Contribution of Both STAT and SRF/TCF to c-fos Promoter Activation by Granulocyte-Macrophage Colony-Stimulating Factor

By Daniel Rajotte, Henry B. Sadowski, André Haman, Kailesh Gopalbhai, Sylvain Meloche, Ling Liu, Gerald Krystal, and Trang Hoang

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic growth factor that has been shown to support cell proliferation in murine fibroblasts engineered to stably express both chains of the human GM-CSF receptor (NIH-GMR). Because the proto-oncogene c-fos is believed to provide a link between short-term signals elicited at the membrane and long-term cellular response, we chose to study the mechanism of GM-CSF–dependent cell regulation using c-fos promoter activity as a molecular marker in both NIH-GMR transfectants and in the CD34+ cell line TF-1. The importance of c-fos and related AP-1 activity in GM-CSF signaling was suggested by a tight correlation between GM-CSF–dependent activation of the c-fos promoter and cell proliferation and by the inhibitory effect of a trans-dominant c-fos mutant on cell growth. To evaluate the contribution of the serum response factor (SRF) associated with the ternary complex factor (TCF) and of STAT proteins to c-fos promoter activation in response to GM-CSF, the SRF binding site (SRE) and/or the STAT binding site (SIE) were inactivated. In serum-free medium, both SRE and SIE are essential to c-fos promoter activation by GM-CSF in NIH-GMR transfectants and in TF-1 cells. No response to GM-CSF was observed when both sites were mutated. The nature of the STAT family member was further investigated by Western blots and DNA retardation assays using an SIE probe. Our data indicate that GM-CSF induced DNA binding of both STAT1 and STAT3 in NIH-GMR and mainly of STAT3 in TF-1 cells. STAT5 tyrosine phosphorylation was also observed in TF-1 cells. Finally, expression of a dominant negative MAPK mutant, ERK192A, resulted in a decrease of both SRE- and SIE-dependent activation of c-fos promoter by GM-CSF, suggesting that STAT1/3 are regulated not only by tyrosine kinases, but also partially by MAPK.

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Previous data from our laboratory and elsewhere indicate that the blast cells of acute myeloblastic leukemia (AML) produce a number of growth factors that include interleukin-1β (IL-1β) and GM-CSF, which contribute to paracrine and autocrine growth stimulation.56-59 Interestingly, STAT-related factors are shown to be constitutively activated in these cells,60 possibly as a consequence of endogenous growth factor production or as a result of constitutively activated Jak kinases. Indeed, a gain of function mutation in the Drosophiila homolog of mammalian Jak kinases, hop Tum-1, was shown to cause lymph gland hypertrophy and hematologic defects.31,32 Together, these observations suggest that the Jak/STAT pathway may be frequently activated in hyperproliferative cells.

A number of reports indicate that GMR increases Ras-dependent nucleotide exchange, Raf-1 activation, and MAPK/ERK tyrosine phosphorylation,10,15,33 but the possibility of pathway cross-talk, as shown for IL-6,44 interferon (IFN),35 and EGF36 has not been addressed. Thus, it is likely that ERK could regulate early response genes not only by activating TCF/Etk-1, but also through modulation of STAT function.

The present study was designed to investigate the biologic relevance of both the Jak/STAT and Ras signalling pathways in response to GM-CSF, using c-fos promoter activity as a molecular marker. Two model systems were chosen. NIH-3T3 transfectants expressing both α and β chains of GMR were used for initial studies, because these were previously shown to respond to GM-CSF. Observations using NIH-GMR were then extended to the CD34+ cell line TF1,17 which was selected as a model system because the cells express functional cell surface receptors for GM-CSF, IL-3, Steel factor (SF), and erythropoietin (Epo) and have retained a normal requirement for hematopoietic growth factors for survival in culture.38 The nature and significance of STAT activation was investigated through site-directed mutagenesis of SIE in the c-fos promoter and through the study of STAT tyrosine phosphorylation and DNA binding activity. Finally, the role of MAPK in regulating the SRE and STAT pathways was directly addressed using a dominant negative ERK192A.

MATERIALS AND METHODS

Cells. The mouse fibroblast cell line NIH-3T3 and the NIH GMR transfectants were subcultured three times weekly in Iscove’s modified Dulbecco’s medium (IMDM; GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; GIBCO). The TF-1 cell line29 was subcultured three times weekly in IMDM supplemented with 10% FCS and 200 µM L-recombinant GM-CSF. Cells were maintained at 37°C in a humidified incubator containing 5% CO2. Where indicated, cells were maintained in serum-free medium supplemented with 1% bovine serum albumin (BSA; Sigma, St Louis, MO) and 180 µg/mL iron-saturated transferrin (TF; Calbiochem, La Jolla, CA), as described previously.28

Plasmids. The GMR cDNA was cloned in the expression vector pME18S. The cDNA for βc (KH97) was cloned in the same vector but without the neomycin-selectable gene. Both were kindly provided by Dr Tosho Kitamura (DNAX, Palo Alto, CA). The catalytically inactive ERK-1 mutant was constructed by changing threonine 192 to alanine in hamster Erk-1 cDNA29 using the altered sites mutagenesis system (Promega, Madison, WI). The mutant cDNA was subcloned into the eukaryotic expression vector pRC-CMV (Invitrogen, San Diego, CA) to create the plasmid pCMV-HEIT192A.

Wild-type or mutated versions of the mouse c-fos promoter were excised by HindIII and SmaI, possibly as a consequence of endogenous growth autocrine growth stimulation.33-34 Interestingly, STAT-receptor complexes were generated by polymerase chain reaction (PCR) using the mutated SRE fos promoter cloned in the pXP2 vector as a template. Oligonucleotides used were 5'-atgtaaagcttgtggcagaatccctcc-3' (mSIE) and 5'-ttggcttcattcc-3' (pXP2-luciferase sequence). The PCR fragment was digested by HindIII-SmaI and inserted in the same sites of the pXP2 vector. RSV-luciferase40 and RSV-GH4 reporter plasmids were used as controls in transient transfections. The pBABE-fos (139-145) construct (kindly provided by Dr P. Jolicoeur, Clinical Research Institute of Montreal, Montreal, Quebec, Canada) was generated by cloning rat c-fos (139-145) cDNA35 into the EcoRI site of the pBARE-puro vector, as described.60

Stable transfection of the genes encoding the α and β chains of GMR in NIH-3T3 cells. The GMRα cDNA and KH97 were cotransfected by the calcium phosphate method in NIH-3T3 at a ratio of 1:10, respectively. Briefly, 1 day before transfection, 200,000 cells were seeded in a 60-mm tissue culture dish. On the day of transfection, 400 µL of DNA-CaPO4 precipitate containing 12 µg of DNA was added to the culture and incubated for 6 hours. Cells were then washed and fed with culture medium. Two days after transfection, the cultures were expanded 1:10 and G418 was added at a concentration of 500 µg/mL. After 14 days in selective medium, independent clones were picked, expanded, and tested for binding. Expression of both transgenes were verified on Northern blots and through cross-linking studies with [125I]-GM-CSF (data not shown). Binding studies with [125I]-GM-CSF were performed on these transfectants as described previously.47 Computer analysis of binding data using the program ALFFIT48 showed the presence of two GM-CSF binding sites; one of high (40 to 60% of total binding) and one of low affinity (1 to 19% of total binding) in all clones.

Retroviral infections. Transfection of the pBABE-puro or pBABE-Δfos (139-145) vectors in BOSC 23 cells were performed as described.29 Retroviruses were collected 48 hours after transfection and filtered through a 22-µm Millipore filter. Retroviral infections were performed 24 hours after seeding 2 x 105 NIH-GMR cells on 35-mm petri dishes. Cells were infected with 200 µL of viral suspension (107 pfu/mL) containing 8 µg/mL polybrene at 37°C. Cultures were agitated periodically and fed after 2 hours with 2 mL IMDM 10% FCS. Two days after infection, cells were incubated in the presence or absence of 800 µmol/L GM-CSF in serum-free medium containing 1% BSA, 180 µg/mL iron-saturated TF, and 10 µg/mL puromycin (Sigma) for selection. Cells were passaged every 2 days until 6 days after selection, when cells were counted or submitted to thymidine incorporation assay.

Evaluation of cell proliferation through membrane filtration of [3H]thymidine-labeled cells. Adherent cells (75,000) were deprived of serum through an overnight incubation in IMDM (GIBCO) supplemented with 1% BSA (Sigma) and 180 µg/mL iron-saturated TF, hereafter referred to as serum-free medium. Cultures were stimulated with GM-CSF at the indicated concentrations for 48 hours and pulsed with [3H]thymidine (DuPont-NEW, Wilmington, DE) at a final concentration of 3 µCi/mL for the last 12 hours. This time point was defined as optimal in kinetic studies of GMR transfectants after stimulation with GM-CSF (data not shown). Cells were then collected and retained on fiberglass filters (Schleicher & Schuell, Keene, NH), which were sequentially washed with 4 mL phosphate-buffered saline (PBS), 4 mL of 10% trichloroacetic acid, 4 mL H2O, and 4 mL of
methanol. Filters were air dried before liquid scintillation (EcoLume; ICN, Costa Mesa, CA) counting.

c-fos promoter-luciferase reporter assay. Wild-type or mutated versions of the fos reporter constructs were transiently transfected in NIH-GMR by the calcium-phosphate method or in TF-1 cells by electroporation. NIH-GMR cells were seeded at a concentration of 2.5 × 10^6 in 60-mm culture dishes. Cells were transfected with 400 μL of Ca₃(PO₄)₂ precipitate containing 6 to 10 μg of plasmid DNA. After 16 hours, the precipitate was removed and the cells were washed once with PBS. Cells were then incubated for 24 hours in serum-free medium in the presence or absence of 20 ng/mL GM-CSF or 10 ng/mL tumor necrosis factor α (TNFα). After incubation, cells were collected and lysed in 120 μL of lysis buffer (25 mmol/L glycylglycine, pH 7.8, 15 mmol/L MgSO₄, 4 mmol/L EGTA, 1% Triton X-100, and 1 mmol/L DTT). For TF-1, 10^7 cells were starved for 16 hours in serum-free medium without GM-CSF and then submitted to electroporation. Briefly, cells were resuspended in 400 μL of serum-free medium containing 10 μg of the fos reporter construct and 20 μg of carrier plasmid DNA. The mixture of cells and plasmid was allowed to stand at room temperature for 10 minutes before applying an electric shock of 250 V and 960 μF using a Genepulsor (Bio-Rad, Hercules, CA). Five minutes after the pulse, cells were dispersed in 10 mL of serum-free media in the presence or absence of 200 pmol/L GM-CSF and then incubated at 37°C for 12 hours. TF-1 cells were then collected and lysed as described for the NIH-GMR transfectants.

Protein concentrations were determined by the method of Bradford (Bio-Rad) and adjusted for sample variation before dosage of luciferase activity in NIH-GMR transfectants. Activities provided by the manufacturer. In parallel, cells were transfected with RSV-GH (2 pg) of serum-free media in the presence or absence of 20 ng/mL GM-CSF and GM-CSF could substitute in part for serum growth factors in supporting cell proliferation (Fig 1A and data not shown). GM-CSF induced a dose-dependent stimulation of thymidine incorporation, with median effective concentrations of 30 to 50 pmol/L that were in the range of the dissociation constant of the GM-CSF receptor in these cells (40 to 100 pmol/L). Previous studies indicate that GM-CSF induces c-fos mRNA accumulation and promoter activity, which are shown elsewhere to play a central role in integrating signalling networks evoked by a variety of extracellular stimuli. Thus, we tested in our transfectants the ability of GM-CSF to activate the murine c-fos promoter linked to the luciferase reporter gene. Our data indicate that GM-CSF induced c-fos mRNA accumulation and promoter activity, which consistently reached a 10- to 18-fold increase at saturating concentrations of GM-CSF (Fig 1A). More importantly, there was a twofold increase in c-fos promoter activity at 24 pmol/L of GM-CSF, which was also the biologically active threshold in the proliferation assay. At 120 pmol/L, the induction of c-fos was near maximal and correlated with a near maximal proliferative response to GM-CSF. Thus, there is a tight correlation between mitogenic response and activation of the c-fos promoter.

To directly address the contribution of c-fos in the response to GM-CSF, we overexpressed, in our NIH-GMR transfectants, a c-fos that lacks the DNA binding domain, Δfos(139-145). This c-fos mutant, which has been shown to associate with c-jun but cannot bind to an AP-1 site, could act in a dominant negative manner on AP-1 binding

RESULTS

GM-CSF–dependent cell proliferation and activation of c-fos promoter activity. In all NIH-GMR transfectants, GM-CSF could substitute in part for serum growth factors in supporting cell proliferation (Fig 1A and data not shown). GM-CSF induced a dose-dependent stimulation of thymidine incorporation, with median effective concentrations of 30 to 50 pmol/L that were in the range of the dissociation constant of the GM-CSF receptor in these cells (40 to 100 pmol/L). Previous studies indicate that GM-CSF induces c-fos mRNA accumulation and promoter activity, which are shown elsewhere to play a central role in integrating signalling networks evoked by a variety of extracellular stimuli. Thus, we tested in our transfectants the ability of GM-CSF to activate the murine c-fos promoter linked to the luciferase reporter gene. Our data indicate that GM-CSF induced c-fos mRNA accumulation and promoter activity, which consistently reached a 10- to 18-fold increase at saturating concentrations of GM-CSF (Fig 1A). More importantly, there was a twofold increase in c-fos promoter activity at 24 pmol/L of GM-CSF, which was also the biologically active threshold in the proliferation assay. At 120 pmol/L, the induction of c-fos was near maximal and correlated with a near maximal proliferative response to GM-CSF. Thus, there is a tight correlation between mitogenic response and activation of the c-fos promoter.
c-fos transcription activity. Δfos(139-145) was transduced into NIH-GMR cells using the pBABE-puro retroviral vector that confers resistance to puromycin. Because the abrogation of AP-1 function could be detrimental to the cells, the effects of Δfos(139-145) were assessed in a transient transduction assay in which GM-CSF stimulation was applied to the cells at the same time as the selective pressure. Control cultures were infected with a retrovirus harboring the vector without insert (Fig 1B). As determined by thymidine incorporation, GM-CSF induced a sixfold to sevenfold increase in cell proliferation in control cultures. However, in cells selected for expression of Δfos(139-145), no proliferative response to GM-CSF was observed. Together, our observations indicate that the presence of a functional AP-1 complex is required for GM-CSF-dependent mitogenesis and suggest that GM-CSF–induced signalling events implicated in regulating c-fos transcription would be of biologic relevance.

**Activation of both SIE and SRE sites of the c-fos promoter by GM-CSF.** To evaluate the contribution of STAT and SRF/TCF on c-fos promoter activation by GM-CSF, we used c-fos promoter constructs carrying inactivating mutations in either SIE or SRE or both (mSIE, mSRE, and mSIE-SRE, respectively). The specificities of the mSIE and mSRE mutations have been characterized previously. Figure 2 shows the structure of each mutant and their response to GM-CSF when linked to a luciferase reporter gene. Both total luciferase activity and fold induction by GM-CSF are shown for each construct. Wild-type c-fos promoter has a low but detectable activity in NIH-GMR cells maintained in serum-free medium. On stimulation with GM-CSF, there was a 15-fold induction for the wild-type fos construct (Fig 2), whereas the activity of RSV promoter used as an external control was not significantly affected (data not shown). Interestingly, the basal level or GM-CSF–induced level of activity of mSIE was higher than that of mSRE, indicating that SRE has a greater contribution than SIE on intrinsic promoter activity, as reported by others. When the basal level of activity for each construct was taken as 1, data shown in Fig 2 indicate that GM-CSF induced a sevenfold and ninefold increase in c-fos promoter activity for mSIE and mSRE.
Luciferase (mV) Fold induction:

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<th>NIH GMR</th>
<th>TF-1</th>
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<tr>
<td>SIE</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>SRE</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>luciferase</td>
<td>9</td>
<td>4</td>
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<tr>
<td>NIH GMR</td>
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<td>TF-1</td>
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Fig 2. GM-CSF activates both the SRE and SIE sites of the fos promoter. The c-fos promoter constructs used are described in the Materials and Methods. NIH-GMR and TF-1 cells were transiently transfected with wild-type or mutated c-fos promoter linked to the luciferase reporter gene together with the RSV-GH control vector. After transfection, cells were incubated for 40 hours in serum-free medium in the presence (E) or absence (W) of 800 pmol/L GM-CSF. After incubation, the level of luciferase activity, in millivolts (mV), was measured as described in the Materials and Methods. Luciferase activity readings for TF-1 were comparable to those presented for duplicate cultures of NIH-GMR cells. For both NIH-GMR and TF-1 cells, data are expressed as the fold increase over control (as described in Fig 1).

respectively. Together, our observations are consistent with the view that both SRE and SIE can independently and additively mediate an increase in c-fos promoter activity in response to GM-CSF.

The significance of both SIE and SRE contribution to c-fos activation was further investigated in TF-1 cells, a bipotent erythroid/monocytic cell line that expresses CD34 and CD33 and requires GM-CSF for survival. Data observed with TF-1 are comparable to those observed with NIH-GMR and underscore the importance of both STATs and SRF in c-fos promoter activation by GM-CSF (Fig 2).

Activation of STAT proteins by GM-CSF. The tyrosine kinase Jak2 is tyrosine phosphorylated and activated after GM-CSF stimulation in hematopoietic cell lines. In our transfectants, GM-CSF elicited a dose-dependent tyrosine phosphorylation of Jak-2 (Fig 3A), indicating a conservation of GMR signalling events between hematopoietic and nonhematopoietic cells. It has been shown that Jak-2 activation in response to GM-CSF is followed by an activation of STAT5 in pro-B cells and monocytes and more recently of STAT1 and 3 in neutrophils. We therefore investigated the nature of STAT proteins activation on GM-CSF stimulation in NIH GMR and TF-1 cells. STAT3 is abundantly expressed in TF-1 and NIH-GMR and become tyrosine phosphorylated in response to GM-CSF (Fig 3B). As expected, STAT5 tyrosine phosphorylation was also detected in TF-1 cells (data not shown). Because of the unexpected induction of STAT3 tyrosine phosphorylation by GM-CSF, the function of STAT3 was further investigated in gel shift assays.

Homodimeric and heterodimeric complexes of activated STAT1 and STAT3 bind with high affinity to oligonucleotide probes containing a synthetic STAT binding site termed m67. This site is derived from the SIE within the human c-fos gene promoter and forms three distinct mobility-shift complexes (SIF-A, -B, and -C) in cell extracts containing STAT1 and STAT3. To assess the activation of these STATs by GMR ectopically expressed in NIH-3T3 cells or by endogenous GMR in TF-1 cells, extracts from untreated or GM-CSF–treated cells were subjected to mobility shift assays with a 32P-labeled m67 probe. We found that SIE DNA-binding activity was rapidly induced by GM-CSF in NIH-GMR cells (Fig 4, compare lanes 1 and 2), in which all three SIF complexes (A, B, and C) were detected. These complexes clearly contained activated STAT1 and/or activated STAT3, because SIF-A and -B were specifically supershifted by the addition of STAT3 antisera (Fig 4, compare lanes 3 and 4) and SIF-B and STAT3 were specifically supershifted by the addition of STAT1 MoAb (Fig 4, compare lanes 5 and 6). The STAT3 antisera was raised against the C-terminus of STAT3 and appears specific for STAT3. The STAT1 MoAb used specifically recognizes STAT1 (alpha/beta). It should also be noted that activation of both STAT1 and STAT3 was observed in all NIH-GMR transfectants tested (data not shown). In TF1 cells, GM-CSF induces mainly
STAT3 homodimers (SIF-A) in a dose-dependent manner (Fig 5). The concentrations of GM-CSF required for optimal activation of STAT1/3 more closely resemble those required for Jak-2 tyrosine phosphorylation than those required for cell proliferation or c-fos promoter activation. Significantly, the latter two events are determined after 48 hours of stimulation, whereas the former are short-term events. The sum of these data clearly indicate a recruitment of both STAT1 and STAT3 on a c-fos SIE in response to GM-CSF in NIH-GMR and of STAT3 in TF-1 cells. This activation of STAT3 and to a lesser extent of STAT1 could well account for the SIE-dependent activation of c-fos promoter by GM-CSF shown in Fig 2.

Role of ERK-1 in the SIE- and SRE-dependent activation of the c-fos promoter. Previous reports have shown a crucial role for ERK in Ras-dependent signalling. More recent studies indicate that ERK could also regulate STAT function, suggesting that STAT may be a target of both Jak and ERK. We therefore addressed the potential contribution of ERK activity in GM-CSF signalling and, more precisely, on the activation of the c-fos promoter. When ERK-1 activity was directly measured by the MBP peptide phosphorylation assay, we observed a rapid and sustained increase in enzymatic activity (Fig 6). This increased activity was detected within 5 minutes after GM-CSF stimulation, peaked at 15 minutes, and remained elevated for at least 3 hours. The activation of ERK-1 was reproducibly detected in all clones tested (data not shown).

ERK-1 phosphorylation at position 192 is crucial for its activation. It has been shown previously that a catalytically inactive ERK-1 carrying a threonine to alanine mutation at position 192 can repress endogenous ERK activity and can inhibit growth factor-induced cell proliferation. Therefore we used this dominant negative ERK to directly address the contribution of ERK activity on GM-CSF-induced c-fos transcription. Hence, NIH-GMR cells were cotransfected with a constitutively active form of ERK-1 and NIH-GMR cells were exposed to GM-CSF in NIH-GMR transfectants. NIH GMR cells were exposed to GM-CSF in NIH-GMR and/or TF-1 cells were starved overnight in serum-free medium and then stimulated with GM-CSF for 10 minutes or left untreated. In (A), NIH-GMR cell extracts were subjected to immunoprecipitation with anti-JAK-2, followed by SDS-PAGE and Western blotting with either an antiphosphotyrosine antibody (4G10) or an anti-JAK-2. In (B), cells were stimulated in 2 nmol/L of GM-CSF. Cell extracts were immunoprecipitated with an antiphosphotyrosine (4G10) followed by SDS-PAGE. Western analysis was performed on the immunoprecipitated extracts (anti-P-Tyr IP) or on total cell extracts (cell lysate) with an anti-STAT3.
with c-fos luciferase reporter constructs and with the mutant ERK192A cloned in the pRC-CMV expression vector. Control cells were cotransfected with the expression vector without insert (Fig 7). The presence of ERK192A resulted in a 50% decrease in wild-type c-fos promoter induction by GM-CSF. An inhibition of the same order of magnitude was also observed for mSIE and mSRE constructs. Cells stimulated with TNFα were used as controls, because TNFα is known to be a weak activator of MAPK and was shown to preferentially activate the ERK-related JNK kinase.

Thus, TNFα promoted cell proliferation and c-fos promoter activity in our transfectants without affecting ERK activity (Table I). As shown in Fig 7, expression of the dominant negative ERK192A did not interfere with TNFα-induced c-fos promoter activity, indicating the specificity of inhibition observed with GM-CSF. Taken together, these data suggest that ERK1 regulates c-fos transcription induced by GM-CSF through both STAT1/3 and SRF/TCF complexes.

**DISCUSSION**

The experiments described above suggest that the regulation of c-fos is important for GM-CSF–dependent signalling and examine the mechanisms whereby ligand-dependent activation of the GM-CSF receptor leads to an increase in c-fos promoter activity. Our data provide direct evidence for a contribution of both STAT1/3 and SRF/TCF to c-fos promoter activation in response to GM-CSF and for an important role of ERK activity in regulating both transcription complexes.

Because the proto-oncogene c-fos is believed to provide a link between short-term signals elicited at the membrane
and long-term cellular responses.\(^59\) we chose to study the molecular basis of GM-CSF--induced cellular responses using c-fos as an indicator. We first sought to address the importance of c-fos in GM-CSF--induced cell proliferation. Our experiments indicate a tight correlation between induction of cell proliferation by GM-CSF and activation of the c-fos promoter. Furthermore, overexpression in NIH-GMR transfectants of a trans-dominant c-fos mutant allowed us to directly address the contribution of AP-1 function in response to GM-CSF. To measure the effects of the \(\Delta\)fos mutant, we used a transient dual-selection proliferation assay. Thus, \(\Delta\)fos was transduced into the cells through retroviral infection that allows for reproducible and high efficiency gene transfer and permits a direct comparison with cells receiving the selective marker alone. Furthermore, infected cells (with \(\Delta\)fos or control vector) were selected simultaneously for puromycin resistance and for proliferative response to GM-CSF in serum-free medium. Under these conditions, cells need both puromycin resistance and a positive response to GM-CSF to survive and proliferate. Hence, this transient selection-proliferation assay may be applicable to the study of genes that irreversibly affect essential cell functions.

It was previously believed that SRE is necessary and sufficient to confer serum response to the c-fos promoter. The contribution of SIE was shown by PDGF stimulation, but was thought to be weak. To quantitate the contribution of either cis-regulating element to the activation of the c-fos promoter by GM-CSF, we chose to use luciferase as a reporter gene for its sensitivity of detection and linearity over a broader range of activity. Thus, the sensitivity of the assay has allowed us to define the important contribution of both SIE and SRE to the GM-CSF response of the c-fos promoter, although SRE appears to be dominant over SIE on intrinsic promoter activity. Interestingly, mutation of SIE consistently results in a threefold to fourfold increase in basal fos promoter activity in unstimulated cells. It is possible that the activity of the c-fos promoter is repressed by factors that are constitutively bound to sequences that are adjacent to SIE, hence the dominance of SRE over SIE on intrinsic promoter activity observed here and elsewhere.\(^16,60\) Mutation or deletion of these sites may provide a direct answer to this question. Despite this constitutive repression, a fos promoter construct that can no longer recruit SRF/TCF is reproducibly induced fourfold to ninefold by GM-CSF, indicating the importance of STAT3 and possibly STAT1 on c-fos promoter activity in response to GM-CSF. Although we have not directly addressed the contribution of other binding sites that have been identified in the c-fos promoter, such as the AP-1--like FAP site and the Ca/CRE, our data indicate an essential role for SIE and SRE in mediating the GM-CSF--dependent activation of c-fos promoter because a construct bearing mutations at both these sites is unresponsive to GM-CSF stimulation. In contrast to previous results, the contribution of both SIE and SRE in our cells more closely resembles the recent observations reported by Hill and Treisman\(^61\) in vitro, as well as the in vivo requirement for all of the regulatory elements of the c-fos promoter in the response evoked by extracellular stimuli in transgenic mice carrying fos promoter-LacZ fusions.\(^62\) The authors suggest that this cooperativity could be attributed to the formation, by the transcription factors, of a nucleation site that directs the assembly of an interdependent transcription complex on chromosomal DNA.

The GM-CSF--inducible complexes that form on a high-affinity SIE probe migrated at the same position as SIF-A, -B, and -C, indicating the formation of STAT1/3 homodimers and heterodimers, which were confirmed by supershifting with specific anti-STAT1 and anti-STAT3, respectively. In monocytes and pro-B cells, GM-CSF was previously shown to activate STAT5 and not STAT1 and STAT3.\(^24,25,63\) However, in murine fibroblasts, our data suggest that GMR can activate both STAT1 and STAT3. Surprisingly, in the CD34+ cell line TF-1, GM-CSF also activates STAT3. Thus, our data are consistent with the view that the recruitment of STAT family members may be more promiscuous than previously anticipated, although we have not ruled out the possibility that it may also depend on the affinity of the receptor for specific STAT proteins. Interestingly, the disruption of STAT1 gene did not affect normal development, but the mice were highly susceptible to viral infection, possibly due to their unresponsiveness to IFN.\(^64,65\) The response to other cytokines that include GH, EGF, or IL-10 was nonetheless unaffected, suggesting a functional redundancy of STAT1 with STAT3 in these pathways. In TF-1 cells, our data clearly suggest that STAT3 rather than STAT1 is activated by GM-CSF. Mutations of SIE results in a 50% decrease in GM-CSF--induced c-fos promoter activity, indicating a direct link between STAT activation by GMR and c-fos transcription in both NIH-GMR and TF-1 cells. Further addressing the importance of STAT3 and STAT5 in signal transduction will require gene ablation in transgenic mice or the use of a dominant negative STAT mutant.

Previous studies have independently shown that GM-CSF induces the tyrosine phosphorylation of p42MAPK and p44MAPK in hematopoietic cells and in GMR-transfected fibroblasts and also activates MAPK in human neutrophils.\(^10,66,67\) Two lines of evidence suggest an important role for the MAPK pathway in GM-CSF signalling. Overexpression of SHC was shown to increase the response of TF-1 cells to GM-CSF,\(^68\) whereas a truncated GMR that can no longer recruit Ras does not suppress apoptosis in transfected BaF3 cells.\(^3\) These data argued for an important role for the

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Table 1. Cell Proliferation, c-fos--Luciferase Activity, and ERK Activity in Response to GM-CSF and TNF

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<tr>
<th></th>
<th>Thyminde Uptake (cpm)</th>
<th>MAPK Activity (pmol/min/mg)</th>
<th>Luciferase Activity Driven by the c-fos Promoter (fold induction)</th>
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<td>0</td>
<td>6,100 ± 1,200</td>
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<tr>
<td>GM-CSF</td>
<td>29,330 ± 3,426</td>
<td>0.72</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>TNF</td>
<td>33,720 ± 3,860</td>
<td>0.15</td>
<td>3 ± 0.5</td>
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Assays for cell proliferation, ERK activity, or c-fos promoter activity in response to GM-CSF or TNF were performed as described in the Materials and Methods. For ERK activity measurements, cells were stimulated with GM-CSF or TNF for 15 minutes. The activation of ERK by GM-CSF and lack of activation by TNF were also observed in all 5 NIH GMR clones tested.
ras-MAPK pathway in cell proliferation and cell survival induced by GM-CSF. Our data indicate that the activations of Jak-2/STAT and Ras/MAPK by GM-CSF converge on the activation of c-fos transcription. More importantly, the use of a dominant negative ERK1 provides direct evidence for a role of ERK activity in STAT and SRF/TCF functions induced by GMR. Hence, our results suggest that GM-CSF-independent activation of ERK activity can regulate both STAT- and SRF/TCF-mediated c-fos promoter activity and that regulation of STAT activity is not attributed solely to tyrosine phosphorylation by Jak kinases, but also to ERK mediated serine phosphorylation (Fig 8). In summary, our data are consistent with the view that the Jak-STAT and Ras-MAPK-TCFISRF pathways can independently and cooperatively contribute to c-fos transcription after ligand-induced activation of GMR. Furthermore, the activation of MAPK may provide the possibility of pathway cross-talk and fine tuning of the response evoked by GM-CSF.

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D Rajotte, HB Sadowski, A Haman, K Gopalbhai, S Meloche, L Liu, G Krystal and T Hoang

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