RAPID COMMUNICATION

Multiple Signaling Pathways Induced by Granulocyte Colony-Stimulating Factor Involving Activation of JAKs, STAT5, and/or STAT3 Are Required for Regulation of Three Distinct Classes of Immediate Early Genes

By Shin-Shay Tian, Peter Tapley, Catherine Sincich, Robert B. Stein, Jon Rosen, and Peter Lamb

Granulocyte colony-stimulating factor (G-CSF) is the major regulator of proliferation and differentiation of neutrophilic granulocyte precursor cells. G-CSF activates multiple signaling molecules, including the JAK1 and JAK2 kinases and the STAT transcription factors. To investigate G-CSF signaling events regulated by the JAK-STAT pathway, we have generated UT7-epo cells stably expressing either wild-type (wt) G-CSF receptor or a series of C-terminal deletion mutants. Gel mobility shift and immunoprecipitation/Western analysis showed that STAT5 is rapidly activated by G-CSF in cells expressing the wt G-CSF receptor, in addition to the previously reported STAT3 and STAT1. Mutants lacking any tyrosine residues in the cytoplasmic domain maintain their ability to activate STAT5 and STAT1 but cannot activate STAT3, implying that STAT5 and STAT1 activation does not require receptor tyrosine phosphorylation. We also observed significant changes in the ratio of STAT1:STAT3:STAT5 activated by various G-CSF receptor C-terminal deletion mutants. These mutant receptors were further used to investigate the role of JAKs and STATs in G-CSF-mediated responses in these cells. We found that JAK activation correlates with G-CSF–induced cell proliferation, whereas STAT activation is not required. We have also identified three classes of G-CSF immediate early genes, whose activation correlates with the activation of distinct JAK-STAT pathways. Our data show that, whereas c-fos is regulated through a pathway independent of STAT activation, oncostatin M, IRF-1, and egr-1 are regulated by an STAT5-dependent pathway and fibrinogen is regulated by an STAT3-dependent pathway. In conclusion, our results suggest that G-CSF regulates its complex biologic activities by selectively activating distinct early response genes through different JAK-STAT signaling molecules.

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cDNA as a template, truncation mutants were constructed by replacing the Eco47III-Xba I fragment, which contains all of the cytoplasmic region and part of the extracellular domain with the corresponding PCR products containing only a portion of the cytoplasmic domain. Paired with a forward primer spanning the region containing the Eco47III site, the following oligonucleotides were used as the reverse PCR primers: MGR1 (A644), 5'-TGA CAC CCG ACC CAG ACC GT-3'; MGR2 (A674), 5'-GAA CCA CTA ACC ACC GTG CGA CCT C-3'; MGR3 (A684), 5'-GCC AAA GAA CAG AAG GAG GTC AA-3'; MGR4 (A703), 5'-GGA CTT GCT GGG CAC CTT CC-3'; MGR4D (A709), 5'-GGA CAA CCT GGT GTA GTG CGA CCT C-3'; MGR3 (A684), 5'-GCC AAA GAA CAG AAG GAG GTC AA-3'; and MGR5 (A738), 5'-CGA CCA CCC CGA GGG TCC GTG GAC T-3'.

**Cell culture and transfection.** UT-7epo cells were a gift from SmithKline Beecham Pharmaceuticals (Dr Connie Erickson-Miller, King of Prussia, PA). The untransfected UT-7epo cells were maintained in Iscove's modified Dulbecco's medium (IMDM; GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and 2 U/mL erythropoietin (Epo; R&D, Minneapolis, MN). Stable cell lines were maintained in the growth medium containing 200 µg/mL of hygromycin. The IL-3-dependent NFS-60 cells were a gift from Dr James Ihle (Memphis, TN). AML-193 cells were obtained from the ATCC (Rockville, MD), and both cell lines were expanded, and subjected to two additional rounds of FACS sorting.

**Proliferation assay.** The proliferation assays were set up in 96-well cell culture plates at 2 × 10^5 cells and 150 µL per well with or without G-CSF at the concentrations indicated. Each point was performed in quadruplicate. Cells were treated for 2 days and then 15 µL of the cell proliferation reagent WST-1 (Boehringer Mannheim, Indianapolis, IN) was added to each well and incubated for 1 hour at 37°C. WST-1 assay measures the overall activity of mitochondrial dehydrogenases in the samples. Absorbance was measured at 450 nm wavelength using a Microplate Autoreader (BIO-TEK Instruments, Inc, Winooski, VT).

**RESULTS**

Generation of stable cell lines expressing G-CSFR mutants. To investigate G-CSF signaling events regulated by the JAK-STAT pathway, we constructed a series of C-terminal deletion mutants that progressively truncate the cytoplasmic domain of the G-CSFR receptor (designated MGR1 (Δ644), MGR2 (Δ674), MGR3 (Δ684), MGR4C (Δ703), MGR4D (Δ709), MGR4 (Δ717), and MGR5 (Δ738), as shown in Fig 1) and have cloned them into the mammalian expression vector pMC1hygro by CellFECTIN (GIBCO-BRL) as suggested by the manufacturer. The transfected cells were cultured for 24 hours and then selected with hygromycin-containing (200 µg/mL) medium for approximately 10 days.

**Preparation of nuclear extracts and gel retardation assays.** Nuclear extracts were prepared as described.12 The oligonucleotides used were m67 and IRF-1,14,15 which are GAS sequences that bind to STAT proteins activated by a variety of cytokines. Gel mobility shift assays and antibody supershift experiments were performed as described.12

**Preparation of cell lysates and immunoblotting.** To examine phosphorylation of STAT proteins and JAK kinases, cells were deprived of cytokines overnight before treatment with G-CSF at 10 ng/mL for 10 minutes. Induced or uninduced cells were lysed and immunoblotting was performed as described.16 The STAT5 antibody was from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The STAT3 antibody, raised against amino acids 688 to 727 of STAT3, and the STAT1 antibody, raised against the C-terminal 36 amino acids of p91, are gifts of Dr J.E. Darnell Jr.20,21 The JAK1 and JAK2 antibodies were from Pharmingen (San Diego, CA) and antiphosphotyrosine antibody 4G10 was from Upstate Biotechnology Inc (Lake Placid, NY). Reverse transcriptase-PCR (RT-PCR). UT-7epo stable cell lines were starved overnight without Epo in growth medium containing 0.5% FBS. The starved cells were either left untreated or treated with G-CSF (10 ng/mL) for 30 minutes or 2 hours in the presence of cycloheximide (10 µg/mL). Total RNA was prepared from 1 × 10^7 cells using the QIAshredder and RNEasy kit (QIAGEN Inc, Chatsworth, CA), and first-strand cDNAs were prepared using the SuperScript Pre amplification System (GIBCO-BRL). The RT-PCR conditions that gave the optimal signal to noise ratio for the induced genes were determined individually using RNA prepared from the MGRwt cells. PCR was performed as suggested by the manufacturer (GIBCO-BRL) for 25 cycles and the annealing temperature was 58°C for all of the primer sets. The human c-fos and GAPDH PCR primers were purchased from Clontech Laboratories, Inc (Palo Alto, CA). The OSM primers (forward primer, 5'-AAC AAC ATC TAC TGC ATG GCC-3' and reverse primer, 5'-ACT GAG TGC ATG AAG CGA TG-3') were synthesized using an Oligo 1000M DNA Synthesizer (Beckman, Fullerton, CA). The PCR products were resolved by 2% agarose gels and visualized by ethidium bromide staining.

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amount of STAT1 in AML-193 cells. Using the IRF-1 GAS response element in an oligonucleotide pull down/Western blot analysis, we confirmed these results; in addition, we showed that STAT5 is also activated strongly by G-CSF in AML-193 cells (Fig 3B). Figure 3C shows the same experiments performed using lysates from NFS-60 cells. An identical pattern of STAT activation is observed. As a positive control for STAT5 activation, NFS-60 cells were also induced with IL-3. We observed activation of STAT5 predominantly, consistent with previous reports (Fig 3C). Gel mobility shift and antibody supershift assays using m67 as a probe showed that the STAT1 homodimer, STAT1/STAT3 heterodimer, and STAT3 homodimer are activated by G-CSF in the MGR4D (Δ709) stable transfectant (shown in the left panel of Fig 3D). Because the m67 probe does not bind to STAT5 efficiently, another probe, IRF-1, was used to analyze the same extracts. Using this probe, STAT5 and STAT1 homodimers were detected (shown in the right panel of Fig 3D). Similar data were obtained from MGRwt cells (data not shown), although the observed level of STAT1 activation was lower, as discussed below. Our data show that the UT-7epo stable cell lines displayed the same pattern of JAK-STAT activation as did myeloid cells expressing endogenous G-CSFR and therefore provide a useful model system for further study.

We prepared nuclear extracts from the stable transfectants expressing wt or mutant G-CSFRs after treatment with either suboptimal or saturating levels of G-CSF for various times. Two STAT-binding elements, m67 (which detects STAT3 homodimer, STAT3/STAT1 heterodimer, and STAT1 homodimer; Fig 4A) and IRF-1 (which detects STAT5 and STAT1 homodimers; Fig 4B), were used as probes for gel
mobility shift assays. Truncation of the G-CSFR to amino acid 708 [MGR4D (Δ709), 4 (Δ717), and 5 (Δ738)] still allows efficient activation of STAT3. However, removal of 6 additional amino acids, including tyrosine 703 [MGR4C (Δ703)] greatly reduced STAT3 activation, but had no effect on STAT1 activation (Fig 4A). Further truncation of the receptor to amino acid 673 [MGR 2 (Δ674)], which removes the Box2 homology region, reduced STAT1 activation to barely detectable levels (Fig 4A and B). STAT5 activation parallels that of STAT1, with the MGR3 (Δ684) mutant showing efficient STAT5 activation and MGR2 (Δ674) showing greatly reduced STAT5 activation (Fig 4B). This finding implies that, unlike STAT3, efficient STAT5 and STAT1 activation does not require receptor tyrosine phosphorylation. These experiments also show that, for the mutants that allow STAT3 and/or STAT3 activation, we do not detect major differences in either the time course of STAT activation or shifts in the G-CSF dose-response curve relative to the wt receptor. However, we did observe significant changes in the ratio of STAT1/STAT3 STAT5 activated by the various C-terminal deletion mutants. For example, STAT1 was only activated slightly in the MGRwt stable transfectant, whereas some mutants, notably MGR3 (Δ684), MGR4C (Δ703) and MGR4D (Δ709) showed dramatically increased levels of both STAT1 homodimer and STAT1/STAT3 heterodimer formation in response to G-CSF (Fig 4A and B).

Analysis of STAT3 and STAT5 activation in the stable cells expressing mutant receptors by immunoprecipitation/antiphosphotyrosine Western blot analysis generally confirmed the results observed in the gel mobility shift assays (Fig 5A and B). Efficient STAT5 activation is evident in cells expressing wt or mutant receptors MGR3 (Δ684)-MGR5 (Δ738), with a very low level being observed in cells expressing MGR2 (Δ674) (Fig 5B). A small amount of STAT3 was activated by the MGR3 (Δ684) mutant, as shown in Fig 5A, which is consistent with the formation of a minor amount of STAT1/STAT3 heterodimer in the MGR3 (Δ684) nuclear extract (Fig 4A). Efficient STAT3 activation is seen in cells expressing wt or mutant receptors MGR4 (Δ717)-5 (Δ738). We also tested the ability of these mutant receptors to activate JAK1 and JAK2 kinases. JAK1 and JAK2 are activated by MGRwt and all of the mutant receptors, with the exception of MGR1 (Δ644) (Fig 5C and D). MGR1 (Δ644) contains only 18 amino acids of the cytoplasmic domain of the receptor, up to the C-terminal boundary of the Box1 homology region. Our data suggest that sequences downstream of Box1 are important for G-CSF–induced JAK activation. The level of JAK1 activation by the MGR2 (Δ674) mutant is substantially reduced, implying that Box2 also contains sequences important for JAK1 activation. Both MGR3 (Δ684) and MGR5 (Δ738) stable transfectants contain a minor population of cells expressing very high level of receptors (Fig 2). This may lead to the activation of these two mutant receptors in the absence of G-CSF, resulting in the somewhat higher basal activation of JAK1 and JAK2 shown in Fig 5C and D.

Activation of early response genes by wt and mutant G-CSFRs. G-CSF induces transcriptional activation of a spe-
Fig 4. Gel mobility shift assay of nuclear extracts from UT-7epo stable cell lines. Cells were not treated (−), treated with 10 U/mL Epo for 10 minutes, or treated with 0.5 or 10 ng/mL G-CSF for 15, 60, or 120 minutes as indicated. Nuclear extracts were prepared and incubated with a radiolabeled m67 (A) or IRF-1 (B) probe and then separated on non-denaturing polyacrylamide gels. The G-CSF–induced DNA-binding complexes are indicated by arrows. The mutant receptors are abbreviated as MGR1 through MGR5.
A STAT3

MGR1 MGR2 MGR3 MGR4 MGR5 WT
- + - + - + - + - +

B STAT5

MGR1 MGR2 MGR3 MGR4 MGR5 WT
- + - + - + - + - +

C JAK1

MGR1 MGR2 MGR3 MGR4 MGR5 WT
- + - + - - - - - -

D JAK2

MGR1 MGR2 MGR3 MGR4 MGR5 WT
- + - + - + + + - +

Fig 5. G-CSF–induced JAK and STAT activation in UT-7epo stable cells. Cells were either not treated (−) or treated with 10 ng/mL G-CSF for 10 minutes and total cell lysates were prepared and incubated with an STAT3 (A), STAT5 (B), JAK1 (C), or JAK2 (D) antibody. Immunoprecipitated proteins were then separated by 8% SDS-PAGE gels and transferred to nitrocellulose. The blots were probed with the antiphosphotyrosine antibody 4G10. The mutant receptors are abbreviated as MGR1 through MGR5.

Specific spectrum of immediate early response genes. To understand whether activation of the different STAT transcription factors discussed above contributes to regulation of early response genes, we have examined G-CSF–induced activation of a panel of these genes. The c-fos, OSM, IRF-1, egr-1, and fibrinogen genes are induced rapidly by G-CSF in cells stably transfected with MGRwt (Fig 6 and data not shown). These genes contain GAS-like response elements in their promoters that could be the binding sites for G-CSF–activated STAT complexes. We therefore examined the ability of each of the mutant receptors to induce expression of these genes. The c-fos proto-oncogene, which is involved in mitogenic response regulated by many growth factors, is efficiently activated by the wt and all of the mutant receptors except MGR1 (Δ644) (Fig 6A). In contrast, two other G-CSF–regulated immediate early genes, OSM (which belongs to the IL-6 cytokine superfamily) and IRF-1 (which is a transcription factor inducible by interferon-γ and IL-6), are induced by all the mutants except MGR1 (Δ644) and MGR2 (Δ674) (Fig 6B and C). We observed a similar activation pattern for another G-CSF–regulated gene, egr-1 (data not shown). We also analyzed the activation of fibrinogen by G-CSF treatment and observed the induction of fibrinogen mRNA in the cell lines expressing MGR4D (Δ709), MGR4 (Δ717), MGR5 (Δ738), and MGRwt (Fig 6D). These are the receptor constructs that contain at least the most N-terminal tyrosine residue, Y703 (Fig 1), and are the only ones capable of activating STAT3 homodimer complex (Fig 4A). By cotransfecting the MGR expression plasmids in a transient transfection reporter assay in HepG2 cells, we have also observed a similar dependence on Y703 for the activation of reporters containing STAT3-selective response elements (data not shown). We have, therefore, identified three classes of immediate early genes regulated by G-CSF that require different regions of the receptor cytoplasmic domain for efficient induction.

Induction of proliferation by wt and mutant G-CSFRs. G-CSF induces short-term proliferation of the MGRwt UT-7epo cells as assessed by either a WST-1 proliferation assay or direct cell counting (Fig 7 and data not shown). To examine whether JAK and STAT activation contributes to this G-CSF–induced proliferative signal, we examined all of the G-CSF receptor-transfected UT-7epo stable cell lines for G-CSF–induced proliferation using the WST-1 assay (Fig 7). G-CSF–induced proliferation of most of the MGR mutant cell lines, including MGR2 (Δ674), which activates both JAK1 and JAK2, cannot activate STAT3 and is only capable of marginally inducing STAT5 (Figs 4 and 5). The only cell line that failed to proliferate in response to G-CSF was the stable cell pool expressing the MGR1 (Δ644) mutant. MGR1 (Δ644) lacks receptor sequences C-terminal of Box1 and does not detectably activate JAK or STAT proteins. To determine if any of the MGR mutants show altered sensitivities to G-CSF, we have performed dose-response and time course analyses of MGRwt and all of the MGR mutant stable cell lines by the WST-1 proliferation assay and cell counting. We did not observe any consistent differences in sensitivity to G-CSF between the wt and mutant receptors (data not shown).
Fig 6. G-CSF–induced activation of immediate early genes in UT-7epo stable cells. Cells were either not treated (–) or treated with 10 ng/mL G-CSF for 30 minutes (A, B, and E) or 2 hours (C and D) in the presence of cycloheximide. RT-PCR was performed as described in the Materials and Methods. The PCR products were resolved by 2% agarose gels and visualized by ethidium bromide staining. The mutant receptors are abbreviated as 1 through 5.

DISCUSSION

We previously reported that G-CSF activates the JAK family members JAK1 and JAK2 and the STAT proteins STAT3 and STAT1. In this report, we showed that STAT5 is also efficiently activated by G-CSF in both murine and human cell lines. STAT5 is also activated by G-CSF in murine primary bone marrow cells (Tian et al, unpublished results). Our analysis of a series of G-CSFR mutants shows that the sequences in the G-CSFR cytoplasmic domain required for JAK activation, efficient STAT3 activation, and efficient STAT5 activation are separable. Activation of JAKs requires a region of the receptor including the Box1 homology domain and sequences immediately downstream. Equivalent regions of several other cytokine receptors have also
been shown to be required for JAK activation. However, JAK activation alone is not sufficient for effective STAT activation in response to G-CSF. STAT5 activation requires additional receptor sequences that include the Box2 homology region. Our data show that STAT5 can be activated by mutant receptors that lack tyrosine residues, indicating that G-CSFR phosphorylation is not required for STAT5 activation. This is different from the Epo receptor system, in which Epo receptor tyrosine phosphorylation is involved in Epo-induced STAT5 activation.28 This finding suggests that activation of a STAT family member can proceed through different mechanisms in the context of different cytokine receptor chains. In contrast, efficient STAT3 activation requires additional receptor sequences that include at least one tyrosine, tyr 703. This is consistent with previous analyses performed using different G-CSFR mutants that also indicated that receptor tyrosines are required for STAT3 activation.27 We also observed dramatic increases in the level of STAT1 activation among some of the mutant receptors relative to the wt receptor, which can only activate a low level of STAT1. The most noticeable increase is in mutants MGR3 (Δ684), MGR4C (Δ703), and MGR4D (Δ709). We have not yet detected any changes in G-CSF–triggered responses due to the higher level of STAT1 activation in these mutants. Further study of these mutants may provide us with insight into the role that STAT1 plays in G-CSF signaling.

STAT5 is known to be the major STAT activated by a variety of cytokines, including IL-2, IL-3, IL-5, granulocyte–macrophage colony-stimulating factor (GM-CSF), thrombopoietin (TPO), and Epo.29-32 In the context of Epo and IL-3 signaling, it has been suggested that STAT5 activation is involved in cell proliferation.33 However, conflicting reports by other investigators indicate that STAT5 activation is not required for Epo- or IL-2–induced proliferation.34 In our experiments, the MGR2 (Δ674) mutant, which can induce STAT5 activation only marginally, retains an ability to stimulate cell proliferation that is indistinguishable from that triggered by the wt receptor. This result suggests that, if STAT5 is required for the G-CSF proliferative signal, a very low level of STAT5 activation is sufficient for transducing that signal. We have also observed that, in NFS-60 cells, the concentration of G-CSF sufficient to give an optimal proliferative signal is much lower than that required for maximal STAT5 activation (Tian et al, unpublished results). However, we are currently unable to distinguish between a model in which very low levels of STAT5 activation are sufficient for transducing a G-CSF proliferative signal and one in which STAT5 activation is not required at all. Our data, together with previous data using less extensive sets of receptor mutants,35 clearly show that STAT3 activation is not required for a proliferative response to G-CSF, whereas JAK activation is required.

Many of the biologic effects of cytokines are thought to be the long-term consequences of the activation of distinct sets of early response genes. We examined induction of several early response genes that are regulated by G-CSF in the UT-7 cell transfectants. We were able to correlate activation of different STAT proteins with the activation of different early response genes and found that the genes fell into three distinct classes. The first class, represented by the c-fos gene, does not require STAT activation for G-CSF induction. The c-fos promoter has many regulatory elements, including a GAS response element, the serum-inducible element (SIE), that has been well characterized.36 37 Our data suggest that the SIE does not play an important role in c-fos induction under these conditions. It remains to be determined which of the other c-fos response elements are important for G-CSF–induced c-fos activation. The second class of early response genes, which consists of OSM, IRF-1 and egr-1, appears to be regulated by G-CSF through the same pathway, because the activation of all three genes correlates well with STAT5 activation. STAT5 activation has recently been suggested to be involved in the transcriptional regulation of OSM by Epo and IL-3.38 We therefore conclude that G-CSF–induced STAT5 activation is likely to be involved directly in the transcriptional regulation of OSM, IRF-1, and egr-1. Both OSM and IRF-1 genes contain palindromic GAS-like response elements in their promoters (OSM, 5'-TTCCAGAA-3'; and IRF-1, 5'-TTCCCGGA-3') that might be the direct binding sites of the G-CSF–activated STAT5 complex. We have not been able to identify any GAS-like response element in the promoter of the published mouse egr-1 gene sequence. One explanation is that a somewhat divergent response element in the egr-1 promoter may be involved in STAT5 regulation. IRF-1 is known to be regulated by interferon γ, which predominantly activates STAT1.39 Although G-CSF–induced STAT1 activation always correlates with STAT5 activation, the level of STAT1 activation is very low in the MGRwt transfectant (Fig 4) relative to the level of STAT5. Thus, STAT1 is unlikely to be a significant player in the transcriptional regulation of OSM, IRF-1, and egr-1 by G-CSF. OSM belongs to the IL-6 cytokine superfamily, and IRF-1 and egr-1 are transcription factors that are known to have many activities.40,41 Taken together, our data suggest that, through activation of diverse targets possessing multiple functions, STAT5 might play a critical role in G-CSF–regulated biologic activities. The third class of induced gene is represented by the fibrinogen gene. Activation of this gene correlates with the ability of mutant receptors to activate STAT3. Fibrinogen contains an imperfect palindromic GAS response element (5'-GTACTG-GAA-3') in its promoter, and it was suggested previously that this response element plays a role in IL-6–regulated fibrinogen induction.42 This is consistent with our observation that fibrinogen induction correlates with STAT3 activation in all of the G-CSFR constructs, because STAT3 is the major STAT activated by IL-6.

Recent studies have indicated that different STAT proteins, activated by different cytokines and growth factors, bind to distinct but related GAS response elements.43 The specificity of STAT binding can be strongly influenced by the spacing of palindromic half sites and by the nature of sequences both flanking and between the half site sequences.44 45 This in turn suggests that each member of the STAT family may activate distinct (although possibly overlapping) genetic programs through its ability to differentially interact with sequences present in promoters. Using mutants of the G-CSFR that separate the ability of G-CSF to activate
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Multiple signaling pathways induced by granulocyte colony-stimulating factor involving activation of JAKs, STAT5, and/or STAT3 are required for regulation of three distinct classes of immediate early genes

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