RAPID COMMUNICATION

Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-3 Induce Rapid Phosphorylation and Activation of the Proto-Oncogene Raf-1 in a Human Factor-Dependent Myeloid Cell Line

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The product of the c-raf-1 proto-oncogene, Raf-1, is a 74,000 dalton cytoplasmic serine/threonine protein kinase that has been implicated as an intermediate in signal transduction mechanisms. In the human factor-dependent myeloid cell line M07, both granulocyte-macrophage colony stimulating factor (GM-CSF) and Interleukin-3 (IL-3) were found to induce rapid, dose-dependent phosphorylation of Raf-1, which resulted in altered Raf-1 mobility in sodium dodecyl sulfate-polyacrylamide gels. The increase in phosphorylation was due primarily to an increase in phosphoserine, with only a minor component (<2%) of phosphotyrosine. PMA (12-phorbol 13-myristate) also induced Raf-1 phosphorylation in M07 cells, but the resulting alteration in electrophoretic mobility was different than that observed after GM-CSF or IL-3. GM-CSF and IL-3 rapidly and transiently increased Raf-1 kinase activity using Histone H1 as a substrate in an immune complex kinase assay in vitro. These results suggest that phosphorylation of Raf-1 could play a role in some aspect of GM-CSF and IL-3 signal transduction.

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exposed to various factors at 37°C for 1 to 60 minutes. After stimulation, cultures were washed with cold phosphate-buffered saline (PBS) and lysed in NP-40 buffer (20 mmol/L tris-HCl, pH 8.0, 137 mmol/L NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40) containing inhibitors (1 mmol/L PMSF, Sigma, 0.15 μg/mL aprotinin (Sigma), 10 mmol/L EDTA, 10 μg/mL leupeptin (Sigma), 100 mmol/L sodium fluoride, and 2 μmol/L sodium orthovanadate) at 4°C for 20 minutes. Insoluble material was removed by centrifugation at 4°C for 15 minutes at 10,000g. Total protein content of the lysate was determined by the Bio-Rad protein assay (Bio-Rad, Rockville, NY).

**Gel electrophoresis and immunoblotting.** Lysates (~150 μg) were mixed 1:1 with 2× sodium dodecyl sulfate (SDS) sample buffer with 2-mercaptoethanol, and heated at 100°C for 5 minutes before SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred from the gel onto a 0.2-mm nitrocellulose filter (Schleicher & Schuell, Keene, NH) and immunoblotted with anti-Raf-1 antibody as described previously. Briefer, 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 immediately after the transfer. The blots were then washed in TBST (TBS with 0.05% Tween 20) and incubated washing in TBST, and developed with nitro blue tetrazolium (NBT) and 5-bromo-0-4 chloro-3 indolylphosphate (BICP) (Promega Biotec). Alfalfa seed extract was added as an exogenous kinase substrate.

**Phosphatase treatment of Raf-1 protein.** The lysates from stimulated or unstimulated cells (~10^7 cells) were preincubated with normal rabbit serum and protein A-Sepharose beads for 2 hours at 4°C. The preincubated lysates were then incubated with anti-Raf-1 antibody and protein A-Sepharose beads were used to collect the antigen-antibody complexes. The immunoprecipitates were washed five times with lysis buffer containing protease and phosphatase inhibitors as described above. For phosphatase treatment, the Raf-1 protein immunoprecipitates were washed five times with 100 mmol/L Tris, pH 9.5, 100 mmol/L NaCl, 5 mmol/L MgCl2, (alkaline phosphatase reaction buffer) containing protease inhibitors (1 mmol/L PMSF, 10 mg/mL leupeptin, and 0.15 U/mL aprotinin), and then incubated with calf intestine alkaline phosphatase (600 U/mL) for 1 hour at 37°C. After washing, treated and untreated immunoprecipitates were electrophoresed by SDS-PAGE and transferred to nitrocellulose filters. Raf-1 proteins were detected by probing the blot with anti-Raf-1 antibody.

**Labeling of cells with [32P] orthophosphate.** MO7 cells were washed three times with phosphate-free RPMI 1640 containing 0.5% BSA and cultured for 2 hours in the phosphate-free medium. After 3 hours of incubation, the cells (5 × 10^7) were washed once with the phosphate-free medium and then incubated for a further 1 hour in 1 mL of this medium containing 2 μCi of carrier-free [32P] orthophosphate (Amersham, Arlington Heights, IL). Labeled cells were cultured with or without growth factors at 37°C for 15 minutes and lysed in NP-40 buffer with inhibitors. Raf-1 was immunoprecipitated, separated by 7.5% SDS-PAGE, and visualized by autoradiography.

**Phosphoamino acid analysis.** The phosphoamino acid composition of [32P]-labeled bands excised from SDS-PAGE gels was determined as described previously. Phosphoserine, phosphothreonine, and phosphotyrosine controls (Sigma) were detected by reaction with ninhydrin, and the radioactive amino acids were detected by autoradiography.

**Immune complex kinase assay.** Raf-1 was immunoprecipitated from control (factor-starved), or CSF-treated MO7 cells. 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 DTT, 10 mmol/L MgCl2, 15 μmol/L adenine triphosphate, [ATP]) containing 10 μCi γ-[32P]-ATP for 10 minutes at 37°C. The immune complexes were then separated by SDS-PAGE. In some experiments, histone H1 (5 μg/assay, Boehringer Mannheim, Indianapolis, IN) was added as an exogenous kinase substrate.

**RESULTS**

**GM-CSF and IL-3 induce altered electrophoretic mobility of Raf-1 in MO7 cells.** MO7 cells were removed from growth factor for 12 to 18 hours, and then treated with medium alone, or physiologic concentrations of GM-CSF or IL-3 for 0 to 60 minutes (Fig 1). These cells were then lysed and equal aliquots of protein were analyzed by SDS-PAGE. Raf-1 was detected by immunoblotting with a polyclonal antibody generated against a Raf-1 synthetic peptide. This antibody detected a major band at approximately 70,000 Kd, and a minor band at approximately 68,000 Kd. Migration of these bands was unchanged in response to medium alone for up to 60 minutes (data not shown). However, after treatment with GM-CSF or IL-3, more slowly migrating forms of Raf-1 were observed in less than 5 minutes, and maximal changes were observed after

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**Fig 1.** GM-CSF and IL-3 alter electrophoretic mobility of Raf-1 in SDS-polyacrylamide gels. MO7 cells were factor deprived for 18 hours, and then treated with GM-CSF (A) or IL-3 (B) (10 ng/mL each) for 0 to 60 minutes. The cells were then lysed, and equal aliquots of protein (150 μg) were analyzed by SDS-PAGE. Raf-1 was detected by immunoblotting with a polyclonal antibody generated against a v-raf synthetic peptide. Molecular weight standards were phosphorylase B (107,000) and BSA (69,000).
GM-CSF AND IL-3 ACTIVATE Raf-1

approximately 15 minutes. Appearance of these slower migrating bands was dependent on the dose of GM-CSF or IL-3, with maximum effects detected at 10 ng/mL of either factor (data not shown). The effects of GM-CSF could be completely abrogated by a neutralizing MoAb to GM-CSF (antibody 3092, data not shown). Specificity of the anti-Raf-1 antibody was evaluated by blocking with the immunizing peptide. Reactivity of the antibody with all of the bands observed (before or after GM-CSF stimulation) could be completely blocked by the addition of Raf-1 synthetic peptide, indicating that all of the observed bands contained authentic Raf-1. The modification of Raf-1 mobility induced by GM-CSF was stable for at least 1 hour (Fig 1).

In murine fibroblasts, more slowly migrating forms of Raf-1 occurring in response to growth factors have been shown to be due to phosphorylation of Raf-1. To determine if the more slowly migrating forms of Raf-1 observed in M07 cells following CSF treatment were due to phosphorylation, Raf-1 was immunoprecipitated from cell lysates obtained before and 15 minutes after CSF stimulation, and the immunoprecipitates were treated with alkaline phosphatase. The slower migrating forms of Raf-1 appearing after CSF stimulation were eliminated by alkaline phosphatase treatment, indicating that the slower migration of Raf-1 in these cells is because of increased phosphorylation (Fig 2).

In vivo labeling and phosphoamino acid analysis of Raf-1. To directly demonstrate changes in phosphorylation of Raf-1 in response to CSFs, M07 cells were labeled in vivo with "P orthophosphate, washed, and treated with media alone, GM-CSF, or IL-3 for 15 minutes. The cells were then lysed and Raf-1 protein detected by immunoprecipitation, SDS-PAGE, and autoradiography. Treatment with either GM-CSF or IL-3 increased "P incorporation into Raf-1 (Fig 3A). To determine which amino acids were phosphorylated in Raf-1, the bands were excised from the polyacrylamide gel, the proteins eluted and hydrolyzed, and phosphoamino acid analysis conducted (Fig 3B). Phosphoserine was the predominant phosphoamino acid present in Raf-1 both before and increased substantially after CSF stimulation. Phosphothreonine was not detected in Raf-1 from unstimulated cells. In multiple experiments, a small fraction (<2%) of the "P was detected in phosphothreonine after CSF stimulation and approximately 5% was in phosphothreonine (measured by quantitative densitometric scanning of the films).

Effects of PMA, sodium orthovanadate, and okadaic acid on Raf-1 phosphorylation in M07 cells. Cells were pretreated with medium alone, sodium orthovanadate (10 μmol/L) or okadaic acid (8 μg/mL) for 2 hours and then exposed to control medium, GM-CSF, IL-3, or PMA. Raf-1 was then detected by immunoblot (Fig 4). As expected, GM-CSF and IL-3 induced the appearance of slowly migrating bands of Raf-1. Pretreatment with the phosphatase inhibitors orthovanadate or okadaic acid did not effect Raf-1 migration in either control or CSF-stimulated cells. In the same experiments, both vanadate and okadaic acid were shown to increase phosphate labeling of other cellular proteins when total cellular lysates were examined, thus indicating that phosphatases were effectively inhibited.
PMA induced a prominent alteration of Raf-1 mobility, inducing a shift of almost all Raf-1 protein to slowly migrating forms, including a very slowly migrating form that was unique to PMA and not observed after CSF treatment (Fig 4). The effects of GM-CSF, IL-3, or PMA were not significantly modulated by pretreatment with vanadate or okadaic acid.

**Immune complex kinase assay.** Raf-1 was immunoprecipitated from M07 cells before and 1 to 15 minutes after treatment with GM-CSF, IL-3, or PMA and assayed as described for autokinase activity and phosphorylation of an exogenous substrate, Histone H1 (Fig 5). When assayed with this method, GM-CSF and IL-3 increased phosphorylation of Histone H1, with activity peaking at 1 to 5 minutes. PMA treatment also markedly increased histone H1 phosphorylation. Immunoprecipitates of the same lysates with pre-immune serum did not have significant kinase activity, indicating that the kinase activity being measured in this assay is because of Raf-1, a Raf-1-associated kinase, or a kinase that is immunologically related to Raf-1.

**DISCUSSION**

Raf-1, the product of the c-raf-1 proto-oncogene, is known to encode a 74-Kd cytoplasmic serine/threonine protein kinase. Treatment of 3T3 fibroblasts with platelet-derived growth factor (PDGF), acidic fibroblast growth factor (FGF), or epidermal growth factor (EGF) has been shown to induce phosphorylation of Raf-1 and to activate Raf-1 kinase, suggesting that Raf-1 might be involved in signal transduction from some growth factor receptors. Additional evidence that Raf-1 may be important in receptor-mediated signal transduction comes from studies with the v-raf oncogene, which has been shown to stimulate proliferation of some cells constitutively, and to cooperate with v-myc in abrogating factor dependence of murine macrophages and the murine FDC-P1 cell line. There is some evidence to suggest that raf may function “downstream” of p21 ras. NIH-3T3 cells stop growing after injection of a specific anti-ras MoAb, and this growth arrest can be relieved by introduction of v-raf. Also, introduction of the v-Harvey-ras oncogene into 3T3 cells has been shown to increase ras phosphorylation. Taken together, these results support a role for c-raf in coupling some growth factor receptors to proliferation, and further suggest that Raf-1 kinase activity is likely to be regulated by phosphorylation.

The expression, function, and phosphorylation of Raf-1 have not been studied in human hematopoietic cells in any detail, although a preliminary report of Raf-1 phosphorylation in the murine cell line FDC-P1 has recently appeared. In the present studies we have shown that Raf-1 is expressed in the human factor-dependent cell line M07 and that it is rapidly phosphorylated in response to either of the two growth factors that stimulate proliferation, GM-CSF and IL-3. We have previously shown that both GM-CSF and IL-3 induce tyrosine phosphorylation of a 70,000 dalton protein (p70) in M07 cells, and presented indirect evidence that p70 might be involved in factor-dependent proliferation. In the current study, we found that the amount of phosphotyrosine detected in Raf-1 after factor stimula-

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**Fig 4. Effects of phosphatase inhibitors on Raf-1 phosphorylation.** M07 cells were pretreated with medium alone, sodium orthovanadate (10 μmol/L) or okadaic acid (8 μg/mL) for 2 hours and then exposed to control medium, GM-CSF (10 ng/mL), IL-3 (10 ng/mL), or PMA (10 μmol/L) for 15 minutes. The cells were then lysed, and Raf-1 was detected by immunoblot.

**Fig 5. Immune-complex kinase assay for Raf-1–associated kinase activity.** Raf-1 was immunoprecipitated from factor-starved M07 cells treated with GM-CSF (10 ng/mL), IL-3 (10 ng/mL), or PMA (10 μmol/L) for 0 to 15 minutes. The immune complexes were collected on protein A-sepharose beads, washed, and incubated with histone H1 (5 μg/assay) and 10 μCi γ-32P-ATP for 10 minutes at 37°C. The immune complex-histone mixtures were then separated by SDS-PAGE. Incorporation of 32P into histone H1 was visualized by autoradiography. Maximum histone H1 kinase activity was observed at 1 minute for both GM-CSF and IL-3 (2.5- and 2.3-fold increase over control by scanning densitometry), and increased activity persisted for at least 15 minutes.
tion was very small, and the increase in phosphorylation of Raf-1 was primarily because of an increase in phosphoserine. Thus, it seems likely that the p70 previously described is not Raf-1, or that Raf-1 is only one component of “p70.” Although the fraction of Raf-1 molecules phosphorylated on tyrosine was less than 2% in response to GM-CSF or IL-3, this could still be important in amplifying the signal from the GM-CSF and IL-3 receptors in MO7 cells, which are expressed at only 50 to 100 receptors/cell (S. Cannistra and J. Griffin, unpublished, December, 1989). The increase in phosphotyrosine in stimulated 3T3 cells is also very small, with phosphoserine again being the major phosphoamino acid.13,14

In murine 3T3 fibroblasts, phosphorylation of Raf-1 has been associated with an increase in Raf-1 kinase activity.13,14 Also, Kovacina et al have recently shown that insulin activates the kinase activity of Raf-1 in HeLa and Chinese hamster ovary cells overexpressing the insulin receptor.20 In MO7 cells, GM-CSF and IL-3 also reproducibly induced a significant increase in phosphorylation of Histone H1 in an immune complex kinase assay. However, the activation of Raf-1 was transient, with maximum activity being detected at 1 to 15 minutes and a slow decrease thereafter. PMA treatment also resulted in an increase in Raf-1-associated histone kinase activity, which persisted for a longer period of time (always greater than 15 minutes). The increase in Raf-1 kinase activity occurs slightly more rapidly than the shift of Raf-1 electrophoretic mobility (compare Figs 1 and 5). It is possible that only a small amount of phosphorylation is required to activate Raf-1 kinase. Also, if several amino acid residues are phosphorylated after CSF treatment, it is possible that phosphorylation of some residues is inhibitory to kinase activity. The alteration in electrophoretic mobility of Raf-1 observed after PMA was different than that observed after GM-CSF or IL-3 in that PMA treatment resulted in a more slowly migrating form of Raf-1. It is not known yet if this is because of increased phosphorylation of the same serine residues, or if GM-CSF and PMA induce phosphorylation of different residues. This question is currently being addressed, and could provide clues as to which residues are critical for Raf-1 activation. The downregulation of Raf-1-associated kinase activity seen after GM-CSF or IL-3 is interesting and suggests that some mechanism must exist to regulate Raf-1 activity in stimulated myeloid cells. It is possible that phosphorylation of certain residues are inhibitory to kinase activity, that phosphorylation alters Raf-1 cellular localization or metabolism, or that phosphatases are activated that dephosphorylate critical residues. We did not note any significant alteration in gel mobility of Raf-1 after treating MO7 cells with two phosphatase inhibitors. In HeLa cells overexpressing the insulin receptor, Kovacina et al have shown that treatment of Raf-1 immune complexes with protein phosphatase 1 (but not CD45, placental tyrosine phosphatase, or protein phosphatase 2A) reduced Raf-1 kinase activity stimulated by insulin.20 Additional information about the mechanisms responsible for the downregulation of Raf-1-associated kinase activity would be particularly helpful in understanding the biologic significance of Raf-1 in CSF signal transduction.

The kinases responsible for Raf-1 phosphorylation in MO7 cells have not been identified. Although GM-CSF and IL-3 are known to induce protein tyrosine kinase activity in hematopoietic cells, the receptors themselves do not appear to be either tyrosine or serine/threonine kinases.27,28 In particular, recent cloning of cDNAs encoding low-affinity receptors for GM-CSF and IL-3 indicate that the predicted amino acid sequence of these receptors is not typical of any known type of kinase.27,28 It has been suggested, therefore, that these receptors associate with other membrane kinases that are then activated following ligand interaction.13 The results presented here suggest that GM-CSF and IL-3 receptors can rapidly activate one or more serine kinase in addition to a tyrosine kinase. Although activation of protein kinase C has been implicated in IL-3 signal transduction in murine cells,29 activation of protein kinase C by GM-CSF or IL-3 has not been reported in human myeloid cells. In the MO7 cell line studied here, PMA is, in fact, inhibitory to cell growth, and reduces GM-CSF and IL-3-associated tyrosine kinase activity.12,30

Overall, our results implicate Raf-1 as part of a kinase cascade involved in signal transduction of both the GM-CSF and IL-3 receptors, and provide additional support for the idea that common pathways are used by different mitogens to promote cell proliferation. Identification of both the kinase responsible for Raf-1 phosphorylation, and the in vivo substrates of Raf-1 kinase will be important in understanding growth regulation of myeloid cells.

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