THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 7, Issue of February 16, pp. 3562–3567, 1996

Printed in U.S.A.

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

(Received for publication, August 18, 1995, and in revised form, November 8, 1995)

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Granulocyte-macrophage colony-stimulating factor (GM-CSF), supports proliferation, differentiation, and functional activation of hemopoietic 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^{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)[1] regulates proliferation and differentiation of hemopoietic 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), fMet-Leu-Phe (FMLP)-stimulated intracellular calcium mobilization (9), and oxyradical (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), Sos1 (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^{fes}) in this process. We demonstrate that, upon GM-CSF stimulation, both STAT proteins and p93^{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^{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 costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This work was supported by Associazione Italiana Ricerca sul Cancro (AIRC), by CNR, Progetto Finalizzato A.C.R.O., and by ISS VII Progetto AIDS.

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[1] The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; rh, recombinant human; IL, interleukin; STAT, signal transducers and activators of transcription; PMN, polymorphonuclear neutrophils; Pipes, 1,4-piperazinediethanesulfonic acid.

the role of STAT proteins in GM-CSF-mediated rapid modulation of gene expression in functionally activated nonproliferating 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. rhIL-3 and rhIL-6 were a gift from Genetic Institute (Cambridge, MA). The Sepharose-protein A was purchased from Sigma. Nitrocellulose filters, horseradish peroxidase-conjugated protein A, molecular weight markers, [α-^{32}P]dCTP, [γ-^{32}P]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 antiserum was obtained as described previously (20, 30). Polyclonal anti-c-*fps/fes* antibody was prepared from serum of a rabbit immunized against a synthetic peptides, which was conjugated to keyhole limpet hemocyanin by gluteraldehyde. The peptide sequences for c-*fps/fes* was LLLQDDRHSTSSSEQEREGG (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 ISGR-3 (GenBank™ accession number M97935): RKSKRNLQDNFQEDC. 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. (Nottingham, 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 30 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 1×10^7 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 rhIL-6 (30 ng/ml).

Western Blot Analysis and Immunoprecipitation Studies—Cells (4×10^7) were incubated with or without GM-CSF (10 ng/ml) at 37 °C for 5 min, extracted with cold DIM buffer (50 mmol/liter Pipes, pH 6.8, 100 mmol/liter NaCl, 5 mM $MgCl_2$, 300 mmol/liter sucrose, 5 mmol/liter EGTA, 2 mmol/liter sodium orthovanadate) plus 1% Triton X-100 and a mixture of protease inhibitors (1 mmol/liter phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.15 units/ml aprotinin, 1 μg/ml pepstatin A) for 20 min at 4 °C, and centrifuged at 15,000 $\times$ *g* for 20 min. The clarified supernatant was precleaned for 1 h with 50 μl of Sepharose protein A (3 mg/sample). The precleared cell lysates were then adsorbed by antisera coupled to protein A-Sepharose. Bound proteins were washed several times in DIM buffer and eluted in boiling Laemmli buffer. Eluted proteins were subjected to 8% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred electrophoretically to nitrocellulose; the filters were incubated with blocking solution (10% low fat milk in 20 mmol/liter Tris-HCl, pH 7.6, and 17 mmol/liter NaCl) for 1 h. Antisera were then added at the same solution, and the incubation was carried out for 2 h at room temperature. For detection, the filters were washed three times (10 min each wash) with phosphate-buffered saline, 0.05% Tween 20 and reacted for 1 h at room temperature with horseradish peroxidase-conjugated protein A. The enzyme was removed by washing as above. The filters were reacted for 1 min with a chemiluminescence reagent (ECL) and exposed to an autoradiography film for 1–15 min. To reprobe, nitrocellulose filters were first stripped of antibody by 62 mmol/liter Tris-HCl, pH 6.7, 2% SDS, 100 mmol/liter B^2-mercaptoethanol.

In Vitro Kinase Assay—Anti-JAK2 and anti-p93^{fes} immunoprecipitates were washed and divided into two equal parts. One part was resuspended in an equal volume of kinase assay buffer (50 mmol/liter NaCl, 5 mmol/liter $MgCl_2$, 5 mmol/liter $MnCl_2$, 0.1 mmol/liter Na_3VO_4, 10 mmol/liter Hepes (pH 7.4) containing [γ-^{32}P]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^{fes} antisera.

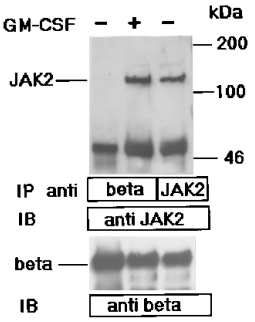

FIG. 1. **JAK2 association with the GM-CSF receptor β common subunit in PMN.** Cell lysates from unstimulated (−) or GM-CSF-stimulated (10 ng/ml for 5 min) (+) PMN were immunoprecipitated with anti-β antiserum. The cells were also immunoprecipitated with anti-JAK2 antiserum to indicate the p130 JAK2 protein. The filter was probed with the anti-JAK2 antiserum (*upper panel*) and reprobed with the anti-β antiserum (*lower panel*). The p130 JAK2 is indicated. *IP*, immunoprecipitated; *IB*, immunoblotted.

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 G GGG CAT TTC CCG TAA ATC and G GGG 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-^{32}P]dCTP. Gel retardation reactions were performed in 13 mmol/liter Hepes, pH 7.6, 80 mmol/liter NaCl, 3 mmol/liter NaF, 3 mmol/liter $NaMo0_4$ 1 mmol/liter dithiothreitol, 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 $\times$ TBE (1 $\times$ 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 $\times$ 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

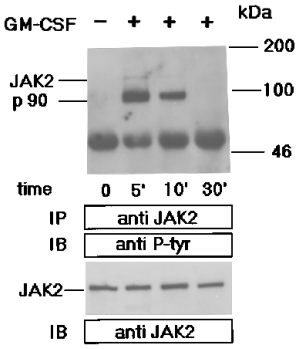

FIG. 2. **Kinetic analysis of GM-CSF-induced JAK2 activation in human PMN.** PMN were incubated in 1640 RPMI medium in the absence or in the presence of GM-CSF (10 ng/ml) for the indicated time, lysed, and immunoprecipitated with the anti-JAK2 antiserum. The filter was probed with 4G10 anti-phosphotyrosine monoclonal antibody (*upper panel*) and reprobed with the antiserum against JAK2 (*lower panel*). The positions of the p130-JAK2 and the p90 phosphotyrosine proteins are indicated. *IP*, immunoprecipitated; *IB*, immunoblotted; *P-tyr*, phosphotyrosine.

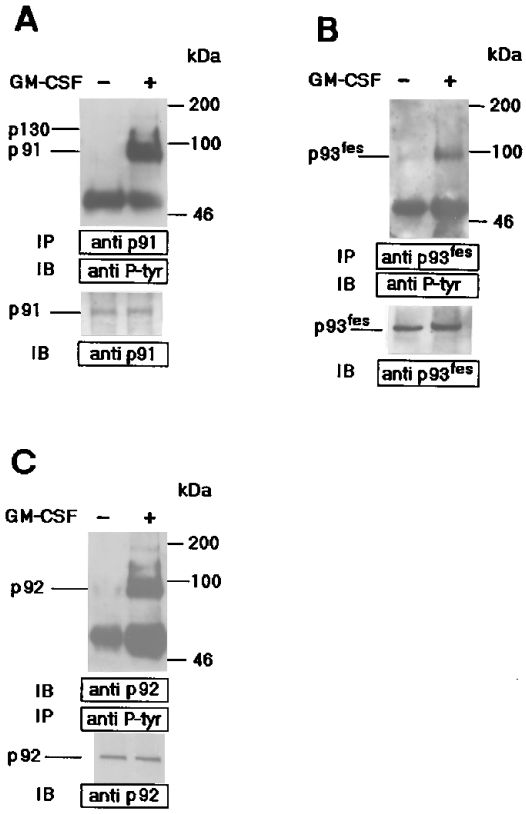

FIG. 3. **GM-CSF-induced tyrosine phosphorylation of STAT-p91, STATp92, and p93^{fes} in PMN.** Untreated (−) and GM-CSF-treated (for 5 min with 10 ng/ml of GM-CSF) (+) PMN were immunoprecipitated with anti-p91 (*A*) anti-p93^{fes} (*B*), and anti-p92 (*C*) antibodies. The filters were probed with 4G10 anti-phosphotyrosine monoclonal antibody (*upper panels*) and reprobed with anti-p91 (*A*), anti-p93^{fes} (*B*), and anti-p91 (*C*) antibodies (*lower panels*). The position of p91, p93^{fes}, and p92 are indicated. *IP*, immunoprecipitated; *IB*, immunoblotted; *P-tyr*, phosphotyrosine.

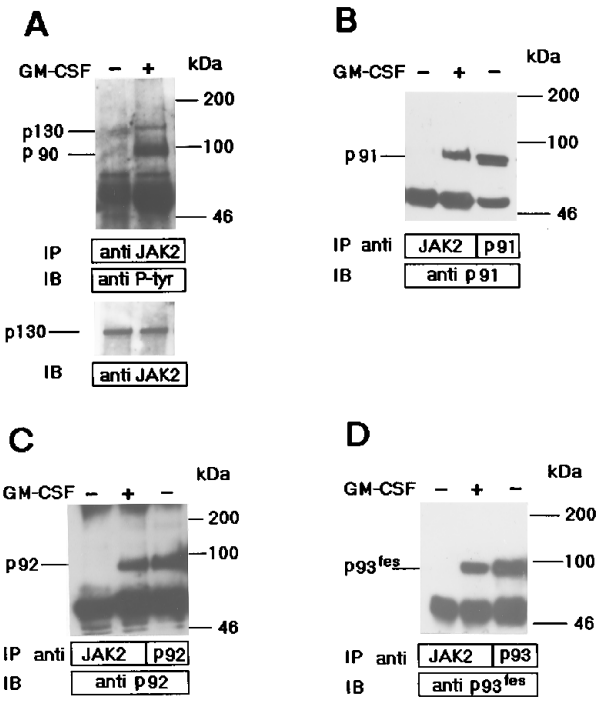

FIG. 4. **p91, p92, and p93^{fes} are physically associated with JAK2.** Unstimulated (−) and GM-CSF-stimulated (+) PMN were lysed and immunoprecipitated with anti-JAK2 antiserum. The immunoprecipitates were divided into four aliquots that were resolved in SDS-polyacrylamide gel and blotted independently. *A*, the filter was probed with 4G10 anti-phosphotyrosine monoclonal antibody (*upper panel*) and reprobed with anti-JAK2 antiserum (*lower panel*). *B*, the filter was probed with anti-p91 antiserum. The cells were also immunoprecipitated with anti-p91 antiserum to indicate the migration of the p91 protein. *C*, the filter was probed with anti-p92 antibody. The cells were also immunoprecipitated with anti-p92 antiserum to indicate the migration of the p92 protein. *D*, the filter was probed with anti-p93^{fes} antiserum. The cells were also immunoprecipitated with anti-p93^{fes} antiserum to indicate the migration of the p93^{fes} protein. The position of p91, p92, and p93^{fes} are indicated. *IP*, immunoprecipitated; *IB*, immunoblotted; *P-tyr*, phosphotyrosine.

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 p93^{fes} was also included in JAK2 co-precipitating p90 phosphoprotein(s). To test these possibilities we first evaluated tyrosine phosphorylation of p91, p92, and p93^{fes} upon GM-CSF stimulation. As shown in Fig. 3, both STAT proteins, p91 and p92, and p93^{fes} become phosphorylated after 5 min of GM-CSF treatment. In addition, in the anti-p91 and -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. 4*A* a large band of approximately 90 kDa can be detected only in GM-CSF-stimulated PMN. The anti-p91,

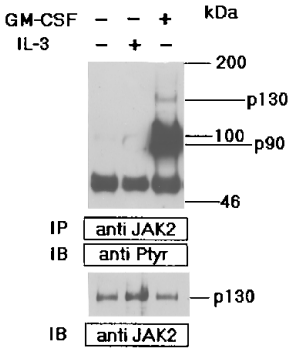

FIG. 5. **GM-CSF-induced JAK2 tyrosine phosphorylation in human PMN.** Cell lysates from unstimulated (−) and IL-3- and GM-CSF-stimulated (10 ng/ml each for 5 min) (+) PMN were immunoprecipitated with anti-JAK2 antiserum. The filter was probed with 4G10 anti-phosphotyrosine monoclonal antibody (*upper panel*) and reprobed with the antiserum against JAK2 (*lower panel*). The positions of the p130-JAK2 and the p90 phosphotyrosine proteins are indicated. *IP*, immunoprecipitated; *IB*, immunoblotted; *P-tyr*, phosphotyrosine.

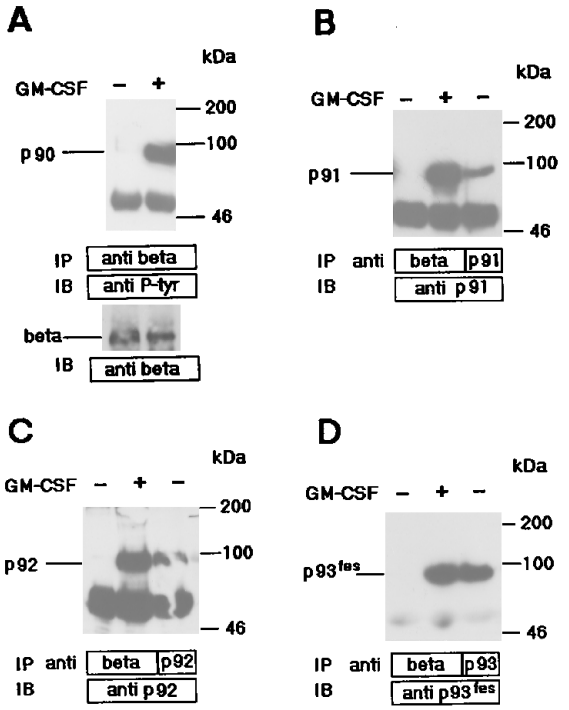

FIG. 6. **p91, p92, and p93^{fes} physically associate with the GM-CSF receptor β common subunit.** Unstimulated (−) and GM-CSF-stimulated (+) PMN were lysed and immunoprecipitated with anti-GM-CSF receptor β common subunit antiserum. The immunoprecipitates were divided into four aliquots that were resolved in SDS-polyacrylamide gel and blotted independently. *A*, the filter was probed with 4G10 anti-phosphotyrosine monoclonal antibody (*upper panel*) and reprobed with anti-GM-CSF receptor β common subunit antiserum (*lower panel*). B, the filter was probed with anti-p91 antiserum. The cells were also immunoprecipitated with anti-p91 antiserum to indicate the migration of the p91 protein. *C*, the filter was probed with anti-p92 antibody. The cells were also immunoprecipitated with anti-p92 antiserum to indicate the migration of the p92 protein. *D*, the filter was probed with anti-p93^{fes} antiserum. The cells were also immunoprecipitated with anti-p93^{fes} antiserum to indicate the migration of the p93^{fes} protein. The positions of p91, p92, and p93^{fes} are indicated. *IP*, immunoprecipitated; *IB*, immunoblotted; *P-tyr*, phosphotyrosine.

anti-p92, and anti-p93^{fes} immunoblots of anti-JAK2 immunoprecipitates from untreated and GM-CSF-treated PMN are shown in Fig. 4, *B*, *C*, and *D*, respectively, demonstrating that the two STAT proteins together with the p93^{fes} are physically associated with JAK2 only upon GM-CSF stimulation. The specificity of these results was further confirmed by the inability 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-JAK2 immunoprecipitates (Fig. 5). The observation that JAK2 physically associates with the β common subunit as well as with p91 and p92 STATs and p93^{fes} 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. 6*A*, 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. 6*B*), anti-p92 (Fig. 6*C*), and anti-p93^{fes} (Fig. 6*D*) antibodies, the two STAT proteins together with the p93^{fes} were found to be physically associated, upon ligand binding, with the β common subunit.

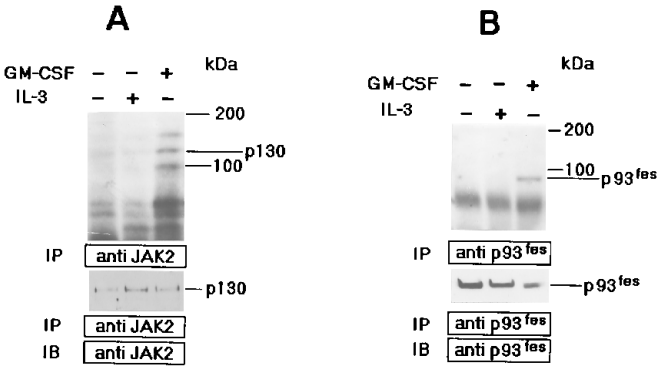

FIG. 7. ***In vitro* JAK2 and p93^{fes} kinase activity following IL-3 and GM-CSF stimulation in human PMN.** Anti-JAK2 (*panel A*) and anti-p93^{fes} (*panel B*) immunoprecipitates from unstimulated (−) and IL-3- and GM-CSF-stimulated (10 ng/ml each for 5 min) (+) PMN were washed and divided in two aliquots. The first one was resuspended in the kinase assay buffer containing [γ-^{32}P]ATP for 30 min at room temperature, washed and eluted with sample buffer for SDS-polyacrylamide gel electrophoresis, separated on 8% gel, and detected by autoradiography (*upper panels*). The second one was separated on 8% gel and probed with anti-JAK2 and anti-p93^{fes} antisera, respectively (*lower panels*). The p130-JAK2 and the p93^{fes} proteins are indicated. *IP*, immunoprecipitated; *IB*, immunoblotted.

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 p93^{fes} correlates with their intrinsic kinase activity. As shown in Fig. 7, anti-JAK2 (*panel A*) and anti-p93^{fes} (*panel B*) immunoprecipitates from GM-CSF-stimulated, but not from IL-3-stimulated, PMN have a detectable *in vitro* kinase activity.

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,

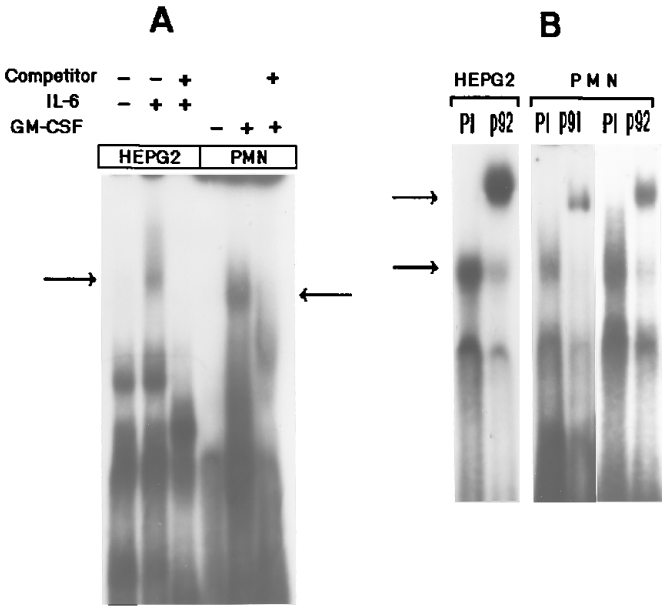

FIG. 8. *A*, induction of DNA-binding activity by GM-CSF and IL-6 in PMN and in HEPG2 cells. Nuclear extracts of untreated (−) or 15-min IL-6-treated HEPG2 cells and GM-CSF-treated PMN (+) were either treated (+) or not (−) treated with a 50-fold excess of unlabeled oligonucleotide (competitor) for 30 min before the addition of radiolabeled oligonucleotide. The complexes were then resolved by nondenaturing polyacrylamide gel electrophoresis. The DNA-binding complexes are indicated. *B*, the GM-CSF-induced DNA-binding complex is antigenically related to p91 and p92. Nuclear extracts from 15-min GM-CSF-treated PMN or IL-6-treated HEPG2 cells were preincubated for 1 h at 4 °C with preimmune serum (*PI*), anti-p91 antiserum, or anti-p92 antibodies before incubation with radiolabeled oligonucleotide and separated on a nondenaturing polyacrylamide gel electrophoresis. The IL-6- and GM-CSF-induced DNA-protein complexes and the supershifted specie are indicated.

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, 63). In agreement with these findings, the present study demonstrates that a set of 90-kDa proteins namely STAT1, STAT3, and p93^{fes} 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. 8*A*, 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. 8*A*) 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, defined 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. 8*B*, 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 appears also when nuclear extract from IL-6-stimulated HEPG2 cells was preincubated with anti-p92 antiserum (Fig. 8*B*) 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^{fes} become phosphorylated upon GM-CSF stimulation and are coimmunoprecipitated by anti-JAK2 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.

Acknowledgment—We thank Prof. G. Borreani for carefully reading this manuscript.

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Granulocyte-Macrophage Colony-stimulating Factor Stimulates JAK2 Signaling Pathway and Rapidly Activates p93, STAT1 p91, and STAT3 p92 in Polymorphonuclear Leukocytes

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J. Biol. Chem. 1996, 271:3562-3567.
doi: 10.1074/jbc.271.7.3562