SCF and G-CSF lead to the synergistic induction of proliferation and gene expression through complementary signaling pathways

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Stem cell factor (SCF) is a potent costimulatory molecule for many cytokines. Its synergy with granulocyte colony-stimulating factor (G-CSF) results in important biologic and clinical effects, although the mechanism by which this occurs remains poorly understood. To investigate this interaction, this study used a retroviral vector to transduce the G-CSF receptor into MØ7e cells, which are known to express the SCF receptor. The transduced G-CSF receptor is functionally active, and the resultant MØ7e-G cells recapitulate the proliferative synergy between SCF and G-CSF. When treated with both cytokines, a marked shortening of the G0/G1 phase of the cell cycle occurs, associated with a suppression of the cyclin-dependent kinase inhibitor p27kip1. In addition, SCF and G-CSF induce the synergistic activation of c-fos, a proto-oncogene involved in propagation of mitogenic signals in hematopoietic cells. G-CSF, but not SCF, induces the tyrosine phosphorylation of STAT1 and STAT3, transcription factors that can mediate the induction of c-fos. However, SCF induces phosphorylation of STAT3 on serine727 (ser727), which is necessary for maximal STAT transcriptional activity, and the combination of SCF and G-CSF leads to complete STAT3 phosphorylation on ser727. The pathways by which SCF and G-CSF lead to serine phosphorylation of STAT3 are distinct and are partially dependent on phosphatidylinositol-3 kinase and ERKs, pathways that are also necessary for the synergistic effects of SCF and G-CSF on proliferation and c-fos induction. Thus, MØ7e-G cells provide a powerful system in which the molecular basis of the synergy between SCF and G-CSF can be dissected. (Blood. 2000;96:3422-3430)

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Material and methods

Cytokines, antibodies, and other reagents

Recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF) (sargramostim) was obtained from Immunex Corporation (Seattle, WA), rhSCF from Biosource International (Camarillo, CA), and rhG-CSF from Amgen (Thousand Oaks, CA). HT-29, murine T cell lymphoma, polybrene, and fetal calf serum (FCS) were purchased from Sigma Chemical Company (St Louis, MO). Antibodies to STAT1 (E-23) and STAT3 (C-20) were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). PD98059 and antibodies to ERK1 were purchased from New England Biolabs (Beverly, MA). Phycoerythrin (PE)-conjugated antibody to c-kit and antibodies to the retinoblastoma protein (Rb) and G-CSF receptor were purchased from Becton Dickinson (San Jose, CA), to p27kip1 from Transduction Laboratories (Lexington, KY), and to p21cip1 from Upstate Biotechnology (Lake Placid, NY). Recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF) (sargramostim) was obtained from Immunex Corporation (Seattle, WA), rhSCF from Biosource International (Camarillo, CA), and rhG-CSF from Amgen (Thousand Oaks, CA). HT-29, murine T cell lymphoma, polybrene, and fetal calf serum (FCS) were purchased from Sigma Chemical Company (St Louis, MO). Antibodies to STAT1 (E-23) and STAT3 (C-20) were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). PD98059 and antibodies to ERK1 were purchased from New England Biolabs (Beverly, MA). Phycoerythrin (PE)-conjugated antibody to c-kit and antibodies to the retinoblastoma protein (Rb) and G-CSF receptor were purchased from Becton Dickinson (San Jose, CA), to p27kip1 from Transduction Laboratories (Lexington, KY), and to p21cip1 from Upstate Biotechnology (Lake Placid, NY).
Biotechnology (Lake Placid, NY). Antibodies to the tyrosine phosphorylated forms of STAT1 and STAT3 and to the ser727-phosphorylated forms of STAT1 and STAT3 were generated as described. RPMI 1640, Dulbecco modified Eagle medium (DMEM), L-glutamine, HEPES, penicillin/streptomycin, and G418 were purchased from Gibco/BRL-Life Technologies (Rockville, MD). Horseradish peroxidase-conjugated antirabbit and antimouse antibodies were purchased from Calbiochem (La Jolla, CA).

Cells

The human factor–dependent myeloid cell line MO7e was obtained from Dr J. Griffin (Dana-Farber Cancer Institute, Boston, MA). Cells were maintained in RPMI 1640 supplemented with 20% (vol/vol) heat-inactivated fetal serum (FCS) and 10 ng/mL GM-CSF. The human 293-derived retroviral packaging cell line 293GPG was obtained from Dr G. Dranoff (Dana-Farber Cancer Institute). This cell line is capable of producing high titer of recombinant Moloney murine leukemia virus particles that incorporate, in a tetracycline-regulated fashion, the vesicular stomatitis virus G (VSV-G) protein.

Whole cell and nuclear extracts

To prepare whole cell extracts, cells were placed on ice, washed once with ice-cold phosphate-buffered saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L NaH2PO4, pH 7.4), and extracted at 4°C for 30 minutes in lysis buffer (10 mmol/L Tris, pH 8.0, 0.5% NP-40, 250 mmol/L NaCl, 10 mmol/L sodium orthovanadate, 100 μmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 μg/mL aprotinin). Insoluble material was removed by centrifugation at 12000g for 5 minutes. To generate nuclear extracts, cells were washed once with ice-cold PBS, then resuspended in 5 mL hypotonic buffer (10 mmol/L Tris, pH 7.4, 10 mmol/L NaCl, and 6 mmol/L MgCl2) and incubated on ice for 5 minutes. The cells were then centrifuged and resuspended in 0.8 mL hypotonic buffer containing 1 mmol/L β-mercaptoethanol, 10 μg/mL PMSF, and 1 mmol/L sodium orthovanadate. Cells were disrupted by shearing in a Dounce homogenizer (type b pestle, 25 strokes), then nuclei were collected by a 10-second centrifugation at 12000g. The supernatant was removed and the nuclear pellet was washed once with hypotonic buffer, then resuspended in 3 volumes of high-salt buffer (20 mmol/L HEPES, pH 7.9, 420 mmol/L NaCl, 25% glycerol, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 1 mmol/L β-mercaptoethanol, 1 mmol/L sodium orthovanadate, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μg/mL aprotinin, and 100 μmol/L PMSF), and rocked at 4°C for 30 minutes. Insoluble material and intact nuclei were removed by centrifugation at 12000g for 3 minutes at 4°C, and the supernatant was recovered and designated the nuclear extract.

Western blotting

Whole cell extracts (100 μg/lane) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and blocked with 5% nonfat dry milk (for anti-STAT, antiphospho-STAT, and antiphosphoSTAT2-STAT7) blots or 5% bovine serum albumin (BSA) (for other blots) in TBST (100 mmol/L Tris, pH 8.0, 0.150 mmol/L NaCl, and 0.05% Tween-20). The primary antibodies were diluted in TBST containing 3% BSA at a dilution of 1:2000 (anti-STAT, antiphosphotyrosine-STAT, and antiphosphosor727-STAT) or 1:500 (others) and incubated with the blots for 1 hour at room temperature. After being washed with TBST extensively, membranes were incubated for 1 hour at room temperature with horseradish peroxidase–conjugated secondary antibody diluted 1:20000 in TBST containing 1% BSA. Membranes were then washed extensively, and bound antibody was detected by chemiluminescence (Renaissance Kit; NEN; Boston, MA). Band intensity was quantitated by densitometry. Membranes were stripped by incubation in 62.5 mmol/L Tris, pH 6.9, containing 2% SDS and 100 mmol/L β-mercaptoethanol for 1 hour at 65°C and then washed in TBST at 4°C overnight.

DNA binding assay

Nuclear extract (2 μL) was mixed with 1 ng 32P-labeled oligonucleotide (5′-AGCCTGATTCCCCAAATGAGGCG-3′) and its complement34 in 10 μL binding buffer (25 mmol/L HEPES, pH 7.9, 100 mmol/L EGTA, 200 mmol/L MgCl2, 500 μmol/L dithiothreitol, 1 μg/mL BSA, 0.2 μg/mL poly dI:dC, 1% Ficoll, and 0.1 μg/mL herring testis DNA). The incubation was performed at room temperature for 15 minutes. For antibody competition, antisera (1:200 dilution) was added at the end of the binding reaction and incubated at 4°C for an additional 15 minutes. The products of the binding reaction were separated on a 4% acrylamide gel in 0.2 × Tris-borate/EDTA, after which the gel was dried and exposed to film.

Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR)

RNA was collected (RNeasy Kit; Qiagen; Valencia, CA) and reverse-transcribed using Superscript IRT (Life Technologies). PCR to amplify c-fos used primers for a 482-bp fragment (sense: 5′-CTACAGGCGT-CATCCT-3′, antisense: 5′-TCTGTCCTGCTTGAAGTGA-3′), and PCR to amplify GAPDH used primers for a 226-bp fragment (Perkin Elmer Applied Biosystems; Branchburg, NJ). Minimal cycle numbers were used to allow quantitative comparisons.

Construction of granulocyte colony-stimulating factor receptor retroviral plasmid

The retroviral vector pMFG was originally constructed in the laboratory of Dr R. C. Mulligan (Harvard Medical School, Boston, MA) and was kindly provided by Dr G. Dranoff. The wild-type Ig-CSF receptor complementary DNA (cDNA), a gift from Dr Seth Corey35 (University of Pittsburgh School of Medicine, Pittsburgh, PA), was amplified by PCR using PfTurbo DNA polymerase (Stratagene; La Jolla, CA) and a set of primers with flanking Xba I and Bgl II restriction site sequences (forward, 5′-ATCCCTAGACT-GCCATGGCAAGGTGGAAACTCG-3′; reverse, 5′-TAATCCA-GATCTGCGCTAGTACCAGCCAGCCCTCC-3′), and then subcloned in frame into pMFG after an intermediate cloning step of the blunt-ended PCR product into the pCR-BluntII-TOPO plasmid (Invitrogen; Carlsbad, CA). Sequence analysis was performed to confirm the fidelity of the pMFG-G-CSF receptor construct.

Retroviral generation and infection of MO7e cells

The pMFG-G-CSF receptor (10 μg) was transfected into 293GPG packaging cells that were at 80% confluence in 10-cm dishes using LipofectAMINE (Gibco/BRL) according to the manufacturer’s recommendations. The day after transfection, the media was replaced with fresh media without antibiotics (Tet-Off media). This allowed the expression of the tetracycline-dependent VSV-G gene, and the subsequent release into the media that were at 80% confluence in 10-cm dishes using LipofectAMINE (Gibco/BRL) according to the manufacturer’s recommendations. The day after transfection, the media was replaced with fresh media without antibiotics (Tet-Off media). This allowed the expression of the tetracycline-dependent VSV-G gene, and the subsequent release into the media.
(wt/vol) sodium citrate as described previously. Data analysis was performed using Multi-Cycle software (Phoenix Flow, San Diego, CA).

Results

High-efficiency transduction of MO7e cells with granulocyte colony-stimulating factor receptor expressing retrovirus

Given the clinical importance of the synergistic interaction between G-CSF and SCF, we generated a model system in which the intracellular signaling events induced by these cytokines could be studied. The hG-CSF receptor was cloned into the retroviral vector pMFG and transduced into MO7e cells, which constitutively express c-kit. After 72 hours, more than 90% of the infected cells were positive for G-CSF receptor surface expression (MO7e-G cells) in 4 independent infection experiments (Figure 1A). The level of expression of the G-CSF receptor in MO7e-G cells was comparable to that in neutrophils isolated from the peripheral blood of healthy donors, indicating that expression of the G-CSF receptor was at physiologic levels (data not shown). Given the level of expression achieved by direct transduction, no further selection or purification steps were necessary. The expression of the G-CSF receptor in MO7e-G cells has remained stable for more than 3 months in culture and had no effect on c-kit expression, because 100% of both MO7e-G and MO7e cells expressed c-kit as measured by direct immunostaining with a PE-conjugated anti-c-kit monoclonal antibody (data not shown).

MO7e-G cells recapitulate the biologic synergy between stem cell factor and granulocyte colony-stimulating factor

To validate MO7e-G cells as a model system for the synergy between SCF and G-CSF, we first assessed the biologic characteristics of these cells. Experimental data suggest that the combination of these cytokines enhances myeloid cell proliferation. To assess the cytokine-dependent growth of these cells, wild-type MO7e and transduced MO7e-G cells were starved for 18 hours and then cultured in the presence or absence of growth factors (Figure 1B). Parental MO7e cells are factor dependent and neither proliferate nor survive without GM-CSF. The expression of the transduced G-CSF receptor enables MO7e-G cells to proliferate in response to G-CSF in the absence of GM-CSF. This was not due to an interaction between G-CSF and the GM-CSF receptor, because wild-type MO7e cells neither proliferated nor survived in response to G-CSF. Moreover, although SCF had a minimal effect on the expression of either MO7e or MO7e-G, the combination of SCF and G-CSF led to a greater-than-additive enhancement of proliferation over that seen with G-CSF alone in MO7e-G cells.

Cotreatment of MO7e-G cells with stem cell factor and granulocyte colony-stimulating factor shortens the G<sub>1</sub>/G<sub>0</sub> phase of the cell cycle

Given the likelihood that the enhanced proliferative state induced by cotreatment with SCF and G-CSF of MO7e-G cells resulted from modifications in cell cycle control, we analyzed the cell cycle distribution induced by these cytokines. Cells were cultured as above, and stained with propidium iodide, after which cell cycle distribution was determined by measuring DNA content with flow cytometry (Figure 2A). SCF induced no significant changes in cell cycle distribution compared to untreated samples. G-CSF induced a significant reduction in the percentage of cells in G<sub>0</sub>/G<sub>1</sub> and a parallel increase in the percentages of cells in S and G<sub>2</sub>/M. However, the combination of G-CSF and SCF led to a significant reduction in the percentage of cells in G<sub>0</sub>/G<sub>1</sub> compared to cells treated with G-CSF alone at all time points (24 hours, P < .01; 48 and 72 hours, P < .05). The duration of each phase was calculated using the doubling times of the cultures and the percentage of cells in each phase of the cell cycle 48 hours after initiation of the culture. No significant difference was found in the duration of S and G<sub>2</sub>/M in cells cultured in G-CSF alone or in combination with SCF. However, a marked shortening of the duration of the G<sub>0</sub>/G<sub>1</sub> was seen in cells cultured with G-CSF and SCF compared to those cultured in G-CSF alone (3.5 versus 8.0 hours; Figure 2B).

Stem cell factor/granulocyte colony-stimulating factor–induced shortening of G<sub>0</sub>/G<sub>1</sub> is associated with a loss of expression of the cyclin-dependent kinase inhibitor p27<sup>Kip-1</sup>

Because the proliferative synergy between SCF and G-CSF is mediated through a shortening of G<sub>0</sub>/G<sub>1</sub>, we investigated the effect
of G-CSF and SCF cotreatment on several key regulatory components of the G₁-S transition. Rb, a critical mediator of the progression from G₁ to S phase, is modulated through phosphorylation by cyclin-dependent kinases (cdks). The regulation of the activity of these cdks is critical for progression through the cell cycle. In particular, cdk inhibitors (cdkis) such as p27 kip-1 or p21 cip-1 block cdk activity and prevent progression from G₁ to S phase. To analyze the effect of SCF and G-CSF on Rb phosphorylation, MO7e-G cells were starved and then cultured in the presence or absence of cytokines. Both SCF and G-CSF induced hyperphosphorylation of Rb, as measured by decreased gel mobility of Rb as seen in a Western blot (Figure 2C). However, the hyperphosphorylation induced by SCF alone was not complete at any of the time points, because the fastest-migrating hypophosphorylated band was always present. By contrast, the combination of SCF and G-CSF, or G-CSF alone, induced complete hyperphosphorylation of Rb. To investigate the role that cdkis play in this cytokine-induced Rb-phosphorylation, p27kip-1 levels were analyzed by Western blot (Figure 2D). SCF alone did not induce a decrease in p27kip-1 protein level. Although G-CSF induced a 22% decrease of p27kip-1 protein level at 24 hours compared to untreated cells (lane 7), the combination of SCF and G-CSF induced a 90% reduction in p27kip-1 protein level (lane 8). No differences were found in the expression of p21cip-1 among the different treatments at any time point (data not shown). Thus, the combination of G-CSF and SCF leads to specific suppression of p27kip-1 in MO7e-G cells.

Costimulation of MO7e-G cells with stem cell factor and granulocyte colony-stimulating factor leads to an increased expression of c-fos

Because cellular function is dictated by the pattern of gene expression, we analyzed the activation of c-fos, a proto-oncogene involved in cell cycle progression, in response to SCF and G-CSF. MO7e-G cells were starved overnight and then treated with SCF or G-CSF alone or in combination for 30 minutes. Total RNA was harvested and analyzed by semiquantitative RT-PCR (Figure 3). Although SCF was unable to induce c-fos expression, G-CSF at high concentrations (1 ng/mL) but not at low concentrations induced modest expression of this proto-oncogene. Costimulation of MO7e-G cells with SCF and G-CSF led to a marked increase in the expression of c-fos compared with cells treated with G-CSF alone. Thus, SCF and G-CSF, which show synergistic effects in inducing cellular proliferation, also lead to marked enhancement in inducing the expression of a proto-oncogene involved in cell cycle progression.

Granulocyte colony-stimulating factor–induced activation of STAT1 and STAT3 is unaffected by stem cell factor

The enhanced activation of c-fos by cotreatment with SCF and G-CSF suggested that one or more signaling pathways activated by these factors was amplified when the cytokines were administered together. In particular, STATs are known to mediate the effects of many cytokines in hematopoietic cells, including G-CSF, and have been found to control cellular proliferation by regulating p27kip-1 expression. Therefore, we explored the possibility that enhanced STAT activation mediated the synergistic effects of SCF and G-CSF on proliferation, p27kip-1 regulation, and c-fos induction. To analyze this, MO7e-G cells were factor starved overnight and treated with SCF, G-CSF, or their combination. Whole cell extracts were obtained and Western blots were performed to detect the activated tyrosine-phosphorylated forms of STAT1 and STAT3. SCF and G-CSF alone led to phosphorylation of STAT1 compared to untreated cells (lane 7), the combination of SCF and G-CSF induced a 90% reduction in p27kip-1 protein level (lane 8). No differences were found in the expression of p21cip-1 among the different treatments at any time point (data not shown). Thus, the combination of G-CSF and SCF leads to specific suppression of p27kip-1 in MO7e-G cells.

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Although SCF did not enhance the tyrosine phosphorylation of STAT1 and STAT3 induced by G-CSF, we considered the possibility that SCF might amplify the actions of G-CSF by increasing STAT translocation or binding to DNA. To address this possibility, nuclear extracts were prepared from cells treated with SCF and G-CSF alone and in combination. The nuclear extracts were incubated with 32P-double-stranded oligonucleotide containing a high-affinity STAT-binding sequence, and protein-DNA complexes were analyzed in an electrophoretic mobility shift assay (Figure 4B). G-CSF treatment led to the induction of 3 protein-DNA complexes (lane 2; complexes Ia, Ib, and Ic) whose specificity was demonstrated by their abolition by inclusion of a 100-fold excess of unlabeled probe in the binding reaction (lane 11). By contrast, a higher mobility nonspecific complex (complex III) was unaffected by excess unlabeled probe. That these complexes contain STAT1 and STAT3 was demonstrated by including antibodies to each of these proteins in the binding reaction. Antibody to STAT1 abolished the high-mobility complex Ic, and antibody to STAT3 diminished complex Ib while inducing formation of a low-mobility band (complex II, lane 8). It is likely that other proteins are participating in the formation of at least some of these complexes because antibodies to STAT1 and STAT3 do not completely abolish all of the protein-DNA complexes. However, antibodies to other STATs did not affect these complexes (data not shown). In contrast to G-CSF, SCF failed to induce formation of any specific protein-DNA complexes (lane 1), consistent with its inability to induce tyrosine phosphorylation of STAT1 or STAT3. Cotreatment with SCF and G-CSF did not lead to any qualitative or quantitative differences in the protein-DNA complexes induced compared to those seen with G-CSF alone (lane 3). Thus, the synergy between SCF and G-CSF is not mediated by increased STAT tyrosine phosphorylation or DNA binding.

**Figure 4.** Tyrosine phosphorylation and DNA binding of STAT1 and STAT3 induced by G-CSF is not affected by SCF. MO7e-G cells were factor starved overnight, then treated for 15 minutes with SCF (100 ng/mL), G-CSF (1 or 100 ng/mL), or both. (A) Total cell extracts were prepared and Western blots were performed to detect total and tyrosine-phosphorylated forms of STAT1 and STAT3. The same membrane was stripped and reprobed for each blot. (B) Nuclear extracts were prepared and binding was assessed to a32P-labeled double-stranded oligonucleotide containing a STAT-binding high-affinity sequence. G-CSF was used at a concentration of 1 ng/mL. Ia, Ib, and Ic indicate specific STAT-DNA complexes; II, “super-shifted” complexes; III, nonspecific complexes.

and STAT3 (Figure 4A; lanes 3 and 4), whereas SCF did not induce any phosphorylation of these STATs (lane 2). The addition of SCF to G-CSF failed to induce any detectable change in the level of tyrosine phosphorylation of either STAT1 or STAT3 compared to that induced by G-CSF alone (lanes 5 and 6). This suggests that the synergy between SCF and G-CSF is not mediated at the level of STAT tyrosine phosphorylation. This is further supported by the finding that SCF did not enhance the phosphorylation of JAK1 and JAK2, the tyrosine kinases activated by G-CSF that mediate STAT tyrosine phosphorylation (data not shown).

The STAT transcription factors can be phosphorylated not only on tyrosine residues, but also on specific serine residues within their transactivation domain (ser727 in STAT1 and STAT3). Although tyrosine phosphorylation directly activates a STAT, ser727 phosphorylation increases the magnitude of the gene transcription mediated by the tyrosine-phosphorylated STATs and is necessary for maximal transcriptional activity. Thus, we tested the possibility that SCF enhanced STAT-dependent gene activation by enhancing phosphorylation of the ser727 residue. MO7e-G cells were factor starved and treated with SCF and G-CSF alone and in combination. Whole cell extracts were prepared and immunoblots were performed with antibodies specific for the tyrosine-phosphorylated and the ser727-phosphorylated form of STAT3 (Figure 5A). Treatment of MO7e-G cells with SCF led to the prominent phosphorylation of STAT3 on ser727, but not on the activating tyrosine705 (lane 2). A Western blot using an antibody that recognizes all forms of STAT3 revealed that ser727-phosphorylated STAT3 migrated slightly slower than the unphosphorylated form (total-STAT3 blot; lane 2). G-CSF treatment led to both tyrosine and ser727 phosphorylation of STAT3, which added a third band in the pattern of migration of STAT3 (lane 3). Combined treatment of MO7e-G cells with SCF and G-CSF induced an increase in ser727 phosphorylation of STAT3 (lane 4), which was greater than that induced by SCF or G-CSF alone. In 4 independent experiments, the phosphorylation of STAT3 on ser727 induced by the combination was 54% greater than that induced by SCF alone.

**Figure 5.** Tyrosine phosphorylation and DNA binding of STAT1 and STAT3. (A) Western blots were prepared and the phosphorylation of STAT3 on ser727 induced by SCF alone (lane 3) compared to that induced by G-CSF alone (lane 4), whereas SCF did not induce any phosphorylation of these STATs (lane 2). The addition of SCF to G-CSF failed to induce any detectable change in the level of tyrosine phosphorylation of either STAT1 or STAT3 compared to that induced by G-CSF alone (lanes 5 and 6). This suggests that the synergy between SCF and G-CSF is not mediated at the level of STAT tyrosine phosphorylation. This is further supported by the finding that SCF did not enhance the phosphorylation of JAK1 and JAK2, the tyrosine kinases activated by G-CSF that mediate STAT tyrosine phosphorylation (data not shown).

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Western blots were performed with the indicated antibodies. The same membrane
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Stem cell factor–induced ser727 phosphorylation of STAT3 is
partially mediated by PI3 kinase and mitogen-activated
protein kinase
Ser727 of STAT3 is contained in the sequence leu-pro-met-ser-pro,
a motif found in substrates of proline-directed serine/threonine
kinases.55,56 However, the identity of the serine/threonine kinase(s)
that phosphorylate this conserved carboxy-terminus serine in STAT
family members has remained elusive. To determine the upstream
signaling events necessary for the serine phosphorylation of STAT3
induced by these cytokines, MO7e-G cells were factor starved and
then incubated for 1 hour prior to cytokine treatment with PD98059 (100 μmol/L), wortmannin (500 nmol/L), or H7 (50 μmol/L);
Western blots were performed with the indicated antibodies. The same membrane
was stripped and reprobed for each blot.

Discussion
Although the synergy between SCF and G-CSF is of great biologic and
clinical importance, the absence of a model system to explore this
interaction at the cellular level has impaired the ability to
understand its mechanism. In this study, we have developed a
system to examine the intracellular events induced by these
molecules alone and in combination by using retroviral transduction to introduce the human G-CSF receptor into the SCF-responsive human hematologic cell line, MO7e. This has demonstrated that the use of VSV-G glycoprotein-pseudotyped retroviruses is a feasible approach toward high efficiency and stable transduction of this human acute megakaryocytic leukemia-derived cell line. This may be an important strategy for the introduction of genes into other human hematologic cells and cell lines, which are difficult to transfect by other means.

The transduced G-CSF receptor is functionally active, in that it supports G-CSF-dependent proliferation of MO7e-G cells, but also recapitulates the proliferative synergy between SCF and G-CSF (Figure 1B). Cell cycle analysis revealed that the enhanced proliferative state induced by SCF and G-CSF cotreatment was associated with a direct effect of these cytokines on cell cycle distribution, specifically a marked shortening of the duration of G0/G1. This is mediated, at least in part, by a marked decrease of expression of the cdki p27kip-1, which is known to set a stoichiometric inhibitory threshold of cdk activity that prevents cdk-induced phosphorylation of Rb and cell cycle progression. Therefore, the loss of p27kip-1 following treatment with SCF and G-CSF may facilitate cell proliferation.

Mitogenic cytokines induce the activation of immediate-early genes, which are necessary for cellular proliferation. These genes are regulated by transcription factors that are activated by cytokine-induced signaling pathways. SCF and G-CSF, which lead to a marked enhancement of proliferation in MO7e-G cells, also lead to synergistic induction of c-fos, a proto-oncogene necessary for cell cycle progression in many systems. The promoter for c-fos contains elements responsive to a number of transcription factors. Among these, we focused on STATs, which are known to be key mediators of cytokine signaling in hematopoietic cells, and have been shown to control cellular proliferation by regulating p27kip-1 expression. As has been reported in other systems, we found that G-CSF induces tyrosine phosphorylation of STAT1 and STAT3 and their subsequent binding to specific DNA elements. By contrast, SCF failed to induce activation of these STATs in MO7e-G cells. The ability of SCF to activate STATs remains controversial. In MO7e cells, SCF has been shown to induce activation of STAT1 in some reports, but not in others. Furthermore, the combination of SCF and G-CSF has been found to activate STAT3 in any cell type, including MO7e. No evidence was found that the synergy between SCF and G-CSF occurs at the level of STAT1 or STAT3 tyrosine phosphorylation, nuclear translocation, or DNA binding (Figure 4).

Although tyrosine phosphorylation is required for STAT dimerization and nuclear translocation, the magnitude of STAT1- or STAT3-mediated (or both) gene induction is modulated by phosphorylation on ser727 within the transactivation domain of both STATs. We found that SCF induces ser727 phosphorylation of STAT3 in MO7e-G cells, as has been reported by others. Furthermore, the combination of SCF and G-CSF maximized the induction of ser727 phosphorylation of STAT3. In fact, all of the STAT3 detectable by Western blot is completely phosphorylated on this residue after treatment with SCF and G-CSF, whereas neither G-CSF nor SCF alone induce this complete shift to the slow-migrating form of STAT3 as has been reported by others. Furthermore, the combination of SCF and G-CSF maximized the induction of ser727 phosphorylation of STAT3.

Because PI3K and ERK-dependent pathways are necessary for the synergistic phosphorylation of STAT3 on ser727, we explored whether inhibition of these pathways would affect gene activation or proliferation elicited by cotreatment with SCF and G-CSF. Simultaneous inhibition of these 2 pathways led to complete abolition of the synergistic induction of c-fos. Furthermore, the
proliferative synergy between SCF and G-CSF was also completely dependent on PI3K and ERK, because the growth of MO7e-G cells in response to SCF and G-CSF was reduced to that seen with G-CSF alone in the presence of inhibitors of these pathways (Figure 6B).

We have described a novel system to explore the signaling events underlying the important biologic interaction between SCF and G-CSF. MO7e-G cells recapitulate the proliferative synergy between SCF and G-CSF and have allowed us to identify a number of intracellular pathways that may mediate their biologic actions. It remains to be determined whether other pathways play a role in the biologic and clinical effects of SCF and G-CSF.

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References


SCF and G-CSF lead to the synergistic induction of proliferation and gene expression through complementary signaling pathways

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