Granulocyte-Macrophage Colony-stimulating Factor Stimulates JAK2 Signaling Pathway and Rapidly Activates p93\textsuperscript{fes}, STAT1 p91, and STAT3 p92 in Polymorphonuclear Leukocytes*

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Granulocyte-macrophage colony-stimulating factor (GM-CSF)\textsuperscript{1} supports proliferation, differentiation, and functional activation of hematopoietic cells by its interaction with a heterodimeric receptor. Although GM-CSF receptor is devoid of tyrosine kinase enzymatic activity, GM-CSF-induced peripheral blood polymorphonuclear leukocytes (PMN) functional activation is mediated by the phosphorylation of a large number of intracellular signaling molecules. We have previously shown that JAK2 becomes tyrosine-phosphorylated in response to GM-CSF in PMN. In the present study we demonstrate that also the signal transducers and activators of transcription (STAT) family members STAT1 p91 and STAT3 p92 and the product of the c-fps/fes protooncogene become tyrosine-phosphorylated upon GM-CSF stimulation and physically associated with both GM-CSF receptor β common subunit and JAK2. Moreover GM-CSF was able to induce JAK2 and p93\textsuperscript{fes} catalytic activity. We also demonstrate that the association of the GM-CSF receptor β common subunit with JAK2 is ligand-dependent.

Finally we demonstrate that GM-CSF induces a DNA-binding complex that contains both p91 and p92. These results identify a new signal transduction pathway activated by GM-CSF and provide a mechanism for rapid activation of gene expression in GM-CSF-stimulated PMN.

Granulocyte-macrophage colony-stimulating factor (GM-CSF)\textsuperscript{1} regulates proliferation and differentiation of hematopoietic progenitor cells and functionally activates polymorphonuclear leukocytes (PMN) (1). In particular, GM-CSF exerts several direct actions on neutrophils, including stimulation of changes in surface expression of both chemotactic receptors and adherence proteins (2–5), as well as hydrogen peroxide production by neutrophils adhered to extracellular matrix components (6, 7). Moreover GM-CSF has indirect effects on neutrophils, such as "priming" these cells for enhanced responses to a number of physiologically relevant stimuli such as ingestion of Staphylococcus aureus (8), serum-opsonized particles (3), antibody-dependent cytotoxicity (3), FMLP-stimulated intracellular calcium mobilization (9), and oxidoregical (2, 10) and platelet-activating factor (11) production as well as leukotriene synthesis (12–14). More recently it has been reported that GM-CSF inhibits programmed cell death both in human eosinophils and neutrophils (15) and that this effect is mediated by tyrosine phosphorylation of intracellular substrates (15).

All GM-CSF effects are mediated by a heterodimeric receptor comprised of a ligand binding subunit, denoted α (16), and of a transducing subunit designated as β (17), which is also shared with interleukin-3 (IL-3) (17) and IL-5 receptor (18). Although GM-CSF receptor does not possess an intrinsic tyrosine kinase domain, several lines of evidence indicate that signaling processes initiated by ligand binding to the receptor induce activation of cellular tyrosine kinases (19). Studies on the biochemical interaction involved in signaling from the GM-CSF receptor have demonstrated that a number of transducing molecules such as Shc (20–22), Grb2 (20), Src1 (20), Ras (23), Raf-1 (24), and mitogen-activated protein kinase (25) become activated upon GM-CSF stimulation. It has also been reported that a nonreceptor tyrosine kinase, the c-fps/fes protooncogene product, is phosphorylated in response to GM-CSF (26). More recently the receptor-associated protein JAK2 (27, 28) has been reported to be rapidly phosphorylated upon GM-CSF receptor activation (29, 30). Recent data suggest that at least two components of latent cytoplasmic proteins termed signal transducers and activators of transcription (STATs) (31), which become activated upon ligand binding, are substrates of JAK family members (32–34). In order to characterize the tyrosine-phosphorylated proteins involved in GM-CSF-mediated PMN activation, we examined the role of two STAT proteins, STAT1 α (p91) and STAT3 (p92) and of c-fps/fes protooncogene product (p93\textsuperscript{fes}) in this process. We demonstrate that, upon GM-CSF stimulation, both STAT proteins and p93\textsuperscript{fes} become tyrosine-phosphorylated and physically associate with GM-CSF receptor β common subunit as well as with JAK2. GM-CSF stimulation was also able to induce p93\textsuperscript{fes} and JAK2 catalytic activity. Moreover we demonstrate that, as previously reported for erythropoietin receptor (35), JAK2 association with the β common subunit is ligand-dependent. Finally we demonstrate that the DNA-binding proteins p91 and p92 are early targets of the GM-CSF-induced DNA-binding complex. These results identify a signal transduction pathway that is activated in response to GM-CSF in human PMN and provide evidence for...
the role of STAT proteins in GM-CSF-mediated rapid modulation of gene expression in functionally activated nonpolarizing cells.

**EXPERIMENTAL PROCEDURES**

Reagents—Cells were cultured in RPMI 1640 medium (Life Technologies, Inc.). Bovine calf serum was from Hyclone Laboratories (Logan, UT). Gelatin was from Difco. rhGM-CSFs were kindly provided by Sandoz Forschungsinstitut, Austria. rhL-3 and rhL-6 were a gift from Genetic Institute (Cambridge, MA). The Sepharose-protein A was purchased from Sigma. Nitrocellulose filters, horseradish peroxidase-conjugated sheep anti-rabbit antibody, molecular weight markers, [α-32P]ATP, and the chemiluminescence reagent (ECL) were from Amersham Corp. Poly(dI-dC):poly(dI-dC) was obtained from Pharmacia (Uppsala, Sweden).

Antisera—Polyclonal anti-IL-3/GM-CSF receptor β common subunit antisera was obtained as described previously (20, 30). Polyclonal anti-c-fps/ fes antibody was prepared from serum of a rabbit immunized against a synthetic peptide, which was conjugated to keyhole limpet hemocyanin by glutaraldehyde. The peptide sequences for c-fps/ fes was LLLQQDRHTSSQEREGG (corresponding to amino acid residues 424–443) (26). The specificity of the antisera was demonstrated by the lack of the immunoprecipitation band in the presence of saturating concentrations of the related peptides (data not shown). Polyclonal antibodies to p91 were generated in rabbits that were injected with a synthetic peptide whose sequence corresponds to residues 84–96 of human transcription factor IRS-3 (GenBank™ accession number M97935): RSKKRNQDNNQFEDC. Peptide conjugation to a carrier protein, injection to rabbits and bleeding were as described previously (36). Anti-phosphotyrosine antibody 4G10, anti-JAK1, and anti-JAK2 antisera were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-STAT3 p92 monoclonal antibodies were obtained from Affinity Research Products Ltd. (London, United Kingdom) and from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany).

Cells—Human neutrophils (PMN) were isolated from venous blood of normal donors by gelatin sedimentation (2.5% gelatin in PBS, pH 7.2, for 20 min at 37°C) followed by Ficoll-Hypaque gradient separation. Contaminating erythrocytes were removed by hypotonic lysis, and the cells were resuspended RPMI 1640 medium at a final concentration of 1 × 10⁸ cells/ml. The percentage of neutrophils in cell preparation used in this study was 97% and cell viability as determined by trypan blue exclusion was 98%.

HEPG2 cells were maintained in RPMI 1640 medium supplemented with 10% bovine calf serum and serum-starved overnight before being treated with rhL-6 (30 ng/ml).

Western Blot Analysis and Immunoprecipitation Studies—Cells (4 × 10⁶) were incubated with or without GM-CSF (10 ng/ml) for 30 min, extracted with cold DIM buffer (50 mmol/liter Pipes, pH 6.8, 100 mmol/liter NaCl, 10 mmol/liter MgCl₂, 5 mmol/liter MnCl₂, 0.1 mmol/liter Na₃VO₄, 5 mmol/liter MnCl₂, 0.1 mmol/liter Na₃VO₄, 10 mmol/liter Hepes (pH 7.4) containing [α-32P]ATP (0.25 μCi/ml) for 30 min at room temperature, washed, eluted with sample buffer for SDS-polyacrylamide gel electrophoresis, separated on 8% gel, and visualized by autoradiography; the other part was separated on 8% gel and probed with anti-JAK2 or anti-p93 antibodies.

Preparation of Nuclear Extract and Gel Retardation Assay—Nuclear extracts from untreated and GM-CSF-treated PMN and untreated and IL-6-treated HEPG2 cells were prepared by Nonidet P40 lysis as described by Sadowski and Gilman (37). The oligonucleotides used were GG CAT TTC CGG TAA ATC and G GG GAT TTA CGG GAA ATG (38). The annealed oligonucleotide was labeled by filling in the overhanging ends with Klenow fragment in the presence of [α-32P]ATP. Gel retardation reactions were performed in 13 mmol/liter Hepes, pH 7.6, 80 mmol/liter NaCl, 3 mmol/liter NaF, 3 mmol/liter NaMoO₄, 1 mmol/liter diithiothreitol, 0.15 mmol/liter EDTA, 0.15 mmol/liter EGTA, and 8% glycerol (including contribution from the nuclear extract) and contained 75 μg/ml poly(dI-dC):poly(dI-dC), approximately 0.3 ng of radiolabeled probe, and 5–10 μg of protein. Reactions were incubated at room temperature for 40 min and then resolved on 4% polyacrylamide gels containing 0.25 × TBE (1 × TBE is 89 mmol/liter Tris borate, 1 mmol/liter EDTA, pH 8) and 5% glycerol. Gels were run at 4°C in 0.25 × TBE at 20 V/cm, dried, and autoradiographed. Oligonucleotide competition was performed by preincubating nuclear extracts with the competitor oligonucleotide (50-fold excess) and poly(dI-dC):poly(dI-dC) for 30 min at room temperature before the addition of labeled probe. Gel mobility shift assays were done with nuclear extract that had been reacted for 1 h at 4°C with the indicated antibodies.

**RESULTS AND DISCUSSION**

GM-CSF plays an important role in host defense by enhancing the functional activities of mature leukocytes and, in particular, neutrophils (2–14). The binding of GM-CSF to its heterodimeric receptor, which is devoid of intrinsic kinase activity, leads to tyrosine phosphorylation of cellular substrates (19). It has been reported that, in growth factor-dependent cell lines, JAK2 is constitutively associated with the β subunit (29–30). Moreover, JAK2 has been shown to be phosphorylated upon growth factor stimulation not only in proliferating cells but also in PMN and eosinophils functionally activated by GM-CSF (30) and IL-5 (38), respectively. Moreover, a ligand-dependent association of JAK2 with erythropoietin receptor has also been reported (35). To further elucidate the interaction between the β subunit and JAK2 we performed co-immunoprecipitation experiments in unstimulated and GM-CSF-stimulated PMN. The results, reported in Fig. 1, demonstrate that in PMN p130 JAK2 physically associates with the β subunit only upon GM-CSF stimulation, suggesting that, under physiological conditions, the association between JAK2 and the receptor may not be constitutive. Kinetic analysis of JAK2 activation, upon GM-CSF stimulation, reported in Fig. 2, demonstrates a transient JAK2 tyrosine phosphorylation peaking at 5 min and disappearing after 10 min.

A likely set of substrates for the JAKs is the family of latent
cytoplasmic transcription factors termed STATs (31). Ligand binding to several cytokine receptors induces tyrosine phosphorylation of STAT family members that, subsequently, translocate to the nucleus, bind to related DNA sequences, and promote transcription (39–52). The anti-phosphotyrosine blot of anti-JAK2 immunoprecipitates from GM-CSF-treated PMN revealed, together with the phosphorylated p130 JAK2, a marked band of phosphotyrosine-containing protein(s) of approximately 90 kDa (Fig. 2). It is known that among the STAT proteins STAT1 and STAT3 exhibit a molecular mass of 91 and 92 kDa, respectively. Therefore we sought to determine whether the JAK2 co-precipitating p90 phosphoprotein(s) included one or both STATs. It has also been demonstrated that a p93 myeloid-specific protein, the product of the protooncogene c-fps/fes, becomes phosphorylated and associated to the GM-CSF receptor β common subunit upon GM-CSF stimulation in a growth factor-dependent cell line (26). Therefore, we tried to assess whether p93fes was also included in JAK2 co-precipitating p90 phosphoprotein(s). To test these possibilities we first evaluated tyrosine phosphorylation of p91, p92, and p93fes upon GM-CSF stimulation. As shown in Fig. 3, both STAT proteins, p91 and p92, and p93fes become phosphorylated after 5 min of GM-CSF treatment. In addition, in the anti-p91 and anti-p92 immunoprecipitates together with the marked band of approximately 90 kDa a faint band of approximately 130 kDa was detected. Taken together these results strongly suggest that at least the two STAT proteins physically associated with JAK2. To confirm this hypothesis, anti-JAK2 co-immunoprecipitates from unstimulated and GM-CSF-stimulated PMN were divided into four aliquots, resolved by SDS-polyacrylamide gel electrophoresis, and independently blotted with the antibodies of interest. In the anti-phosphotyrosine immunoblot reported in Fig. 4A a large band of approximately 90 kDa can be detected only in GM-CSF-stimulated PMN. The anti-p91,
immunoprecipitated; p130-JAK2 and the p90 phosphotyrosine proteins are indicated. CSF receptor stimulated (1) PMN were lysed and immunoprecipitated with anti-GM-CSF receptor β common subunit antiserum. The immunoprecipitates from unstimulated and GM-CSF-stimulated PMN, shown in Fig. 6A, demonstrates the presence of approximately 90-kDa tyrosine-phosphorylated protein(s) only in GM-CSF-stimulated cells. Moreover when aliquots of the same samples were resolved by SDS-polyacrylamide gel electrophoresis and independently blotted with the anti-p91 (Fig. 6B), anti-p92 (Fig. 6C), and anti-p93β (Fig. 6D) antibodies, the two STAT proteins together with the p93β were found to be physically associated, upon ligand binding, with the β common subunit. Tyrosine phosphorylation of various tyrosine kinases is commonly associated with the activation of their catalytic activity (53). An in vitro kinase assay was performed to examine whether phosphorylation of JAK2 and p93β correlates with their intrinsic kinase activity. As shown in Fig. 7, anti-JAK2 (panel A) and anti-p93β (panel B) immunoprecipitates from GM-CSF-stimulated, but not from IL-3-stimulated, PMN have a detectable activity of anti-JAK1 antiserum to co-immunoprecipitate these proteins (data not shown). Moreover the correlation between the biochemical events induced by GM-CSF stimulation and its biological effects on PMN was supported by the observation that, upon IL-3 stimulation, neither functional activation (data not shown) or protein tyrosine phosphorylation were detected in anti-JAK1 immunoprecipitates (Fig. 5). The observation that JAK2 physically associates with the β common subunit as well as with p91 and p92 STATs and p93β implies that the latter three proteins are also directly or indirectly, via JAK2, associated with the β common. To evaluate this hypothesis anti-beta co-immunoprecipitation experiments were performed. The anti-phosphotyrosine immunoblot of anti-β common immunoprecipitates from unstimulated and GM-CSF-stimulated PMN, shown in Fig. 6A, demonstrates the presence of approximately 90-kDa tyrosine-phosphorylated protein(s) only in GM-CSF-stimulated cells. Moreover when aliquots of the same samples were resolved by SDS-polyacrylamide gel electrophoresis and independently blotted with the anti-p91 (Fig. 6B), anti-p92 (Fig. 6C), and anti-p93β (Fig. 6D) antibodies, the two STAT proteins together with the p93β were found to be physically associated, upon ligand binding, with the β common subunit.

PMN are terminally differentiated cells and do not undergo proliferation; however, tyrosine phosphorylation of intracellular substrates has been implicated in a number of functional activities such as superoxide anion production (54–58); regulation of integrin surface expression, leading to adherence of PMN to endothelial cells (59); regulation of microvascular permeability, leading to migration of PMN into inflammatory tissue (60, 61); and modulation of apoptotic process (15). The role of protein tyrosine phosphorylation in physiological agonist-mediated or GM-CSF-mediated PMN activation is further supported by the observation that PMN biological responses are prevented by the addition of tyrosine kinase inhibitors (15, 55, 56).
56, 59, 62). It has been shown that, in PMN, GM-CSF causes a rapid tyrosine phosphorylation of intracellular molecules including both 90- and 130-kDa proteins (15, 55, 62). In agreement with these findings, our present study demonstrates that a set of 90-kDa proteins namely STAT1, STAT3, and p93<sup>−</sup> and a 130-kDa protein, identified as JAK2, become phosphorylated upon GM-CSF stimulation. Therefore, it is reasonable to assume that at least some PMN functional activities may be regulated by the JAK/STAT signaling pathway.

It has been reported that treatment of cells with different cytokines results in rapid STAT protein phosphorylation. Activated STATs (one or more) form dimers that migrate in the nucleus and form stable complexes with specific DNA sequences (response elements) and stimulate transcription (31). Three discrete complexes between activated STAT proteins and DNA response elements have been demonstrated upon EGF treatment (50). These complexes seem to be formed by STAT1 or STAT3 homodimers or by heterodimers between the two STATs (50). In contrast, in interferon-γ (44), IL-6 (50), and GM-CSF-treated cells (51), only one complex can be detected containing either the STAT1 (45) or the STAT3 homodimers (51). The rapid tyrosine phosphorylation of p91 and p92 observed in GM-CSF-stimulated PMN led us to evaluate, by gel retardation assay, the formation of DNA-protein complexes in nuclear extract of untreated and treated cells. As shown in Fig. 8A, both in IL-6-treated HEPG2 cells and in GM-CSF-treated PMN a DNA-protein complex appears. Moreover, when the same nuclear extracts were incubated with an excess of unlabeled oligonucleotide, both the IL-6- and GM-CSF-induced complexes are competed (Fig. 8A) demonstrating its sequence specificity. Moreover it is also clear that the DNA-binding complex observed in IL-6-stimulated HEPG2 cells shows a slower migration than that observed in GM-CSF-stimulated PMN. It has been reported that in HEPG2 cells IL-6 induces only the formation of a major complex, designated also as SIF-A (40, 45), corresponding to the complex containing p92 homodimers (50). In contrast, EGF-activated proteins have been shown to form three complexes, designated as SIF-A, SIF-B, and SIF-C (50), with the serum-inducible element of c-fos (in its mutated, hyperactive form) (40, 45). Therefore, it is possible that the faster migrating complex observed in GM-CSF-stimulated PMN contains either STAT1-STAT3 heterodimers and/or STAT1 homodimers. We thus tested the GM-CSF-induced complex for reactivity with anti-STAT1 p91 and anti-STAT3 p92 antibodies. As shown in Fig. 8B, when anti-p91 and anti-p92 antisera were added to GM-CSF-treated nuclear extract, a new band, which was not present in the binding reaction with preimmune serum, appeared in the upper part of the gel, thus demonstrating the formation of a supershifted species. A supershifted species also appears when nuclear extract from IL-6-stimulated HEPG2 cells was preincubated with anti-p92 antisera (Fig. 8B). The presence of the supershifted complex observed both in anti-p91 and anti-p92-pretreated nuclear extract suggests that, in PMN, GM-CSF can rapidly modulate gene expression by the induction of a DNA-binding complex containing p91 and p92 heterodimer.

It has been shown that tyrosine-phosphorylated proteins are involved in GM-CSF-mediated PMN functional activation and c-fos gene transcription (55). Moreover the role of tyrosine kinases in controlling GM-CSF-induced c-fos gene expression in PMN, has been demonstrated by the use of a tyrosine kinase inhibitor (55). Our finding that at least two of the STAT proteins that become phosphorylated upon GM-CSF stimulation are involved in the formation of a complex with the serum-inducible elements of c-fos, supports the hypothesis that GM-CSF can regulate the transcription of this gene via STAT1 and STAT3 activation.

In conclusion, our study demonstrates that in PMN both STAT1 p91 and STAT3 p92 and the myeloid-specific p93<sup>−</sup> become phosphorylated upon GM-CSF stimulation and are co-immunoprecipitated by anti-j-STAT1 and anti-β common subunit antibodies, and that GM-CSF induces the formation of a DNA-protein complex containing both p91 and p92.

The redundancy of growth factors inducing the same DNA-responsive element to stimulate both cell proliferation and functional activation raises the question of how their specificity can be determined. The answer could be obtained by the identification of more genes whose transcription can be activated by the binding of known or unknown proteins.

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