquots of cells were treated with 1 mL of cold acid solution (0.5 mol/L NaCl/0.2 mol/L acetic acid, pH 2.5) for 5 minutes, followed by washing twice in cold
PBS. As previously reported, this acid treatment removes greater than 90% of cell surface bound GM-CSF.\textsuperscript{21}

\textit{Raf-1 immune complex kinase assay}. Raf-1 was immunoprecipitated from control (factor-starved) or CSF-treated M07 cells.\textsuperscript{17} The immune complexes were collected on protein A Sepharose beads, washed, and incubated in a kinase buffer (25 mmol/L HEPES, pH 7.4, 1 mmol/L diithiothreitol [DTT], 10 mmol/L MgCl\textsubscript{2}, 15 mmol/L ATP) containing 10 \muCi \gamma\textsuperscript{32}P-ATP and histone H1 (5 \mug/assay; Boehringer Mannheim, Indianapolis, IN) as an exogenous kinase substrate\textsuperscript{23}\textsuperscript{34} for 10 minutes at 20°C. The immune complexes were then separated by SDS-PAGE and the dried gel exposed to x-ray film to visualize incorporation of \textsuperscript{32}P into histone H1.

\section*{RESULTS}

\textsuperscript{125}I-GM-CSF internalization is temperature-dependent. We have previously shown that internalization of \textsuperscript{125}I-GM-CSF after binding to neutrophils is temperature-dependent, with no detectable internalization at 4°C.\textsuperscript{22} These studies were repeated with the factor-dependent cell line, M07 (Fig 1A). At 37°C, 30% to 40% of total GM-CSF was acid-resistant (internalized) at each concentration of \textsuperscript{125}I-GM-CSF. Although the binding of \textsuperscript{125}I-GM-CSF was less at 4°C than at 37°C over the 90-minute time period, as expected, no internalization was detected in multiple experiments. In similar experiments, M07 cells were cultured for 0 to 90 minutes at both 4°C and 37°C with a single concentration of \textsuperscript{125}I-GM-CSF, 1.0 ng/mL, and the amount of internalized ligand was measured as above. Again, no internalization of \textsuperscript{125}I-GM-CSF was detected at 4°C, while at 37°C up to one-third of the total cell-associated counts were acid-resistant. These results indicate that internalization of \textsuperscript{125}I-GM-CSF does not occur in M07 cells at 4°C, consistent with our previous results in neutrophils.\textsuperscript{22} M07 cells differ from neutrophils in that the rate and completeness of internalization is lower.\textsuperscript{23}

\textbf{GM-CSF induces activation of protein tyrosine kinase activity at 4°C.} We have previously shown that GM-CSF and IL-3 rapidly induce activation of a receptor-associated tyrosine kinase in MO7 cells, resulting in tyrosine phosphorylation of several cytoplasmic substrates that can be readily identified by immunoblotting with an antiphosphotyrosine MoAb.\textsuperscript{4} To determine if GM-CSF receptor-associated activation of tyrosine kinase activity was temperature-dependent, and thereby potentially dependent on receptor internalization, antiphosphotyrosine immunoblots were performed on MO7 cells treated with GM-CSF at either 4°C (wet ice) or 37°C for 5 to 90 minutes (Fig 2). Increased tyrosine phosphorylation of multiple substrates (pp150, pp93, pp70, and pp42 can be detected in Fig 2) was observed at either temperature in multiple experiments, although with slightly different kinetics. Peak tyrosine phosphorylation of these substrates was observed after 5 to 15 minutes at 37°C, but at greater than 30 minutes at 4°C. At both temperatures, however, onset of tyrosine phosphorylation was evident in less than 5 minutes. At 37°C, the extent of tyrosine phosphorylation of substrates rapidly declined after 15 to 30 minutes, and we have previously shown that this decline can be eliminated by inhibiting tyrosine phosphatases with orthovanadate. In contrast, at 4°C, no evidence of a decline in tyrosine phosphorylation was observed for up to 90 minutes, indicating either that tyrosine phosphatases were not induced or that tyrosine kinase activity remained higher than tyrosine phosphatase activity. Several techniques were used to ensure that no warming of samples occurred during preparation. In particular, in most experiments, cells were lysed directly in boiling SDS sample buffer, avoiding any manipulation, including centrifugation, that could induce warming. In other experiments, cells were first washed with cold PBS, centrifuged in a refrigerated microfuge, and lysed in standard lysis buffer. The results were identical with either method, indicating that GM-CSF can induce tyrosine kinase activity in MO7 cells equally well at either 37°C or 4°C. Similar results were obtained when the MO7 cells were stimulated with IL-3 (data not shown). In other experiments, MO7 cells were pretreated with sodium azide (0.1%) for 30 minutes to...
GM-CSF stimulates increased protein tyrosine phosphorylation at either 4°C or 37°C in MO7 cells. Cells were factor-deprived overnight and then stimulated with GM-CSF (10 ng/mL) on wet ice (4°C) or at 37°C, and lysed at the indicated times directly into boiling 2X sample buffer. Changes in protein tyrosine phosphorylation were detected by immunoblotting. The time after GM-CSF stimulation at which the cells were lysed at either temperature is shown. Molecular weight markers on the left are in kilodaltons.

This treatment substantially reduced GM-CSF-stimulated tyrosine phosphorylation at both 37°C and 4°C.

Similar studies were performed with neutrophils, but antiphosphotyrosine immunoblots were of inconsistent quality when the cells were lysed directly in boiling sample buffer. Pretreatment of neutrophils with the protease inhibitor disopropylfluorophosphate (DFP) before stimulation with GM-CSF, followed by lysis in either sample buffer or standard lysis buffer (see Materials and Methods), gave the best results (Fig 3) and showed that increased tyrosine phosphorylation of the same substrates was evident after GM-CSF stimulation at either 37°C or 4°C. The kinetics of dephosphorylation were not detectably different in neutrophils at 37°C or 4°C, but it is possible that pretreatment with DFP may have affected the phosphatases involved.

**GM-CSF does not induce hyperphosphorylation or activation of Raf-1 at 4°C.** We have previously shown that both GM-CSF and IL-3 rapidly induce phosphorylation and activation of the cytoplasmic serine/threonine kinase Raf-1...
in MO7 cells at 37°C. The relationship of tyrosine kinase activation to Raf-1 phosphorylation is unclear. In MO7 cells, most of the Raf-1 phosphorylation is phosphoserine, rather than phosphotyrosine. To determine if signal transduction at 4°C involved Raf-1 in addition to tyrosine kinase activity, the extent of phosphorylation and enzyme activity of Raf-1 were compared after GM-CSF treatment at either 37°C or 4°C in both MO7 cells and neutrophils (Fig 4A and B). Increased phosphorylation of Raf-1, as shown by a shift in mobility in SDS-PAGE, was readily detected at 37°C, but not at 4°C. Similarly, when Raf-1-associated kinase activity was measured using histone H1 as a substrate in an in vitro kinase assay, a twofold to fivefold increase in Raf-1-associated kinase activity was detected after treating MO7 cells with GM-CSF for 5 to 15 minutes at 37°C in multiple experiments, as we have previously shown.17 In control experiments, if anti-Raf-1 antibody was omitted from the immunoprecipitation, the washed protein A beads precipitated only a minimal level of background kinase activity, which was not increased by GM-CSF treatment of the cells. In contrast, no increase was detected in four experiments when the cells were stimulated at 4°C (data not shown). Similar results were observed in DFP-pretreated neutrophils (data not shown). Thus, in contrast to the temperature-independent activation of tyrosine kinase activity induced by GM-CSF, activation and hyperphosphorylation of Raf-1 was temperature-dependent.

DISCUSSION

The GM-CSF receptor is widely expressed on both hematopoietic and nonhematopoietic cells. Recent studies suggest that the receptor is structurally complex, being composed of at least two chains,9,20 and may also be associated with other hematopoietic growth factor receptors in some cells.28,35 The binding of GM-CSF to its receptor is known to be rapidly followed by a number of biochemical events, but the details of signal transduction remain largely unknown. In the neutrophil, GM-CSF induces Na\(^+\) influx, release of arachidonic acid, and generation lipoxigenase products such as leukotriene B\(_4\).36,38 These events are inhibited by pertussis toxin, suggesting that some initial aspects of signal transduction are coupled to the receptor through a G protein.38 Also, in both neutrophils and immature myeloid cells, GM-CSF has been shown to rapidly induce tyrosine phosphorylation of several proteins.4,10 Because neither of the known chains of the receptor have a predicted amino acid structure consistent with intrinsic tyrosine kinase activity, it is likely that the receptor activates a tyrosine kinase that is either associated with the receptor or linked to the receptor through another mechanism, such as a G protein. The tyrosine kinase activation is likely to be a very proximal event in signal transduction because introduction of other known tyrosine kinase receptors, such as the EGF receptor11,12 or c-fms,39 converts cells from IL-3- or GM-CSF-dependency to EGF- or M-CSF-dependency, respectively.

![Fig 4. GM-CSF stimulates a shift in the electrophoretic mobility of Raf-1 at 37°C but not at 4°C in both MO7 cells and normal neutrophils. Factor-deprived MO7 cells (A) or freshly prepared neutrophils (B) were cultured with GM-CSF (10 ng/mL) for the indicated times, lysed as described in the legends to Figs 2 and 3, and Raf-1 detected by immunoblotting with a monospecific antipeptide antibody recognizing human Raf-1. The slower mobility of Raf-1 in SDS-PAGE after GM-CSF treatment was shown to be due to phosphorylation by treating immune complexes with alkaline phosphatase as previously described.19 The representative experiment shown was performed with the same cells used to generate the data shown in Figs 2 and 3. In the absence of GM-CSF, no shift in Raf-1 mobility was detected (not shown).](image-url)
In contrast to the induction of tyrosine kinase activity, the role of receptor internalization in GM-CSF signal transduction is unclear. In neutrophils, GM-CSF induces rapid and complete receptor internalization, followed by degradation of the ligand. The intracellular fate of the receptor has not been determined because antireceptor antibodies are not yet available. PMA also induces receptor downmodulation by itself and, in the presence of GM-CSF, PMA accelerates ligand/receptor internalization. In early myeloid cells, such as the M07 cell line studied here, the rate of receptor internalization in response to either ligand or PMA is slower than in neutrophils. Interestingly, the rate of receptor internalization is inversely correlated with GM-CSF.

The intracellular fate of the receptor has not been followed indirectly by measuring internalization of radiolabeled ligand complexed to the receptor. In neutrophils, we have previously shown that ligand/receptor internalization is associated with nearly complete loss of the receptor from the cell surface that persists for at least 4 hours. In both M07 cells and neutrophils, internalization of the ligand/receptor complex is exquisitely temperature-dependent and does not occur at 4°C. In contrast, GM-CSF activates tyrosine kinase activity as well, or better, at 4°C than at 37°C, resulting in the phosphorylation of the same substrates. Interestingly, dephosphorylation of these substrates in M07 cells was inhibited at 4°C. The differences in rate of dephosphorylation between 37°C and 4°C were less obvious for neutrophils than for M07 cells. This finding may be simply due to differences in preparation of the cells. Neutrophils were pretreated with DFP to block protease activity before stimulation, and it is possible that DFP might reduce the rate of dephosphorylation of substrates. Due to a lower level of protease activity in M07 cells, DFP pretreatment is not necessary. We have previously shown that GM-CSF–induced tyrosine phosphorylation in M07 cells is controlled by the opposing actions of a receptor-activated tyrosine kinase and a tyrosine phosphatase. Inhibition of tyrosine phosphatase activity with sodium orthovanadate enhances and prolongs GM-CSF–induced tyrosine phosphorylation of most of the substrates, and also enhances factor-dependent cell proliferation. The kinetics of tyrosine phosphorylation observed at 4°C were remarkably similar to those observed at 37°C in the presence of sodium orthovanadate. It is possible that the tyrosine phosphatase that serves to regulate GM-CSF receptor signal transduction is relatively inactive at 4°C. Alternatively, the tyrosine kinase activity may exceed tyrosine phosphatase activity for a more prolonged period of time at 4°C than at 37°C. Overall, these results show that receptor internalization can be at least partially separated from receptor activation. Further, the results indicate that balance of tyrosine kinase activity and tyrosine phosphatase activity is kinetically different at 4°C than at 37°C, and this may facilitate investigations into the identity of the various substrates that are phosphorylated on tyrosine.

Methods to chemically block receptor internalization would be useful to further investigate receptor function. We found that sodium azide, which is commonly used to inhibit internalization of many types of receptors for binding studies, inhibits GM-CSF–stimulated protein tyrosine phosphorylation at both 37°C and 4°C. However, because sodium azide acts through depletion of intracellular ATP, it is quite possible that the kinases are nonfunctional due to lack of ATP. Azide has previously been reported to inhibit the insulin receptor tyrosine kinase through a similar mechanism.

Our observation that the GM-CSF receptor–associated tyrosine kinase is active at 4°C is not unanticipated. Although many types of enzymatic activity are diminished at 4°C, tyrosine kinases are typically only minimally sensitive to temperature. For example, tyrosine kinase activity associated with the platelet-derived growth factor receptor, the epidermal growth factor receptor, the insulin receptor, the insulin-like growth factor II receptor, and the M-CSF receptor are all active at 4°C. The activation of tyrosine kinase activity at 4°C by the GM-CSF receptor is, however, the first example of such activity by a growth factor receptor that is not itself a tyrosine kinase.

In contrast to activation of tyrosine kinase activity, hyperphosphorylation and activation of the serine/threonine kinase Raf-1 was greatly diminished at 4°C in the M07 cell line or in neutrophils. This result may occur because phosphorylation of Raf-1 requires receptor internalization, but is more likely to be due to the possibility that the kinase that phosphorylates and activates Raf-1 in myeloid cells is inactive at 4°C. Raf-1 is known to be activated by mitogens in many different cell lineages and may be a common link in signal transduction pathways between various growth factor receptors and the nucleus. Our results indirectly show that phosphorylation of Raf-1 is either distal to, or unrelated to, activation of tyrosine kinases by GM-CSF in myeloid cells, and directly show that activation of tyrosine kinases does not require prior activation of Raf-1. This latter conclusion is consistent with those obtained from several previously studied cell systems in which Raf-1 is activated, because the growth factor receptors themselves have been tyrosine kinases.

The studies presented here do not address whether or not receptor internalization is required for proliferation, because the cells do not proliferate at 4°C. It is possible that receptor internalization is required for proliferation, and that the activation of tyrosine kinase activity is unrelated to cell growth. Again, however, this possibility is unlikely because of the previous demonstrations that the functions of the GM-CSF and IL-3 receptors that support proliferation of murine factor-dependent cell lines can be replaced by known tyrosine kinase receptors such as the EGF and M-CSF receptors. Taken together, these data suggest...
that the initial events of signal transduction do not require receptor internalization.

Receptor internalization has been observed to occur after the binding of several other hematopoietic growth factors to their receptors. The best studied example is the M-CSF receptor, c-fms. M-CSF/c-fms complexes are internalized within minutes, enter endosomes, and are degraded. After downmodulation, there is a refractory period of several hours during which new receptors are synthesized and reaccumulate at the cell surface. PMA also induces receptor downmodulation, but through a different mechanism than ligand, because PMA does not induce receptor autophosphorylation on tyrosine, and under conditions in which protein kinase C is itself downmodulated by chronic PMA exposure, M-CSF is still able to induce receptor internalization. Interestingly, v-fms is relatively refractory to downmodulation by either M-CSF or PMA.

In the presence of M-CSF, the intrinsic tyrosine kinase of c-fms can be activated at 4°C, and the substrates that are phosphorylated on tyrosine at 4°C are similar to those phosphorylated at 37°C. Neither the receptor nor the ligand are internalized at 4°C, indicating that signal transduction is normally initiated before, and does not require, internalization.

The results presented here suggest that the functional consequences of GM-CSF receptor internalization may be similar in many ways to c-fms internalization. In both cases, receptor internalization is not required for activation of tyrosine kinase activity, can be induced by either ligand or PMA, and probably serves primarily to limit receptor activation by directing the receptor/ligand complex to endosomes for degradation. Further studies are warranted to correlate the structure of the GM-CSF receptor complexes with both signal transduction and internalization.

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