Interferon-inducible Protein 10 and Macrophage Inflammatory Protein-1α Inhibit Growth Factor Stimulation of Raf-1 Kinase Activity and Protein Synthesis in a Human Growth Factor-dependent Hematopoietic Cell Line*

Susan M. Aronica‡§, Charlie Mantel‡§, Rene Gonin†, Mark S. Marshall‡§, Andreas Sarris**, Scott Cooper‡§, Nancy Hague‡§, Xian-feng Zhang‡‡, and Hal E. Broxmeyer‡§§§¶¶

From the Departments of ‡Medicine (Hematology/Oncology), §Medicine (Biostatistics), ¶¶Microbiology/Immunology, †Biochemistry/Molecular Biology, and the ‡‡Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202 the **Department of Hematology, MD Anderson Cancer Center, The University of Texas Medical Center, Houston, Texas 77030, and the ††Department of Medicine, Harvard University Medical School, Boston, Massachusetts 02114

Stimulatory cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF) and steel factor (SLF), act in a synergistic manner to stimulate the growth of hematopoietic progenitor cells, an effect also demonstrated for the growth factor-dependent human hematopoietic cell line MO7e. While little is known about the mechanisms responsible for mediating synergistic interactions of cytokins, Raf-1, a component of the MAP kinase signaling pathway, is thought to play a role in the stimulatory response evoked by several cytokines, including SLF and GM-CSF. Interferon-inducible protein-10 (IP-10) and macrophage inflammatory protein-1α (MIP-1α) are members of the family of suppressive cytokines. Prior exposure of hematopoietic cells to chemokines, including IP-10, inhibits the synergistic action of growth factors on stimulating cell proliferation. Treatment of MO7e cells with the potent inhibitor of cAMP-dependent protein kinase A increases in intracellular cAMP levels, or with cAMP analogs inhibited the synergistic action of GM-CSF and steel factor (SLF) similar to interferon-inducible protein-1α (MIP-1α) and macrophage inflammatory protein-10 (IP-10). In addition, treatment of cells with a potent inhibitor of cAMP-dependent protein kinase A blocked the suppressive action of MIP-1α and IP-10 on Raf-1 kinase activity and on MO7e cell proliferation. The ability of IP-10 and MIP-1α to antagonize the synergistic actions of GM-CSF and SLF appears to involve inactivation of Raf-1 and the down-regulation of protein synthesis. Our findings suggest that both MIP-1α and IP-10 mediate their suppressive effects in MO7e cells by stimulating increases in intracellular cAMP levels and activating protein kinase A, a mechanism we believe to be unique to these chemokines and not one applied to all growth suppressive members of the chemokine superfamily (for example, interferon-inducible protein factor 4).

This article is coordinated by a major stimulatory cytokine, transforming growth factor-β (TGF-β), which is known to stimulate the growth of hematopoietic progenitor cells; thus, the stimulatory response evoked by several cytokines, including TGF-β, is coordinately regulated. Several stimulatory cytokines, including GM-CSF and M-CSF, promote proliferation directly stimulating Raf-1 phosphorylation and activation of receptor kinases, including mSOS and GRB-2. The activation of ras (4). Activated Raf is thought to be a major stimulatory pathway within target cells which are necessary for synergistic stimulation of cell proliferation.

The chemokine family of cytokines includes macrophage inflammatory protein 1-α (MIP-1α) and interferon inducible protein-10 (IP-10) (6). These molecules suppress the synergistic action of combinations of stimulatory cytokines on hematopoietic progenitor cell growth (7–10). Other suppressive chemo-

* These studies were supported by United States Public Health Service Grants R37CA36464, RO1HL46549, and RO1HL49202 from the National Cancer Institute and the National Institutes of Health (to H. E. B.) and American Cancer Society Grant BE-210 (to M. S. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

††† To whom correspondence should be addressed: Walther Oncology Center, Indiana University School of Medicine, 975 W. Walnut St., Rm. 501, Indianapolis, IN 46202-5121. Tel.: 317-274-7510; Fax: 317-274-7592.

 Vol. 270, No. 37, Issue of September 15, pp. 21998–22007, 1995
Printed in U.S.A.
Chemokines include interleukin 8 (IL-8), platelet factor 4 (PF4), MIP-2α, and macrophage chemotactic and activating factor (MCAF; also designated MCP-1) (8–10). Members of the chemokine family which do not suppress progenitor cell growth include MIP-1β and MIP-2α, GRO-α, and RANTES (7–9). While the suppressive effects of chemokines have been characterized, the cellular mechanisms through which growth inhibition is carried out have not been elucidated. Part of the difficulty is that chemokine suppression of growth factor action generally occurs during synergistic stimulation of cell proliferation. Therefore, studying the mechanism of action of specific chemokines is often limited to hematopoietic systems which not only display synergistic growth effects in response to growth factors but can be used readily for various biochemical analyses. Due to the rarity of hematopoietic stem and progenitor cells and the difficulty of isolating enough purified cells of this type for biochemical analyses, growth factor-dependent cell lines have been used (11). Growth arrest resulting from serum deprivation or growth factor deprivation is often associated with profound declines in protein synthesis rates for many cell systems (12). Since growth suppression mediated by chemokines may likely trigger responses similar to those evoked by factor deprivation, we set out to determine whether cytokine or chemokine treatment could alter protein synthesis rates in MØ7e cells.

We and others have shown previously that treatment of MØ7e cells with either GM-CSF or SLF results in increased phosphorylation and Raf-1 kinase activity (13, 14). More recently, we have shown that treatment of MØ7e cells with SLF results in the physical association between Ras and Raf-1 during an event considered necessary for activation of Ras and Raf-1. Since SLF is known to synergize with a number of growth promoting cell growth, we investigated whether Raf-1 kinase activity could be influenced by growth factors in the presence of chemokines. Given that repression of inactivated threonine residue in the Raf-1 protein kinase A (16), we also predicted that growth factors could alter Raf-1 kinase activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture medium (TC-199) was purchased from Biowhittaker (Walkersville, MD). Fetal bovine serum (HyClone Laboratories, Logan, UT). [32P]ATP ([γ-32P]ATP) was purchased from DuPont NEN (Boston, MA). [3H]Leucine ([3H]leucine) was purchased from Linomac (Lake Placid, NY). Rabbit anti-MAP kinase (ERK1) antibody, which recognizes human ERK1, was kindly provided by Santa Cruz Biotechnology, Santa Cruz, CA.

**Cells**—The human factor-dependent cell line MØ7e was obtained from Genetics Institute (Boston, MA). Biological characteristics and culture conditions for the MØ7e cell line have been described (17). MØ7e cells were maintained in RPMI 1640 culture medium supplemented with 20% FBS and 100 units/ml rhuGM-CSF. Prior to growth factor or chemokine treatment, MØ7e cells were washed with RPMI 1640 and "factor starved" in serum-free RPMI 1640 supplemented with 0.5% bovine serum albumin for 16–18 h at 37 °C.

**Proliferation of MØ7e Colony Forming Cells (CFC)**—The percent MØ7e CFC in S-phase were estimated by the high specific activity tritiated thymidine ([3H]Htdr) kill assay. Factor starved MØ7e cells were pretreated at 37 °C for 1 h with control diluent or 50 ng/ml of a specific chemokine. Cells were washed two times and treated with buffered control medium or 50 µCi of high specific activity [3H]Htdr (20 Ci/mmol; DuPont NEN) at 37 °C for 30 min prior to washing twice. Treated cells were then plated at 1.25 × 105 cells/ml in 0.3% agar culture medium with 10% FBS and in the presence of 100 units/ml rhuGM-CSF and 50 ng/ml rhuSLF. Colonies were scored after 7–8 days of incubation at 5% CO2 and lowered (5%) O2 conditions conducive for detection of the suppressive effects of MIP-1α on MØ7e cell proliferation (18).

**Indication of Chemokines and Ligand Binding Assays**—The specific ligand binding affinity and binding capacity of several chemokines were determined for MØ7e cells. Carrier-free rhuMIP-1α and rhuMIP-1β were radiolabeled with [125I]Iodide using the Bolton-Hunter (diiodo-) reagent method, as described previously (19). [32P]ATP was purchased from Amersham Corp. IL-8 was purchased from Peprotech Inc. (Rocky Hills, NJ). rhuGRO-α was a kind gift from Dr. M. P. Beattmann (Immunex Corporation). 125 I-Interleukin-8 (human) was purchased from Amersham Corp. A commercially available [3H]cAMP assay kit (Amersham Corp.) was used to measure cAMP content of samples, as per kit instructions. Incubations were carried out at 4 °C for 4 h, and charcoal-dextran-treated samples were analyzed by liquid scintillation counting.

**Immunoprecipitation**—Factor-starved MØ7e cells were washed, resuspended in phosphate-free RPMI 1640 medium containing 0.1% bovine serum albumin, and incubated for 1 h at 37 °C. Cells were then stained on ice. A commercially available 2 [3H]cAMP assay kit (Amersham Corp.) was used to measure cAMP content of samples, as per kit instructions. Incubations were carried out at 4 °C for 4 h, and charcoal-dextran-treated samples were analyzed by liquid scintillation counting.

**Chemokine Inhibition of Raf-1 Kinase Activity**

Cells were resuspended in PBS containing 5 µCi/ml [3H]leucine into a protein assay kit (Pierce). Incorporation of [3H]Leucine into protein was determined for each sample by precipitating equivalent amounts of total protein (150 µg) onto glass fiber filter discs using ice-cold trichloroacetic acid, as described (20). Total counts of [3H]Leucine present within precipitated protein and in the total protein of whole cell lysate aliquots were determined by liquid scintillation counting.

Treatment of cells with the protein synthesis inhibitor cycloheximide (21) served as a means to determine the percentage of counts which represented ongoing protein synthesis in control, untreated cells. cAMP Assay—Factor-starved MØ7e cells were plated at a density of 5 × 105 cells/well in 24-well tissue culture plates. Following treatment with various agents, cells were harvested at different time points and collected by centrifugation at 500 × g. Cell pellets were resuspended in 150 µl of cold extraction buffer (50 mM Tris-HCl, pH 7.5, 4 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin) and sonicated on ice. Samples were removed and analyzed for total protein content using a protein assay kit (Pierce).

2. A. Sarris, unpublished observations.
resuspended at 3 x 10^6 cells/ml in phosphate-free medium containing carrier-free [^32P]orthophosphate at 1.0 µCi/ml for 2 h. Radiolabeled cells were treated with chemokines and/or growth factors and then placed directly into lysis buffer. Lysates were centrifuged to remove insoluble particles, and aliquots were normalized for protein content prior to denaturation. Immunoprecipitations were conducted by combining 150 µg of each sample with 5 µg of rabbit anti-MAP kinase (ERK1) antibody and incubating on ice for 1.5 h. Raf-1-antibody complexes were collected by protein G-coated Sepharose beads. Immunoprecipitates were washed twice with 1 ml of high LiCl buffer (0.5 M LiCl, 100 mM Tris-HCl, pH 7.6), once with low LiCl buffer (0.1 M LiCl, 100 mM Tris-HCl, pH 7.6), and twice with lysis buffer. ^32P-Labeled immunoprecipitates were used directly in Raf-1 kinase assay as substrate, analyzed by SDS-PAGE and subsequent autoradiography and immunoblotting for Raf-1 content.

**Immunoblotting**—Raf-1 immunoprecipitates, or reaction mixtures from Raf-1 kinase activity assays, were combined with SDS protein sample buffer containing β-mercaptoethanol, boiled for 5 min, and the protein samples were separated by SDS-PAGE. Following electrophoresis, proteins were transferred to PVDF membrane (Millipore, Bedford, MA) using a Bio-Rad transblot apparatus (Hercules, CA) and then exposed to film. Following autoradiography, membranes were probed for specific proteins using a horseradish peroxidase-based detection system. All incubations were carried out at room temperature. Briefly, residual PVDF-binding sites were blocked by incubation of blots with a 5% milk solution in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) for 1.5 h. Blots were then incubated with primary antibody (anti-Raf-1 Ab, 1:5000, anti-Mek1 Ab, 1:2500) in TBS (TBS plus 0.05% Tween 20) for 1 h. Following two wash steps with TBST, blots were incubated with protein G-coated Sepharose beads. Following two wash steps with TBST, blots were incubated with an ECL Western blotting detection kit (Amersham Corp.) in TBST for 1 h. Blots were washed two times and placed in a solution containing a 1:1 mixture of a 3% hydrogen peroxide solution and an ECL Western blotting detection kit (Amersham Corp.) for 1 min. Blots were drained of excess liquid, wrapped in thin plastic wrap, and exposed to film. Protein content was analyzed by means of the angular transformation (24) or by Student’s t test. Statistical Analysis of Protein Synthesis and cAMP Data—The statistical analyses were conducted using StatView software for Apple Macintosh. 

**Results**

**Chemokine Inhibition of Raf-1 Kinase Activity**

MO7e cells were pulse treated for 1 h at 37°C with control medium (McCoy’s) or chemokines (50 ng/ml) plus SDF (50 ng/ml) responsive MO7e CFC were determined as described under “Experimental Procedure.” Results are based on control colony numbers of 89 ± 8 to 515 ± 40 for 3–13 separate experiments.

| Treatments | Percentage MO7e CFC in S-phase | No. of experiments |
|------------|--------------------------------|-------------------|
| Control medium | 62 ± 3 | 13 |
| rhuIP-10 | 31 ± 5α | 10 |
| rhu or rmuMIP-1α | 25 ± 4α | 13 |
| hPFP4 | 41 ± 2α | 12 |
| rhuFL-8 | 24 ± 5α | 12 |
| rhuMIP-1β | 60 ± 3 | 3 |
| rhuGRO-α | 63 ± 3 | 3 |

* Significant difference from control medium, p < 0.01.

**MO7e CFC**—We had previously demonstrated that the human CD14+ monocytes differentiate into macrophages in vitro in the presence of GM-CSF and IL-4 (23) and that stimulation with colony-stimulating factors (CSFs) induces a high proliferative response (24). This preclearing step served to remove active MAP kinase from the assay sample, since MAP kinase is known to phosphorylate Mek1 (22). In order to satisfy the normality assumptions (Gaussian distribution), the percentage data were transformed by means of the angular transformation (24). This transformation is particularly suited to transform percentage data to normality. As a check, x^2 tests for goodness-of-fit (25, 26) were also conducted. Raf-1 and GST-Mek1 phosphorilation data were analyzed using analysis of variance, with experiments as blocks and GM-CSF, SDF, GM-CSF + SDF, and Control as effects. The means of GM-CSF, SDF, and Basal Levels of Leucine Incorporation Are Inhibited by Var.
Chemokine Inhibition of Raf-1 Kinase Activity

Suppressed by IP-10, MIP-1α, and Cholera Toxin—While the ability of specific chemokines to suppress the growth of hematopoietic progenitor cells and MO7e cells has been well documented (8–10, 18, 30), studies relating relative binding affinity to suppressive activity for each chemokine have not been reported for MO7e cells. In order to determine whether the ability of a chemokine to suppress cell growth or protein synthesis in MO7e cells might be related to ligand binding, we set out to determine the ligand binding affinity and capacity of binding sites on the surface of MO7e cells for several chemokines. rhuMIP-1α bound specifically to MO7e cells with a relatively high affinity, with a calculated dissociation constant of 1.2 nM and a capacity of 2,266 binding sites/cell (Table II). This observation is consistent with the results of our previous study in which the dissociation constant for MIP-1α resuspended in an acetonitrile:ether solution was determined to be 1.0 nM (31). We found that binding of chemokines specifically to the surface of MO7e cells was dependent on the number of binding sites determined by the differential elution of radiolabeled MIP-1α and IL-8 from protein-A affinity columns. Similar results were obtained in each of three separate experiments.

Growth Factor-stimulated Increases in Protein Synthesis Are Suppressed by IP-10, MIP-1α, and Cholera Toxin—Treatment of MO7e cells with the growth factors GM-CSF (100 units/ml) and SLF (50 ng/ml) resulted in significant 40–65% increases in the level of [3H]leucine incorporation within 12 h (Fig. 1). IP-10 (50 ng/ml) or MIP-1α (50 ng/ml) exposed to cycloheximide for 1 h, washed with PBS, and then received control vehicle only for the remainder of the treatment duration. Whole cell lysates were prepared and analyzed for protein content. [3H]leucine incorporation was determined for lysate aliquots (150 µg/sample) by trichloroacetic precipitation labeled proteins onto glass fiber filters and counting the amount of [3H]leucine present in dried filters by liquid scintillation counting. Each point represents the mean of three separate determinations. Incorporation levels were significantly lower than control values (p < 0.05) for all groups treated with IP-10, MIP-1α, PF4 at concentrations of 50 µM or greater. In contrast, treatment with MIP-1β at 50 ng/ml did not significantly effect basal levels of leucine incorporation. Similar results were obtained in each of three separate experiments.

Since cholera toxin, which acts by increasing cellular levels of cAMP, could mimic the action of IP-10 and MIP-1α in antagonizing the stimulatory effects of GM-CSF plus SLF on protein synthesis levels, Pretreatment with IP-10 for 15 min prior to stimulation with GM-CSF plus SLF blocked the increases in protein synthesis (70–80%) (Fig. 3). Administration of IP-10 30 min prior to growth factor treatment suppressed the level of [3H]leucine incorporation down to control levels (Fig. 3). IP-10 and MIP-1α Stimulate Significant Increases in cAMP—Since cholera toxin, which acts by increasing cellular levels of cAMP, could mimic the action of IP-10 and MIP-1α in antagonizing the stimulatory effects of GM-CSF plus SLF on protein synthesis levels, we determined whether these or other chemokines could be acting by altering intracellular cAMP levels. Whole cell lysates were prepared from factor-starved MO7e cells treated for different times with various chemokines or cholera toxin, and then assayed directly for cAMP content. Treatment of MO7e cells with 50 ng/ml IP-10 or MIP-1α evoked significant (p < 0.05) 3–4-fold increases, respectively, in cAMP levels within 2 h (Fig. 4). This observation is consistent with results of our recent study in which ACN-treated rhuMIP-1α was shown to significantly increase cAMP in MO7e cells, while rhuRANTES was not (31). Treatment with cholera toxin (1 µg/ml) stimulated increases in cAMP 5–6-fold higher than the maximal level stimulated by either chemokine (not shown). In contrast to these results, treatment with other chemokines,
Chemokine Inhibition of Raf-1 Kinase Activity

FIG. 2. Chemokine pretreatment antagonizes the stimulatory action of GM-CSF plus SLF on protein synthesis levels. Factor-starved MO7e cells maintained in leucine-free RPMI supplemented with [3H]leucine (5 µCi/ml) were treated for the indicated durations with either 100 units/ml GM-CSF plus 50 ng/ml SLF (GM+SLF), 50 ng/ml IP-10 (IP-10), 50 ng/ml MIP-1α (panel A), 1 µg/ml cholera toxin (C.T., panel B), 50 ng/ml IL-8 (panel C), 50 ng/ml PF4 (panel C), IP-10 for 1 h prior to GM+SLF (IP-10+GM+SLF, panel A), MIP-1α for 1 h prior to GM+SLF (MIP-1α+GM+SLF, panel A), cholera toxin (1 µg/ml) for 1 h prior to GM+SLF (CT+GM+SLF, panel B), IL-8 for 1 h prior to GM+SLF (IL-8+GM+SLF, panel C), MIP-1β for 1 h prior to GM+SLF (MIP-1β+GM+SLF, panel C), or PF4 for 1 h prior to GM+SLF (PF4+GM+SLF, panel C). Cell lysates were analyzed for protein content and [3H]leucine incorporation, as described in the legend to Fig. 1. Each point represents the mean of three separate determinations. Incorporation levels for the GM-CSF plus SLF treatment group were significantly higher than controls at 12, 18, and 24 h for experiments shown in panels A–C (p < 0.05). Incorporation levels for MIP-1α, IP-10, or cholera toxin pretreatment groups were significantly less than those for the GM-CSF plus SLF group at 12, 18, and 24 h (p < 0.05).

TABLE II
Chemokine binding affinity and capacity in MO7e cells

Factor-starved MO7e cells resuspended in PBS containing 0.5% BSA were incubated in the presence of various concentrations of 125I-labeled chemokines (range 10−0.1 nM) +/- a 100X molar excess of unlabeled chemokine for each concentration. Microfuge tubes were maintained on ice at 4 °C for 2 h. Specific ligand binding, as measured by radioactivity, was determined for each chemokine as described under “Experimental Procedures.” Each ligand dissociation constant and binding capacity were determined from Scatchard plot analysis of each individual experiment using the non-linear kinetics curve-fitting program Enzfitter. Each value represents the mean +/- S.D. of observations obtained from three separate experiments.

| Chemokine   | Dissociation constant $K_d$ (nM) | Binding capacity (no. sites/MO7e cell) |
|-------------|----------------------------------|----------------------------------------|
| rhuMIP-1α   | 1.22 ± 0.4                       | 2,266 ± 202                             |
| rhuIL-8     | 1.32 ± 0.5                       | 1,954 ± 223                             |
| rhuMIP-1β   | 3.90 ± 1.4                       | 2,421 ± 384                             |

including PF4, IL-8, MIP-1β, and GRO-α, failed to stimulate any changes in cellular cAMP levels (Fig. 4).

Increased Raf-1 Phosphorylation Stimulated by Growth Factors Is Antagonized by Chemokine Pretreatment—Since GM-CSF and SLF each alone are believed to exert at least part of their effects within target cells through an activation of the Ras/Raf-1/MAP kinase cascade (13, 15, 32, 33), we set out to determine whether treatment with the growth factors GM-CSF and SLF, alone or in combination with various chemokines, could alter the phosphorylation state of the Raf-1 kinase protein. Factor-starved MO7e cells maintained in phosphate-free medium were incubated with [32P]ATP in the presence of various growth factors and/or chemokines. [32P]-Labeled proteins were immunoprecipitated from whole cell lysates using anti-Raf-1 antibodies, separated by SDS-PAGE, transferred to PVDF membranes, and exposed to film. Raf-1 appears as a single band at an approximate molecular weight of 74 kDa, as indicated by the arrow (Fig. 5, A–C). Treatment of factor-starved MO7e cells with the combination of GM-CSF (100 units/ml) and SLF (50 ng/ml) synergistically increased the phosphorylation of Raf-1 (Fig. 5A, lanes 1–4). Pretreatment with IP-10 (50 ng/ml) or MIP-1α (50 ng/ml) for 1 h prior to growth factor treatment greatly reduced the increase in Raf-1 phosphorylation (Fig. 5A, lanes 5 and 6). Pretreatment with IP-10 for 15 min was less effective than the 1-h pretreatment duration at blocking Raf-1 phosphorylation (Fig. 5A, lane 7).
Fig. 3. Inhibitory effectiveness of IP-10 is related to pre-treatment duration. Factor-starved MO7e cells were treated with IP-10 at the same time as 100 units/ml GM-CSF plus 50 ng/ml SLF (IP-10(0)+G+S), or for 15 min (IP(15)+G+S), 30 min (IP(30)+G+S) or 45 min (IP(45)+G+S) prior to treatment with GM-CSF plus SLF in the presence of [3H]leucine. Cell lysates were analyzed for protein content and [3H]leucine incorporation, as described in the legend to Fig. 1. Each point represents the mean of duplicate determinations. Similar results were obtained in each of three separate experiments.

Fig. 4. IP-10 and MIP-1α significantly increase cAMP levels in MO7e cells. Factor-starved MO7e cells were treated for the indicated times with IP-10 (50 or 100 ng/ml), MIP-1α (50 or 100 ng/ml), PF4 (100 ng/ml), IL-8 (50 ng/ml), MIP-1β (50 ng/ml), or 1 µg/ml cholera toxin (not shown). Cell lysates were analyzed directly for protein content and for cAMP content, using a commercially available [3H]cAMP assay kit (Amersham). Each sample was assayed in duplicate. Each bar represents the mean ± S.E. of separate determinations obtained from three separate experiments. cAMP levels evoked by treatment with either concentrations of IP-10 or MIP-1α were significantly higher than control levels at 2 and 4 h (p < 0.05). cAMP levels of cells treated with PF4, IL-8, or MIP-1β did not differ significantly from control levels.

Pretreatment with cholera toxin (1 µg/ml) or forskolin (50 µM) for 1 h also reduced the increases in phosphorylation of Raf-1 stimulated by GM-CSF and SLF (Fig. 5A, lanes 8 and 9). In contrast to these results, pretreatment of cells with 50 ng/ml GRO-α, MIP-1β, or IL-8 failed to block phosphorylation of Raf-1 stimulated by GM-CSF plus SLF (Fig. 5B). Immunoblot analysis of PVDF membranes for Raf-1 protein using anti-Raf-1 antibodies demonstrated equivalent protein loading between lanes (Fig. 5C). Results of densitometric analysis of phospho-

Effect of chemokine and cytokine treatment on Raf-1 phosphorylation. Factor-starved MO7e cells (3 × 10⁶ cells/ml) were cultured with [32P]orthophosphate in phosphate-free RPMI, as described under “Experimental Procedures.” A, cells were treated for 10 min with 100 units/ml GM-CSF (lane 2), 50 ng/ml SLF (lane 3), or the combination of GM-CSF plus SLF (lane 4), or were treated with 50 ng/ml IP-10 (lane 5), 50 ng/ml MIP-1α (lane 6), 1 µg/ml cholera toxin (lane 8), or 50 µM forskolin (lane 9) 1 h prior to a 10 min treatment with GM-CSF plus SLF. Cells were also treated with 50 ng/ml IP-10 for 15 min prior to GM-CSF plus SLF (lane 7). B, MO7e cells were treated for 10 min with GM-CSF (100 units/ml) plus SLF (50 ng/ml) (lane 2), for 1 h with 50 ng/ml GRO-α (lane 4), 50 ng/ml MIP-1β (lane 5), 50 ng/ml PF4 (lane 6), 50 ng/ml IL-8 (lane 7), or were treated for 1 h with 50 ng/ml GRO-α (lane 3), 50 ng/ml MIP-1β (lane 8), or 50 ng/ml IL-8 (lane 9) prior to a 10-min treatment with GM-CSF plus SLF. Control cells (lane 1, panels A and B) received vehicle only. Raf-1 proteins were immunoprecipitated from whole cell lysates by anti-Raf-1 antibodies, separated by 12% SDS-PAGE, transferred to PVDF membrane, and the intensity of 32P-labeling visualized by autoradiography. At left is indicated the position of the molecular weight markers. Raf-1 appears as a single band at approximately 74 kDa, as indicated by the position of the arrow. C, Raf-1 protein content was determined by immunoblotting PVDF membranes used for 32P analysis with anti-Raf-1 antibodies and horseradish peroxidase-linked protein G. Raf-1 proteins were visualized upon exposure of ECL-treated membranes to film, as described under “Experimental Procedures.” Treatment groups are the same as in panel A.
Chemokine Inhibition of Raf-1 Kinase Activity

**Table III**

| Treatment               | N  | Mean area | Standard deviation | Minimum | Maximum |
|-------------------------|----|-----------|--------------------|---------|---------|
| Control                 | 15 | 3.45      | 1.69               | 1.19    | 6.27    |
| GM-CSF                  | 11 | 6.26      | 1.78               | 2.98    | 8.63    |
| SLF                     | 11 | 6.84      | 1.63               | 2.84    | 8.71    |
| GM-CSF + SLF            | 15 | 15.89*    | 3.42               | 9.84    | 20.98   |
| MIP-1α                  | 6  | 3.97      | 1.81               | 2.09    | 6.80    |
| IP-10                   | 6  | 3.91      | 1.47               | 2.57    | 6.44    |
| MIP-1α + GM-CSF + SLF   | 15 | 7.22      | 2.25               | 3.23    | 10.35   |
| IP-10 + GM-CSF + SLF    | 13 | 8.19      | 2.78               | 3.81    | 12.04   |

a N = number of separate determinations in each group.

b Denstometry measurements for each group are expressed as the mean area under the peak, corresponding to band intensity on the autoradiogram, expressed as a percentage of the total area under the curve analyzed by the densitometer within each experiment. Values were normalized to protein content prior to statistical analysis.

c Significant difference from Control mean (Dunnett, p < 0.05).

d Synergistic interaction between GM-CSF and SLF (F, p < 0.001).

e Significant difference from GM-CSF + SLF mean (p < 0.05).

Results of densitometric analysis of phosphorylated protein bands are presented in the summary statistics of Tables III and IV.

Growth Factor Stimulation of Raf-1 Kinase Activity Is Suppressed by Chemokine Pretreatment — We determined whether the actions of IP-10 and MIP-1α on Raf-1 phosphorylation were related to changes in kinase activity. Raf-1 protein bands were immunoprecipitated from M07e whole cell lysates, separated by SDS-PAGE and blotted to polyvinylidene difluoride membranes, as described under “Experimental Procedures.” Intensity of [32P]orthophosphate-labeled proteins were visualized by autoradiography of membranes and differences described under “Experimental Procedures.” Intensity of densitometric analysis of phosphorylated protein bands are presented in the summary statistics of Tables III and IV.

Significant difference from Control mean (Dunnett, p < 0.05). For both Raf-1 and MAP kinase, the means of GM-CSF + SLF were normalized to Raf-1 or Mek1 protein content prior to statistical analysis. For both Raf-1 and MAP kinase, the means of GM-CSF + SLF values were significantly higher than the mean IP-10 + GM-CSF + SLF (Dunnett, p < 0.05) for the results of our studies suggested a correlation between changes in cellular cAMP levels and the suppressive activities of cAMP-dependent protein kinase A. cAMP values were normalized to Raf-1 and MAP kinase activity. Since Raf-1 is known to be inhibited directly by the action of cAMP-dependent protein kinase A, we made use of the specific protein kinase A inhibitor PKI (34) to block the action of protein kinase A within M07e cells. As expected, treatment of M07e cells with GM-CSF in combination with PKI resulted in a decrease in Raf-1 kinase activity, as shown by the increased phosphorylation of the GST-Mek1 substrate in a Raf-1 kinase assay (Fig. 7, lower band, lane 2). Pretreatment with PKI (10 μg/ml) had no effect upon the ability of GM-CSF plus SLF to activate Raf-1 (lane 3). Pretreatment of M07e cells with MIP-1α prior to growth factor treatment resulted in a decrease in the phosphorylation and activity of Raf-1 (lane 5). Pretreatment of M07e cells with MIP-1α in combination with PKI, however, resulted in a block to the suppressive action of MIP-1α and allowed for the activation of Raf-1 by GM-CSF and SLF, as evidenced by increased Mek1 phosphorylation (Fig. 7, lane 6).

In addition to examining the ability of PKI to block the inhibitory effects of MIP-1α on Raf-1 activation, we set out to determine whether PKI could also block the growth suppres-
MIP-1α and IP-10 were blocked completely upon coincubation to stimulation by the combination of GM-CSF (100 units/ml) for 1 h prior to a 10-min treatment with GM-CSF plus SLF. Control (lane 4), MIP-1β of cells with either chemokine and 10 µg/ml PKI (Table V). In

and 50 ng/ml SLF (Table V). The suppressive activity of PF4, and IL-8 significantly of MO7e cells for 1 h at 37 °C in vitro.

Membranes with anti-Mek1 antibodies, horseradish peroxidase-linked bodies. Immune complexes were collected on protein G-Sepharose beads, washed, and incubated with GST-Mek1 (1 µg) and 0.1 mM ATP for 30 min at 30 °C. Immunocomplexes were subjected to SDS-PAGE, transferred to PVDF membranes, and 32P incorporation with results of Raf-1 kinase assays and MO7e CFC thymidine incorporation buffer conditions and the amount of Raf-1. We report here that part of Raf-1 kinase activity stimulated by growth factor treatment, while simultaneously increasing cAMP levels by cAMP may not be applied as a general inhibitory mechanism for all suppressive chemokines, but as one which is limited to a particular subset. ACN treatment of cells with agents which serve to increase cAMP levels, such as cholera toxin and forskolin, and by the cAMP analog 8-bromo-cAMP. These results are consistent with those we reported in a recent study in which cAMP and camp analogs were shown to inhibit the number of MO7e CFC in cycle, in a manner similar to the suppressive action of ACN-treated MIP-1α (31). Taken together with results of Raf-1 kinase assays and MO7e CFC thymidine kill assays in which we demonstrated that the suppressive action of MIP-1α and IP-10 are blocked by the protein kinase A inhibitor PKI, our observations strongly suggest that part of the inhibitory mechanism employed by MIP-1α and IP-10 relate directly to alterations in intracellular cAMP levels. However, our observations that rhuIL-8 and PF4 fail to alter cAMP levels, fail to block activation of Raf-1 stimulated by growth factors, and do not appear to be sensitive to inhibition of protein kinase A in MO7e cells indicate that inactivation of Raf-1 by cAMP may not be applied as a general inhibitory mechanism for all suppressive chemokines, but as one which is limited to a few members of the chemokine superfamily.

A receptor which binds MIP-1α with high affinity has recently been cloned (38). Based upon sequence information, this receptor is thought to be comprised of seven membrane-spanning domains, a protein structure which is consistent with membrane-bound receptors coupled through G-proteins to adenyl cyclase and other effector molecules (38). While this observation is of interest, neither direct nor indirect activation of adenyl cyclase has been reported for any of the chemokine...
Chemokine Inhibition of Raf-1 Kinase Activity

TABLE IV
Densitometric analysis of Raf-1 kinase assay data

| Treatment                  | N  | Mean area | Standard deviation | Minimum | Maximum |
|----------------------------|----|-----------|--------------------|---------|---------|
| Control                    | 12 | 4.04      | 1.81               | 0.95    | 7.69    |
| GM-CSF                     | 8  | 6.52      | 2.46               | 1.11    | 10.51   |
| SLF                        | 5  | 7.42      | 3.17               | 1.90    | 14.08   |
| GM-CSF + SLF               | 5  | 18.77     | 6.21               | 10.75   | 30.67   |
| IP-10                      | 5  | 4.29      | 2.94               | 1.01    | 7.36    |
| MIP-1α + GM-CSF + SLF      | 5  | 8.68      | 3.67               | 3.00    | 16.78   |
| IP-10 + GM-CSF + SLF       | 10 | 9.58      | 5.99               | 4.04    | 14.93   |

* N = number of separate determinations in each group.
* Significant difference from Control mean (Dunnett, p < 0.05).
* Synergistic interaction between GM-CSF and SLF (F, p < 0.001).
* Significant difference from GM-CSF + SLF mean (p < 0.05).

Our observation that both growth suppressive chemokines, such as MIP-1α and IL-8, and non-suppressive chemokines, such as MIP-1β, can bind with moderately high affinity to MO7e cells indicates that the degree of suppressive activity may not be related directly to the number or specificity of chemokine-binding sites, but rather to the effector pathways activated in response to ligand binding. However, the 2-3-fold difference between binding affinities for MIP-1α and MIP-1β may account for the ability of excess MIP-1β to block the suppressive action of MIP-1α, since we have shown that greater concentrations of MIP-1β are required in order to block MIP-1α suppressive action (18).

We have demonstrated that GM-CSF and SLF can stimulate increases in protein synthesis in MO7e cells. This provides an additional model system through which the growth promoting effects of cytokines can be examined. By making use of protein synthesis regulation as an assay system, we have shown that pretreatment of MO7e cells with the chemokines IP-10 and MIP-1α, and IL-8 and to a lesser extent, can block the stimulatory effects of GM-CSF and SLF. Since declines in protein synthesis are associated with quiescent or growth-arrested cells, and since chemokines alter protein synthesis, it is clear that the suppression of the growth promoting effects of GM-CSF and SLF on hematopoietic cells (13-15) may be linked to growth suppression. Although these chemokines to block the stimulatory effects of growth factors appears to require a minimal pretreatment action, it is likely that stimulatory and inhibitory pathways that are utilized by cytokines and chemokines may share several key components which, depending upon how they are modified, may ultimately lead to growth activation or suppression. Although Raf-1 activation of MAP kinase is thought to play a role in the growth promoting effects of GM-CSF and SLF on hematopoietic cells (13-15), the connections to factors downstream of MAP kinase which are ultimately responsible for activation of cell growth have not been completely identified. Since active cell growth and division are associated with periods of increased protein synthesis, it is likely that a key component of growth regulation by cytokines may reside in the activation of the protein synthesis machinery within target cells. Activation of the initiation factor eIF-4E, as an example, is thought to represent at least one of the rate-limiting steps in the stimulation of protein synthesis in eukaryotic systems (39). What is of particular interest regarding eIF-4E is that it can be phosphorylated and activated through an unknown mechanism directly upon exposure to active Ras and, in a manner similar to Raf-1, can be activated in response to phosphorylation by protein kinase C (40). Since exposure of hematopoietic cells and MO7e cells to cAMP has been shown to be inhibitory for cell growth (30, 31, 41), it may be through inactivation of eIF-4E or related proteins that protein synthesis is shut down and cell growth is halted. Conversely, activation of protein synthesis in...
Chemokine Inhibition of Raf-1 Kinase Activity

TABLE V

Influence of PKI on abrogation of the suppression of MO7e CFC in S-phase by MIP-1α, IP-10, IL-8, and PF4

MO7e cells were pulse treated for 1 h at 37 °C with control medium (McCoy’s) or chemokines (50 ng/ml) in the presence or absence of PKI (10 μg/ml) prior to pulse treatment of MO7e cells +/- high specific activity [3H]Tdr. Percent GM-CSF (100 units/ml) plus SLF (50 ng/ml) responsive MO7e CFC were determined as described under “Experimental Procedures.”

| Treatment       | CFC +[3H]Tdr –PKI | CFC +[3H]Tdr +PKI | CFC +[3H]Tdr –PKI | CFC +[3H]Tdr +PKI | %CFC in S-phase +PKI
|------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Control          | 337 ± 7           | 318 ± 6           | 146 ± 10          | 125 ± 8           | 57 ± 14           |
| MIP-1α           | 318 ± 14          | 317 ± 12          | 220 ± 9           | 115 ± 2           | 31 ± 6            |
| IP-10            | 316 ± 9           | 308 ± 7           | 210 ± 10          | 122 ± 7           | 34 ± 6            |
| IL-8             | 315 ± 15          | 315 ± 10          | 200 ± 1           | 213 ± 6           | 37 ± 2            |
| PF4              | 312 ± 13          | 309 ± 6           | 206 ± 8           | 210 ± 6           | 34 ± 6            |

* CFC values represent the mean +/- S.E. of the number of responsive colonies observed for three separate determinations.

13. Miyazawa, K., Hendrie, P. C., Mantel, C., Wood, K., Ashman, L. K., and Broxmeyer, H. E. (1991) Exp. Hematol. 19, 1110–1120
14. Kanakura, Y., Duker, B., Wood, K. W., Mammen, H. J., Okuda, K., Roberts, T. M., and Griffin, J. D. (1991) Blood 77, 243–246
15. Tauchi, T., Feng, G.-S., Marshall, M. S., Shen, R., Mantel, C., Pawson, T., and Broxmeyer, H. E. (1994) J. Biol. Chem. 269, 25205–25212
16. Wu, J., Dent, P., Jelink, T., Wolfman, A., Weber, M. J., and Sturgill, T. W. (1991) Science 262, 1065–1068
17. Hendrie, P. C., Miyazawa, K., Yang, Y.-C., Langefeld, C. D., and Broxmeyer, H. E. (1991) Exp. Hematol. 19, 1091–1097
18. Broxmeyer, H. E., Benninger, S. M., Buga, N., Hendrie, P., Cooper, S., Mantel, C., Cornetta, K., Vadas, M. M., Dainiak, N., Schechter, A., and Najman, A. (1991) Inserm EuroTEXT Libby, Paris
19. Oh, K. O., Monia, B. P., Conia, H., Murray, R., Kom, Y. J., Bruheim, J., and Broxmeyer, H. E. (1994) J. Immunol. 147, 2978–2983
20. Miyazawa, K., Hendrie, P. C., Mantel, C., Wood, K., Ashman, L. K., and Broxmeyer, H. E. (1992) Adv. Hematol. 358, 417–421
21. Lu, L., Krueger, J., and Ravetch, J. V. (1993) J. Biol. Chem. 268, 7785–7800
22. Nishida, E., and Gotoh, Y. (1993) Trends Biochem. Sci. 18, 126–131
23. Neote, K., DiGregorio, D., Mak, J. Y., Horuk, R., and Schall, T. J. (1993) J. Biol. Chem. 268, 2586–2594
24. Snedecor, G. W., and Cochran, W. G. (1989) Statistical Methods, 8th Ed., pp. 191–216.
25. SAS Proprietary Software (1989) Cary, NC
26. Shapiro, S. S., and Francia, R. S. (1972) Biometrics 71, 335–342
27. Snedecor, G. W., and Cochran, W. G. (1989) Biostatistical Analysis, 2nd Ed., Prentice-Hall, pp. 194–195, 2nd Ed., Prentice-Hall, Edgewood Cliffs, NJ
28. SAS Proprietary Software (1989) Cary, NC
29. Reference Manual, Stata Release 3.1 (1993) Stata Corporation, College Station, TX
30. Hendrie, P. C. and Broxmeyer, H. E. (1994) Immunopharmacology 16, 547–560
31. Mantel, C., Aronica, S., Luo, Z., Marshall, M. S., Kim, Y. J., Cooper, S., Hague, S., and Broxmeyer, H. E. (1993) EMBO J. 12, 3358–3365
32. Kanakura, Y., Duker, B., Broxmeyer, H. E., Kwon, B. S., Pratt, G. X., Banerjee, P., Brautigan, D. L., Rapp, U. R., and Druker, B. J. (1995) Blood 85, 99–101
33. Kyriakis, J. M., App, H., Zhang, X., Banerjee, P., Brautigan, D. L., Rapp, U. R., and Druker, B. J. (1995) Mol. Cell. Biol. 15, 2342–2350
34. Rinker-Schaeffer, C. W., Austin, V., Zimmer, S., and Rhoads, R. E. (1992) Science 256, 1065–1068
35. Chuang, E., Barnard, D., Hettich, L., Zhang, X.-F., Avruch, J., and Marshall, M. S. (1994) Mol. Cell. Biol. 14, 5318–5325
36. Neote, K., DiGregorio, D., Mak, J. Y., Horuk, R., and Schall, T. J. (1993) Cell 72, 415–425
37. Kaspar, R. L., Rychlik, W., White, M. W., Rhoads, R. E., and Morris, D. R. (1993) J. Biol. Chem. 268, 3619–3622
38. Rinker-Schaeffer, C. W., Austin, V., Zimmer, S., and Rhoads, R. E. (1992) J. Biol. Chem. 267, 10659–10664
39. Rinker-Schaeffer, C. W., Austin, V., Zimmer, S., and Rhoads, R. E. (1992) Mol. Biol. Cell 12, 2351–2355
40. Ito, T., Jagus, R., and May, W. S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7455–7459
41. Ito, T., Jagus, R., and May, W. S. (1994) Exp. Hematol. 22, 763a

Acknowledgments – We thank Dr. Zhi-jun Luo, for the kind gift of the GST-Mek1 fusion protein and protein kinase inhibitor, and Dr. Young Kim for his assistance with these experiments.