Distinct Regions of the Granulocyte Colony-Stimulating Factor Receptor Are Required for Tyrosine Phosphorylation of the Signaling Molecules JAK2, Stat3, and p42, p44MAPK

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The protein tyrosine kinases JAK1 and JAK2 are phosphorylated on tyrosine after the interaction of granulocyte colony-stimulating factor (G-CSF) with its transmembrane receptor. So too is Stat3, a member of the STAT family of transcriptional activators thought to be activated by the JAK kinases. Truncated G-CSF receptor (G-CSF-R) mutants were used to determine the different regions of the cytoplasmic domain necessary for tyrosine phosphorylation of the signaling molecules JAK2, Stat3, and p42, p44MAPK. We have shown that G-CSF-induced tyrosine phosphorylation and kinase activation of JAK2 requires the membrane proximal 57 amino acids of the cytoplasmic domain. In contrast, maximal Stat3 tyrosine phosphorylation required amino acids 96 to 183 of the G-CSF-R cytoplasmic domain. Stat3 DNA binding could occur with a receptor truncated 96 amino acids from the transmembrane domain and containing a single tyrosine residue, but was reduced in comparison with the full-length receptor. Together with the tyrosine phosphorylation of Stat3, this finding suggests that additional Stat3 binding sites are present in the full-length receptor. Because the G-CSF-R can signal a mitogenic response in the absence of the carboxyl-125 amino acids of the receptor, Stat3 does not appear to be required for proliferation. MAP kinase tyrosine phosphorylation correlated with both the proliferative response and JAK2 activation.

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MATERIALS AND METHODS

G-CSF-R mutants. BAF/BO3 cell lines transfected with either the full-length G-CSF-R cDNA (HGR-183) or constructs containing truncated G-CSF-R cDNAs (HGR-96, HGR-57, and HGR-26) were obtained from the Immuneex Corp (Seattle, WA). HGR-26 expresses a mutant receptor truncated 26 amino acids carboxyl to the transmembrane domain (amino acid 655). HGR-57 expresses a receptor truncated 57 amino acids from the transmembrane domain (amino acid 686). HGR-96 expresses a receptor truncated 96 amino acids from the transmembrane domain (amino acid 725) (Fig 1). Cell lines used were pooled, stable transfectants.

Cell culture. BAF/BO3 cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics. HGR-183 and HGR-96 were maintained in medium containing 10 ng/mL recombinant human G-CSF (rhG-CSF; kindly provided by Amgen, Thousand Oaks, CA). HGR-57 was maintained in medium containing 10 ng/mL rhG-CSF and 2% WEHI-3B D− conditioned medium (as a source of murine IL-3). Although HGR-57 can be maintained in G-CSF alone, it grows considerably slower than the other cell lines. HGR-26 was maintained in medium containing 10% WEHI-3B D− conditioned medium. The murine macrophage cell line BAC1.2F5 was maintained in Dulbecco's modified Eagle's medium of intracellular substrates by the JAK family of protein tyrosine kinases. JAK1 and JAK2 are rapidly tyrosine-phosphorylated in response to G-CSF. The JAK kinases are thought to induce tyrosine phosphorylation of STAT molecules (signal transducers and activators of transcription), resulting in their activation and translocation to the nucleus. G-CSF–induced Stat3 and Stat1 activation in AML-193 cells has been reported by Tian et al. It is unclear whether there is a link between the JAK kinases and the G-CSF mitogenic response.

The Ras/MAP kinase pathway has been shown to be activated in response to G-CSF in proliferative cell lines. The link between the G-CSF-R and the Ras/MAP kinase cascade has still to be elucidated, but, in the absence of an intrinsic kinase domain in the G-CSF-R, it is likely to be initiated by an associated protein tyrosine kinase.

For this study, we used truncated mutants of the G-CSF-R to delineate the regions of the cytoplasmic domain required for activation of the G-CSF-R signaling molecules, JAK2, Stat3, and MAP kinase.
Fig 1. Schematic diagram of the G-CSF-R cytoplasmic domain showing the Box homology domains and tyrosine residues (Y). The truncated receptor mutants expressed in the BAF/B03 cell lines (HGR-26, HGR-57, and HGR-96) are depicted and the corresponding relative proliferative response to G-CSF (our unpublished data) is shown on the right. ++, maximum incorporation of 33,000 cpm; +, maximum incorporation of 15,000 cpm.

(DMEM) supplemented with 10% fetal bovine serum and 30% L-cell-conditioned medium.

Antisera. The monoclonal antibody to the G-CSF-R extracellular domain, LMM741, was coupled to CNBr-activated Sepharose (5 mg antibody/mL of Sepharose). A total of 15 μL/mL of a 50% slurry of lysate was used for immunoprecipitation. The rabbit antiserum to the G-CSF-R, R55, was used for immunoblotting at 0.4 μg/mL. JAK2 antiserum was obtained from Upstate Biotechnology Inc (UBI; Lake Placid, NY) and was used at 2 μL/mL lysate for immunoprecipitation and 1:1,000 for immunoblotting. Antiphosphotyrosine antibody (4G10) was obtained from UBI. It was used at a concentration of 1.0 μg/mL for immunoprecipitation. Agarose-conjugated antiphosphotyrosine antibody (4G10; UBI) was used at 15 μL/mL cell lysate for immunoprecipitation. Anti-Stat3 (Western blotting) and anti-Stat3 antibodies were obtained from Transduction Laboratories (Lexington, KY). Anti-Stat3 antibody (gel shift assays) and p42, p44MAPK were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA).

Preparation of cell lysates. BAF/B03 cell lines were starved of growth factor for 4.5 to 5.5 hours before incubation with either rhG-CSF (100 ng/mL) or medium for 10 minutes. Cells were then centrifuged at 4°C and lysed in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% [vol/vol] Triton X-100, 2 mmol/L EDTA) containing the addition of 0.2 to 1.0 mmol/L Na3VO4, 1 mmol/L NaF, 1 μmol/L pepstatin, 1 mmol/L phenylmethylsulphonyl fluoride, 1 mmol/L, 1,10-phenanthroline, leupeptin (10 μg/mL), and aprotonin (10 μg/mL). Lysates were centrifuged at 13,000 rpm in an Eppendorf benchtop centrifuge for 10 minutes at 4°C to remove nuclei and cell debris and the supernatant was retained for protein analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Antibody binding was visualized with either peroxidase-conjugated swine antirabbit IgG (1:1,000; Dako Corp, Carpinteria, CA) or peroxidase-conjugated rabbit antianimal IgG (1:1,000; Dako) and the enhanced chemiluminescence (ECL) system (Amersham, Little Chalfont, Buckinghamshire, UK), according to the manufacturers’ instructions.

In vitro kinase assay. Immunoprecipitates were washed and suspended in an equal volume of kinase assay buffer containing [γ-32P]ATP (0.25 μCi/mL) for 30 minutes at room temperature. Immunoprecipitates were analyzed by SDS/6% PAGE. The gels were treated with 1 mol/L KOH 55°C for 2 hours to remove phosphoserine and phosphothreonine. Radioactive bands were visualized with IMAGEQUANT software on a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA).

Electrophoretic mobility shift assays. Complementary strands of oligonucleotides containing the high-affinity SIF (c-sis-inducible factor) binding site m67I7 (5'GATCGACATTTCCCGTAAATCG) with overhanging Sau3a ends, synthesized on an Applied Biosystems (Foster City, CA) DNA Synthesizer, were annealed and labeled by fill-in reactions with Klenow DNA polymerase and dNTPs in the presence of [α-32P]-dATP. Protein extracts were prepared from BAF/B03 cell lines starved of serum and growth factor for 5.5 hours before incubation with either rhG-CSF (100 ng/mL) or medium for 10 minutes. BAC1.2F5 cells were starved of growth factor for 18 to 22 hours before incubation with rhCSF-1 (5,000 U/mL; kindly provided by Chiron, Emeryville, CA) for 5 minutes. After incubation of the cells in hypotonic buffer A (10 mmol/L HEPS, pH 7.9, 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L DTT), the cells were lysed by 30 strokes in a glass dounce homogenizer in hypotonic buffer A with the addition of 50 mmol/L NaF, 1 mmol/L Na3VO4, 0.5 mmol/L phenylmethylsulphonyl fluoride, 1 μmol/L leupeptin, and 2 mmol/L aprotonin. The lysed cells were incubated on ice for 20 minutes. Nuclei and membrane debris were removed by centrifugation. The binding reactions contained 8 μg protein 1 ng of labeled DNA, and 1.5 μg sonicated salmon sperm carrier DNA. In some experiments, protein samples were preincubated with an excess of unlabeled probe or antibodies to either Stat1 or Stat3. The proteins were allowed to bind to the DNA for 15 minutes at room temperature before being loaded onto a nondenaturing 5% polyacrylamide gel containing 0.5× TBE (final concentration, 45 mmol/L Tris-borate, 1 mmol/L EDTA) and electrophoresed in 0.5× TBE for about 3 hours at 110 V. The gel was then dried and subjected to autoradiography.

RESULTS

Activation of JAK2 requires the membrane proximal region of the G-CSF-R cytoplasmic domain. Uptake of tritiated thymidine in response to G-CSF confirmed that the mitogenic response of the BAF/B03 cells expressing the truncated receptor mutants remained the same as previously reported. HGR-183 and HGR-96 cells proliferated in response to G-CSF. HGR-57 cells had a reduced proliferative
response and HGR-26 cells did not proliferate in response
to G-CSF (Fig 1). Immunoprecipitation of G-CSF-R pro-
teins and blotting with G-CSF-R antiserum shows the rela-
tive receptor expression on the four cell lines (Fig 2A). 125I-
labeled rhG-CSF binding to HGR-26, HGR-57, HGR-96, and HGR-183 gave specific binding of 7,908, 17,138, 8,967, and 1,623 cpm per 10^6 cells, respectively. The greater re-
ceptor expression in HGR-57 may have arisen as a compensa-
tory mechanism under G-CSF growth conditions. The lower
molecular weight band in Fig 2A is likely to be due to a
differently glycosylated form of the receptor' (Layton and
Nicholson, manuscript submitted).

The JAK2 response of the mutant cell lines was investi-
gated. G-CSF stimulation of BAF/BO3 cells expressing the
full-length G-CSF-R (HGR-183) resulted in the tyrosine
phosphorylation of JAK2 (Fig 2B). JAK2 tyrosine phosphor-
ylation was also observed with HGR-96 and HGR-57 cells
but not with HGR-26 cells. We were unable to detect tyrosine
phosphorylation of JAK1 in this cell system despite detection
of the JAK1 protein (data not shown).

The G-CSF-induced kinase activity of JAK2 was as-
se ssed using an in vitro autophosphorylation assay. JAK2
kinase activity was induced by G-CSF stimulation of HGR-
183, HGR-96, and HGR-57 cells but not of HGR-26 cells
(Fig 2C). Thus, the region of the cytoplasmic domain re-
quired for tyrosine phosphorylation of JAK2 correlated with
the region required for kinase activation.

**Maximal tyrosine phosphorylation of Stat3 requires amino
acids 96 to 183 of the G-CSF-R cytoplasmic domain.** To
determine whether Stat3 is tyrosine phosphorylated in re-
sponse to G-CSF, antiphosphotyrosine immunoprecipitates
were analyzed by immunoblotting with Stat3 antiserum (Fig
3). Stat3 tyrosine phosphorylation was only observed with
the full-length G-CSF-R. We were unable to detect tyrosine
phosphorylation of Stat1α (Stat91) in response to G-CSF,
despite detection of the Stat1α protein (data not shown).

**G-CSF-induced binding of Stat proteins to DNA requires
amino acids 57 to 183 of the G-CSF-R cytoplasmic domain.**
Phosphorylated STAT protein complexes are believed to mi-
grate to the nucleus and bind to specific DNA sequences,
thus leading to gene activation. One DNA sequence that
is recognized by STATs is the high-affinity SIF binding
sequence, m67. G-CSF induction of m67-binding STATs
was examined in electrophoretic mobility shift assays. A
CSF-1-stimulated macrophage cell line (BAC1.2F5) was
used as a control to indicate the relative positions of Stat3/
Stat1 DNA complexes on the gel. G-CSF stimulation of

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**Fig 2.** (A) Receptor expression in the transfected BAF/BO3 cell
lines. HGR-26, HGR-57, HGR-96, and HGR-183 were grown under
the conditions described in Materials and Methods and lysed. G-CSF-R
proteins were immunoprecipitated (IP) with the anti-G-CSF-R mono-
clonal antibody LMM741 and separated on 6% SDS-PAGE. Relative
levels of receptor expression were then analyzed by Western blot
with polyclonal rabbit serum to the G-CSF-R. (B) JAK2 tyrosine
phosphorylation in the G-CSF-R deletion mutants. HGR-26, HGR-
57, HGR-96, and HGR-183 were serum and growth factor starved before
 incubation with rhG-CSF (+) or medium (−) for 10 minutes (B and C).
Cells were lysed and phosphorylated proteins were immunoprecipita-
ted with antiphosphotyrosine antibody 4G10 (PY). Immunoprecipi-
tated proteins were separated on 6% SDS-PAGE and analyzed by
Western blot with anti-JAK2 serum. (C) JAK2 kinase activity in the
G-CSF-R deletion mutants. Cells lysates were not precleared. JAK2
proteins were immunoprecipitated from fresh lysates with anti-JAK2
serum. Immunoprecipitates were then subjected to an in vitro kinase
assay. The mobilities of the prestained molecular weight markers are
shown on the left of each panel. The figure is representative of three
experiments.
HGR-183 and HGR-96 cells induced STAT proteins that bound to m67 (Fig 4). The interactions were specifically competed by an excess of unlabeled m67 probe. The addition of Stat3 antiserum resulted in a new slower-migrating band, indicating that Stat3 was the major component of the DNA complex. The addition of Stat1 antiserum also resulted in a new slower-migrating band on the gel but had no effect on the major Stat3 band. Faint bands corresponding to the control Stat1:Stat3 heterodimer and Stat1 homodimer bands were visible on the original film. However, the band intensity did not appear to fully account for the Stat1 antibody shift, leaving the option of a third unidentified STAT in the DNA complexes. The DNA binding observed with HGR-96 lysates was reduced in comparison to lysates from cells expressing the full-length receptor (Fig 4). Induction of STAT binding to the SIF binding sequence was not observed with HGR-57 and HGR-26. Cells were starved and stimulated under the same conditions as were used for the experiments in Fig 3.

MAP kinase activation correlates with JAK activation and proliferation in BAF/BO3 cells. G-CSF induction of p42, p44 MAPK tyrosine phosphorylation was examined to investigate the relationship between the JAK/STAT pathway and the MAP kinase response. Tyrosine phosphorylation of p42, p44 MAPK was clearly induced with G-CSF stimulation of HGR-57 and HGR-96 and was maximal with HGR-183 (Fig 5). The apparent weak induction of MAP kinase tyrosine phosphorylation with mutant HGR-26 was observed in two of three experiments.

DISCUSSION

The results clearly show that G-CSF-induced tyrosine phosphorylation and kinase activation of JAK2 require only the first 57 amino acids of the G-CSF-R cytoplasmic domain that encompass the Box 1 and Box 2 homology regions. Constitutive association of the JAK kinases has been observed with many of the hematopoietin receptors, including the association of JAK1 with the G-CSF-R. The exceptions are the erythropoietin and growth hormone receptors, in which association appears to be induced by ligand bind-

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**Fig 4.** Induction of Stat3 DNA binding in the G-CSF-R deletion mutants. HGR-26, HGR-57, HGR-96, and HGR-183 were serum and growth factor starved for 5.5 hours before incubation with rhG-CSF (+) or medium (-) for 10 minutes. Cells were then subjected to an electrophoretic mobility shift assay. Aliquots of total protein extracts were incubated with the radiolabeled m67 DNA and unlabeled m67 probe, Stat3, and Stat1 antibodies as indicated. The complexes were then subjected to electrophoresis and autoradiography. The murine macrophage cell line BAC1.2F5 (C) was incubated with rhCSF-1 for 5 minutes. The position of the three DNA binding complexes are indicated by arrows. The upper band corresponds to a Stat3 homodimer, the middle band to a Stat3:Stat1 heterodimer, and the lower band to a Stat1 homodimer. The experiment was repeated twice with the same results.
G-CSF: JAK2 activation. Deletion analysis of gp130 has shown that the Box 1 and Box 2 regions are required for mitogenesis and JAK2 activation. Furthermore, the Box 1 region resulted in the G-CSF-R and that this association occurs within the first 57 amino acids of the G-CSF-R cytoplasmic domain. Whether the association is direct or via an adaptor molecule(s) is unclear at present.

Although the 26 membrane proximal amino acids containing Box 1 were not sufficient for JAK2 activation, there is evidence from related receptors that Box 1 is required for JAK2 activation. Deletion analysis of gp130 has shown that IL-6 activation and tyrosine phosphorylation of JAK2 requires both the Box 1 and Box 2 regions. Furthermore, mutation of two of the conserved proline residues in the gp130 Box 1 region resulted in loss of both the proliferative response and JAK2 kinase activation. Similarly, three prolines in the IL-5 receptor α chain Box 1 region were required for mitogenesis and JAK2 activation in response to IL-5. It is interesting to note that mutation of the Box 1 prolines in the murine G-CSF-R cytoplasmic domain not only results in loss of the proliferative response but also in the loss of myeloperoxidase gene transcription mediated by the carboxyl-terminal region. Hypothetically, mutation of the prolines may prevent JAK2 association and/or activation and hence gene transcription mediated by JAK2 tyrosine phosphorylation of both the receptor and subsequently associated molecules such as Stat3. The conserved proline residues may also be structurally required to bring the individual cytoplasmic domains and associated JAK kinases into close proximity on receptor dimerization.

High levels of Stat3 tyrosine phosphorylation detectable by Western blot required amino acids 96 to 183 of the G-CSF-R cytoplasmic domain. It seems likely that the STAT molecules associate with phosphotyrosine residues in the cytokine receptors via Src homology 2 (SH2) domain. This region of the G-CSF-R cytoplasmic domain contains three of the four tyrosine residues. When the BAF/BO3 cells were starved for a shorter period of time (4.5 hours instead of 5.5 hours), some degree of induction of Stat3 tyrosine phosphorylation was observed with HGR-57 and HGR-96 (data not shown). However, under these conditions, maximal tyrosine phosphorylation of Stat3 still required the full-length receptor.

Induction of Stat3 binding to the m67 probe was observed with both the HGR-96 receptor and the full-length receptor. This is particularly interesting with respect to a recent report defining a specific Stat3 binding motif (YXXQ). There is one YXXQ motif in the G-CSF-R that is present in the HGR-96 receptor (Y704). The Stat3 DNA binding activity observed with HGR-96 supports the observation that this tyrosine motif is able to mediate Stat3 activation. However, compared with the full-length G-CSF-R, Stat3 DNA binding activity in HGR-96 was reduced. Because maximal tyrosine phosphorylation of Stat3 was also observed with the full-length receptor, it seems likely that one or more of the additional three tyrosine residues in the full-length receptor can also bind Stat3.

The discrepancy between the relative lack of Stat3 tyrosine phosphorylation and the induction of Stat3 DNA binding with HGR-96 reflects the greater sensitivity of the gel-shift assays. This is further evident from the apparent lack of Stat1 tyrosine phosphorylation detectable by Western blot despite detection of Stat1 in the DNA complexes.

Although the primary G-CSF STAT response is activation of Stat3, the partial mobility shift observed with Stat1 antisemur is consistent with the observations of Tian et al, who identified two DNA-binding complexes, the major complex being a Stat3 homodimer and the minor complex being a Stat3:Stat1 heterodimer. After tyrosine phosphorylation, Stat3 may be induced to dissociate from the receptor complex through an increased affinity for the SH2 domains of either Stat3 or Stat1.

Because the membrane proximal 57 amino acids of the G-CSF-R are sufficient for G-CSF–induced proliferation, activation of Stat3 is probably not essential for proliferation in this cell line (Fig 6). However, although HGR-57 proliferates in response to G-CSF, it does so more slowly than HGR-96. JAK2 may therefore contribute to proliferation in HGR-
Fig 6. Proposed model for JAK2 signaling from the G-CSF-R. JAK2 activation in response to G-CSF requires the first 57 amino acids of the cytoplasmic domain. Stat3 is hypothesized to be downstream in the JAK kinase pathway and requires the carboxy-terminal region of the receptor for its activation, possibly due to its interaction with one or more of the tyrosine residues. This suggests that the JAK2/Stat3 pathway is not required for the G-CSF mitogenic response. The Box 1, 2, and 3 homology regions are indicated by shaded boxes.

96 cells by phosphorylating the receptor on tyrosine 704, enabling the recruitment of SH2-containing signaling molecules (Stat3 and possibly others). The recent finding that the proto-oncogene product p95EBrv associates with JAK2 after granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation of cells suggests that JAK2 may feed into pathways other than the JAK/STAT cascade. Tyrosine phosphorylation of MAP kinase is absolutely required for its activation. It is clear from data presented in Fig 5 that tyrosine phosphorylation of p42, p44, requires the first 57 amino acids of the cytoplasmic domain. The intensity of the response appears to increase with receptor length, particularly when the relative receptor level is taken into account. This is in contrast with levels of JAK2 activation that did not increase with increasing receptor length in the different cell lines, suggesting that amino acids 57 to 183 did not contribute to JAK2 activation. The variation in MAP kinase response between the full-length receptor and the truncated mutants indicates that several pathways may be initiated at the receptor level that then converge in the activation of MAP kinase. These data also support the proposition that MAP kinase is involved in cell proliferation as the membrane proximal 57 amino acids are the minimum length of cytoplasmic domain that can be used to deliver a mitogenic signal. We have observed that G-CSF cannot induce proliferation in the transfected BAF/BO3 cells in the absence of serum. This indicates that at least two signals are required for proliferation in these cells.

It is unclear whether JAK2 contributes to MAP kinase activation. The observation that G-CSF activates both JAK2 and MAP kinase in mutant HGR-57 is consistent with this possibility. In contrast, JAK2 activation and mitogenesis in response to GM-CSF requires the membrane proximal 62 amino acids of the GM-CSF receptor β chain but Shc, Ras, and MAP kinase activation require a distal region of the receptor. A recent report showed that JAK2 is critical for erythropoietin-induced mitogenesis in DAER cells. However, the effect on the proliferative response to IL-3 in the same cell line was not nearly as profound. This suggests that JAK2 can deliver a mitogenic signal but that its contribution to proliferation will vary between the different cytokine responses.

ACKNOWLEDGMENT

We thank L. Paradisi for expert assistance with the mobility shift assays, J. Stickland for figure production, Amgen for rhG-CSF, and Immunex for providing the BAF/BO3 cells containing the G-CSF-R mutants. We thank F. Walker and A.F. Wilks for reviewing the manuscript. We also thank D. Bowtell for helpful discussions.

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