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Convergence of Signaling by Interleukin-3, Granulocyte-Macrophage Colony-stimulating Factor, and Mast Cell Growth Factor on JAK2 Tyrosine Kinase*

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Maria F. Brizzi‡, Maria G. Zini‡, Maria G. Aronica‡§, Janna M. Blechman¶, Yosef Yarden¶, and Luigi Pegoraro‡‖

From the ‡Dipartimento di Scienze Biomediche e Oncologia Umana, Università di Torino, 10126 Torino, Italy and the ¶Department of Chemical Immunology, the Weizman Institute of Science, 76100 Rehovot, Israel

Mast cell growth factor (MGF) (also called stem cell factor) synergizes with several lymphokines, including interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF), to promote proliferation and differentiation of certain hemopoietic progenitor cells. Although similar patterns of tyrosine-phosphorylated proteins characterize cells stimulated by MGF, IL-3, and GM-CSF, only the MGF receptor is a tyrosine kinase, and the heterodimeric receptors for IL-3 and GM-CSF share a common β subunit that is devoid of enzymatic activity. Here we show that signaling pathways utilized by all three cytokines include the cytoplasmic tyrosine kinase JAK2. Analysis of several factor-dependent myeloid cell lines indicated that JAK2 is physically associated with the common β subunit and with MGF receptor (c-Kit) even prior to ligand binding. However, each of the ligands induced elevated tyrosine phosphorylation of JAK2 and a consequent increase in its catalytic activity. These results demonstrate for the first time the convergence within the same myeloid cells of signaling pathways originating in two distinct lymphokine receptors and a tyrosine kinase receptor on activation of a cytoplasmic tyrosine kinase.

Mast cell growth factor (MGF)[1] (also called stem cell factor, steel factor, and c-Kit ligand) has been implicated in a number of important developmental roles (1–3) and is a potent costimulating cytokine that acts synergistically with hemopoietic colony-stimulating factors, such as granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL-3), to stimulate colony formation of hemopoietic progenitor cells (1). MGF exerts its effects by binding to the c-*kit* proto-oncogene product that belongs to the tyrosine kinase receptor family (4, 5). An important feature of this family is the presence of an intrinsic tyrosine kinase domain and consensus sequences for recognition of SH2 domains through which physical association of the receptor with intracellular signaling proteins occurs (6, 7). In contrast IL-3 and GM-CSF are members of a family of hemopoietic growth factors (8) that stimulate proliferation and differentiation of hemopoietic progenitor cells and functional activation of mature cells (9). These factors bind to an heterodimeric receptor comprised of a ligand binding subunit, denoted α (10, 11), and a common transducing subunit designated as β (12), which is also shared with IL-5 receptor (13). Receptors for most of the cytokines, including IL-3 and GM-CSF, that function in the hemopoietic system are similar in structure and thus belong to the cytokine receptor family (8). Although none of these receptors possesses intrinsic tyrosine kinase domains, several lines of evidence have indicated that signaling processes initiated by ligand binding to the receptors involve tyrosine phosphorylation of the receptor components and activation of cellular tyrosine kinases (14). Studies on the biochemical interaction involved in signaling from the IL-3/GM-CSF receptor have suggested that some pathways involved in their signal transduction are shared with MGF (15–17). In particular, IL-3, GM-CSF, and MGF activate a common pattern of transcriptional factors (18) and also a number of transducing molecules such as Ras (17–20), Raf-1 (16, 21, 22), and mitogen-activated protein kinase (16, 22). It has also been reported that these factors induce the phosphorylation of the p52^{shc} and that, following its tyrosine phosphorylation, p52^{shc} associates with Grb2 (23–25) and Sos1 (26, 27). Moreover it has been reported that GM-CSF induces tyrosine phosphorylation of the c-*fps/fes* proto-oncogene product (28), a nonreceptor protein-tyrosine kinase.

Receptor-associated proteins such as JAK kinases 1 and 2 (29, 30), which are rapidly phosphorylated after receptor activation, have been recently shown to be involved in the early signal transduction events elicited by several cytokines such as erythropoietin (31), IL-3 (32), interleukin 6 (IL-6) (33, 34), granulocyte colony-stimulating factor (35), and GM-CSF (36). The most striking features in the JAK family are the presence of two kinase domains and the lack of readily detectable SH2 or SH3 domains, which participate in the functions of a variety of cytoplasmic protein tyrosine kinases (29, 30). In the present study we evaluated JAK2 activation in GM-CSF-stimulated proliferation and functional activation of human myeloid cells. To assess whether JAK2 participates in the signaling pathway shared by GM-CSF and MGF, we have also studied JAK2 tyrosine phosphorylation upon MGF stimulation. We found that GM-CSF induces a rapid tyrosine phosphorylation of JAK2 kinase both in myeloid cell lines such as M-07e, TF-1, and AML-193, which proliferate in response to this factor, as well as in polymorphonuclear neutrophils (PMN) that become functionally activated upon GM-CSF treatment. We also demon-

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§ Fellow of Associazione Italiana Ricerca sul Cancro.

‖ To whom correspondence should be addressed: Dipartimento di Scienze Biomediche e Oncologia Umana, Sezione Clinica, Università di Torino, Via Genova 3, 10126, Torino, Italy. Tel.: 39-11-663-5539; Fax: 39-11-663-7520.

[1] The abbreviations used are: MGF, mast cell growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; PAGE, polyacrylamide gel electrophoresis; STAT, signal transducers and activators of transcription; PMN, polymorphonuclear neutrophils; BCS, bovine calf serum; IMDM, Iscove's modified Dulbecco's medium; Pipes, 1,4-piperazinediethanesulfonic acid.

strated that JAK2 phosphorylation occurs not only in response to GM-CSF but also in response to MGF. In agreement with these findings JAK2 kinase activity was increased by both GM-CSF and MGF. Finally co-immunoprecipitation of JAK2 with the β common subunit and with the MGF receptor demonstrated that physical association exists prior to cytokine stimulation.

EXPERIMENTAL PROCEDURES

Reagents—Iscove's modified Dulbecco's medium (IMDM) (Life Technologies, Inc.) supplemented with 5% bovine calf serum (BCS) (Hyclone Laboratories, Logan, UT) was the culture medium throughout. Gelatin was from Difco. MGF was a gift from Dr. S. Gillis (Immunex, Seattle, WA). Recombinant human IL-3 and recombinant human GM-CSF were kindly provided by Sandoz Forschungsinstitut, Austria. The protein A-Sepharose was purchased from Sigma. Nitrocellulose filters, horseradish peroxidase-conjugated protein A, molecular weight markers, [γ-^{32}P]ATP, and the chemiluminescence reagent (ECL) were from Amersham Corp.

Antisera—Polyclonal anti-IL-3/GM-CSF receptor β common subunit antibody was prepared from serum of a rabbit immunized against a synthetic peptide that was conjugated to keyhole limpet hemocyanin by glutaraldehyde. The peptide sequence was ELPPIEGRSPRSPRNNPVPPE corresponding to amino acid residues 769–789 of KH97, the cytoplasmic domain near the C terminus (12). The specificity of the antiserum was demonstrated by the lack of the immunoprecipitation band in the presence of saturating concentrations of the peptide (data not shown). The anti-phosphotyrosine antibody 4G10 and the anti-JAK2 antiserum were obtained from Upstate Biotechnology, Inc. K27 and K44 anti-Kit monoclonal antibodies were obtained as described previously (37).

Cell Lines—M-07e and TF-1 cell lines, both dependent on IL-3, GM-CSF, and MGF (38, 39), were grown in IMDM supplemented with 5% BCS. Five ng/ml of recombinant human IL-3 were routinely added every 3 days. Growth factor deprivation was achieved by washing the cells twice and culturing them for 18 h in serum-free IMDM. AML-193 (40) and Chinese hamster ovary c-Kit-transfected cells (T-18) (41) were grown in IMDM supplemented by 5% BCS. Growth arrest was obtained by extensive washing and BCS deprivation for 18 h. PMN were isolated from venous blood of normal donors by gelatin sedimentation (2.5% gelatin in phosphate-buffered saline, pH 7.2, for 30 min at 37 °C) followed by Ficoll-Hypaque gradient separation.

Western Blot Analysis and Immunoprecipitation Studies—Cells (2×10^7), serum- and growth factor-starved for 18 h, were stimulated at 37 °C, extracted with cold DIM buffer (50 mM Pipes, pH 6.8, 100 mM NaCl, 5 mM $MgCl_2$, 300 mM sucrose, 5 mM EGTA, 2 mM sodium orthovanadate) plus 1% Triton X-100 and a mixture of protease inhibitors (1 mmol/liter phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.15 unit/ml aprotinin, 1 μg/ml pepstatin A) for 20 min at 4 °C, and centrifuged at 15,000 × *g* for 20 min. The clarified supernatant was precleaned for 1 h with 50 μl of protein A-Sepharose (3 mg/sample). The precleaned cell lysates were then adsorbed by antisera coupled to protein A-Sepharose. Bound protein was washed several times in DIM buffer and eluted in boiling Laemmli buffer. Eluted protein was subjected to 8% SDS-PAGE. Proteins were then transferred electrophoretically to nitrocellulose; the filters were incubated with blocking solution (10% low fat milk in 20 mM Tris-HCl, pH 7.6, and 17 mM 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 for each wash) with phosphate-buffered saline, 0.05% Tween 20 and reacted for 1 hour 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, the nitrocellulose was first stripped of antibody by 62.5 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM β_2-mercaptoethanol.

In Vitro Kinase Assay—Anti-JAK2 immunoprecipitates were washed and divided into two equal parts. One part was resuspended in an equal volume of kinase assay buffer (50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM Na_3VO_4, 10 mM 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-PAGE, separated on 8% gel, and visualized by autoradiography; the other part was separated on 8% gel and probed with anti-JAK2 antiserum.

RESULTS AND DISCUSSION

In the present study we evaluated JAK2 phosphorylation upon GM-CSF stimulation in different human myeloid cells, namely the growth factor dependent M-07e and TF-1 cell lines, which grow and proliferate in response to IL-3, GM-CSF, and MGF; the factor-dependent AML-193 cell line, which proliferates in response to IL-3 and GM-CSF; and PMN, which become functionally activated upon GM-CSF treatment (9) and in c-Kit-transfected Chinese hamster ovary cells (T-18). All myeloid cells, including PMN, were found to express IL-3/GM-CSF receptor β common subunit (data not shown). Kinetic analysis of JAK2 activation, upon GM-CSF and IL-3 stimulation, showed a rapid and transient JAK2 tyrosine phosphorylation (data not shown). The results depicted in Fig. 1 demonstrate that JAK2 tyrosine phosphorylation, assessed 10 min after GM-CSF stimulation, occurs in M-07e, TF-1, and AML-193 cells and in GM-CSF-stimulated PMN. By contrast, in T-18 cells, no activation of JAK2 in response to GM-CSF was detectable, in agreement with the absence of IL-3 and GM-CSF receptors. JAK2 tyrosine phosphorylation upon GM-CSF stimulation has been reported to be correlated with the induction of mitogenesis (36). Our finding that JAK2 is tyrosine-phosphorylated in GM-CSF-stimulated PMN suggests that JAK2 is also involved in the functional activation of mature myeloid cells. It has been demonstrated that a subset of substrates is phosphorylated in response to IL-3 and GM-CSF (42, 43) even when cells are stimulated at 4 °C, suggesting that the tyrosine kinase responsible for these phosphorylations is closely associated with the β subunit before ligand binding (43). Data reported in Fig. 2 demonstrate that JAK2 is physically associated with the β subunit both in stimulated and unstimulated M-07e and TF-1 cells. This suggests that JAK2 could be the proposed tyrosine kinase associated with the receptor prior to ligand binding (43). Our findings are in agreement with previous observations that erythropoietin and GM-CSF receptors and the granulocyte colony-stimulating factor receptor associate with JAK2 and JAK1, respectively, prior to ligand binding (31, 35, 36) and with the hypothesis (36) that the changes in the conformational status of the receptor-JAK2 complex, due to ligand stimulation, lead to JAK2 kinase activation. However, we cannot rule out the possibility that ligand-independent association of JAK2 with IL-3/GM-CSF receptor β common subunit, observed in our leuke-

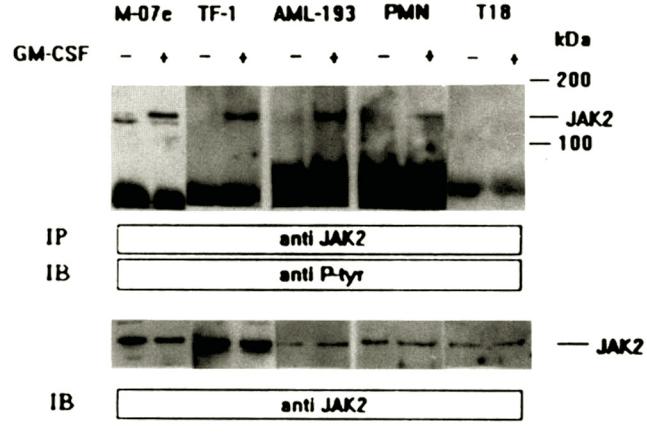

FIG. 1. **GM-CSF-dependent JAK2 tyrosine phosphorylation.** M-07e, TF-1, AML-193, PMN, and T-18 cells were starved in serum-free IMDM medium for 18 h and stimulated with (*plus sign*) or without (*minus sign*) GM-CSF (25 ng/ml) for 10 min. The cells were then lysed and immunoprecipitated with the anti-JAK2 antiserum. SDS-PAGE was done as described under "Experimental Procedures." The filter was immunoblotted with 4G10 anti-phosphotyrosine monoclonal antibody (*upper panel*) and reprobed with the anti-JAK2 antiserum (*lower panel*). *IP*, immunoprecipitated; *IB*, immunoblotted; *P-tyr*, phosphotyrosine.

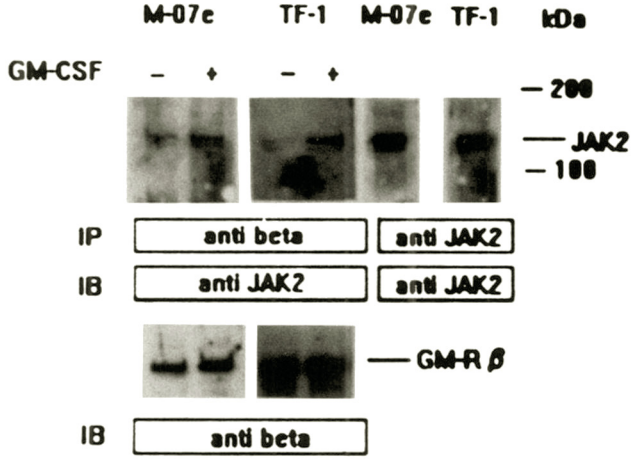

FIG. 2. **JAK2 associates with the IL-3/GM-CSF receptor β common subunit.** Cell lysates from unstimulated (*minus sign*) or GM-CSF-stimulated (25 ng/ml for 10 min) (*plus sign*) M-07e and TF-1 cells were immunoprecipitated with anti-β antiserum. The cells were also immunoprecipitated with anti-JAK2 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*). *IP*, immunoprecipitated; *IB*, immunoblotted; *GM-R*, GM-CSF receptor β subunit.

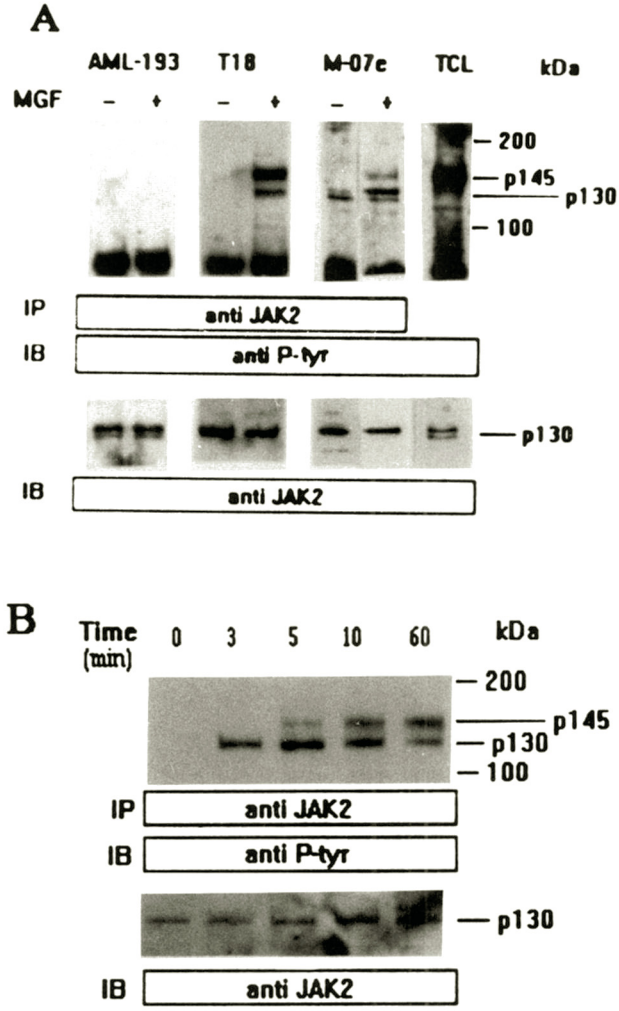

FIG. 3. **MGF-dependent JAK2 tyrosine phosphorylation.** *A*, cell lysates from unstimulated (*minus sign*) and MGF-stimulated (25 ng/ml for 10 min) (*plus sign*) AML-193, T-18, and M-07e cells were immunoprecipitated with the anti-JAK2 antiserum. The filter was probed with 4G10 anti-phosphotyrosine monoclonal antibody (*upper panel*) and reprobed with the antiserum against JAK2 (*lower panel*). The total cell lysate (TCL) of M-07e cells stimulated with MGF was used to indicate the tyrosine-phosphorylated $p145^{kit}$ *protein. B*, M-07e cells deprived of growth factor for 18 h were stimulated with MGF (25 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 and the p145 phosphotyrosines are indicated. *P-tyr*, phosphotyrosine; *IP*, immunoprecipitated; *IB*, immunoblotted.

mic cell lines, depends on their inability to reach the quiescent phase (G_0) following growth factor deprivation (44).

The proliferation and differentiation of hemopoietic cells are regulated by a network of cytokines that may interact in a synergistic or antagonistic manner (9). However, their relationship and mechanism of action are only poorly understood. Thus, one of the central quests in dissecting the mechanisms underlying the interaction among these factors has been to identify shared signaling intermediates. JAK2 kinase is commonly involved in the transducing pathways of receptors belonging to the hemopoietic growth factor family (31–35). This led us to investigate whether JAK2 is also activated by the tyrosine kinase receptor c-Kit, which plays a central role in the regulation of hemopoiesis. The results depicted in Fig. 3*A* demonstrate that when AML-193, M-07e, and T-18 cells are stimulated with MGF, the p130 JAK2 protein becomes phosphorylated both in M-07e and T-18 but not in AML-193. This observation is consistent with the absence of MGF receptor on AML-193 cells (17). The time course of JAK2 phosphorylation on M-07e cells demonstrates (Fig. 3*B*) that JAK2 becomes phosphorylated very rapidly after 3 min following MGF stimulation and remains still phosphorylated after 60 min.

MGF receptor is a p145 protein that becomes phosphorylated upon ligand binding (41). As shown in Fig. 3*A*, an anti-phosphotyrosine blot of anti-JAK2 immunoprecipitates from MGF-treated M-07e and T-18 cells revealed, together with the phosphorylated JAK2 protein, a phosphotyrosine-containing protein of approximately 145 kDa. Therefore we tried to determine whether the JAK2 co-precipitating p145 phosphoprotein was indeed c-Kit. Thus we performed an anti-phosphotyrosine immunoprecipitation of cell lysates from unstimulated and MGF-stimulated M-07e cells. The phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and then immunoblotted with anti-phosphotyrosine antibody. The results shown in Fig. 4*A* demonstrate the presence of two phosphorylated proteins of approximately 145 and 130 kDa. The filter was reblotted with anti-JAK2 antiserum and then with anti-Kit antibody. As shown in Fig. 4*A*, immunoblot with anti-JAK2 antiserum revealed the presence of the same p130 protein whereas immunoblot with anti-Kit antibody revealed the presence of the 145 protein. Moreover anti-Kit and anti-JAK2 immunoprecipitates from unstimulated and MGF-stimulated M-07e and TF-1 cells were resolved on SDS-PAGE and blotted with anti-JAK2 (Fig. 4*B*) or anti-Kit (Fig. 4*C*) antibodies, respectively. As can be seen, both in unstimulated and stimulated cells JAK2 co-precipitates with MGF receptor suggesting that a ligand independent association of JAK2 with MGF receptor is present. Tyrosine phosphorylation of various tyrosine kinases is commonly associated with the activation of their catalytic activity (45). As described previously for other receptors such as erythropoietin, IL-6, and GM-CSF receptors (31, 33–35), JAK2 kinase activity is stimulated upon ligand binding. An *in vitro* kinase assay was thus performed to examine whether phosphorylation of JAK2 correlates with its intrinsic kinase activity. As shown in Fig. 5, anti-JAK2 immunoprecipitates from GM-CSF- and MGF-stimulated cells have a detectable *in vitro* kinase activity. The same result was obtained from IL-3-stimulated cells (data not shown). The major product of phosphorylation was a p130 protein that comigrates with JAK2. JAK2 activity

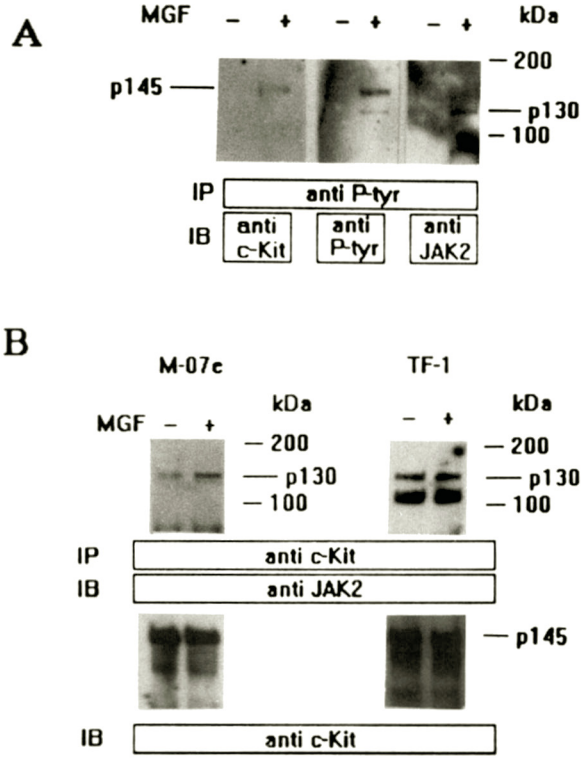

FIG. 4. **JAK2 associates with the MGF receptor.** *A*, M-07e cells were growth factor deprived for 18 h, and then unstimulated (*minus sign*) and MGF-stimulated (25 ng/ml for 10 min) (*plus sign*) cells were lysed and immunoprecipitated with 4G10 anti-phosphotyrosine monoclonal antibody. The filter was probed with 4G10 anti-phosphotyrosine antibody and reprobed with the anti-JAK2 antiserum and then with the anti-Kit antibody (K27). *B*, M-07e and TF-1 cells were starved for 18 h, and then unstimulated (minus sign) and MGF-stimulated cells (25 ng/ml for 10 min) (*plus sign*) were lysed and immunoprecipitated with the anti-Kit antibody (K44). The filter was probed with the anti-JAK2 antiserum and reprobed with the anti-Kit antibody (K27). *C*, M-07e and TF-1 cells, growth factor deprived for 18 h, were unstimulated (*minus sign*) and MGF-stimulated (25 ng/ml for 10 min) (*plus sign*) and then lysed and immunoprecipitated with the anti-JAK2 antiserum. Cell lysate was also immunoprecipitated with the anti-Kit antibody (K44) to indicate the $p145^{Kit}$ protein. The filter was probed with the anti-Kit antibody (K27) (*upper panel*) and reprobed with the anti-JAK2 antiserum (*lower panel*). The positions of the p130 and the p145 phosphotyrosines are indicated. *IP*, immunoprecipitated; *IB*, immunoblotted; *P-tyr*, phosphotyrosine.

was found to be lower in unstimulated than in GM-CSF- and MGF-stimulated cells.

Latent cytoplasmic proteins termed STATs (signal transducers and activators of transcription) (46) have been reported to

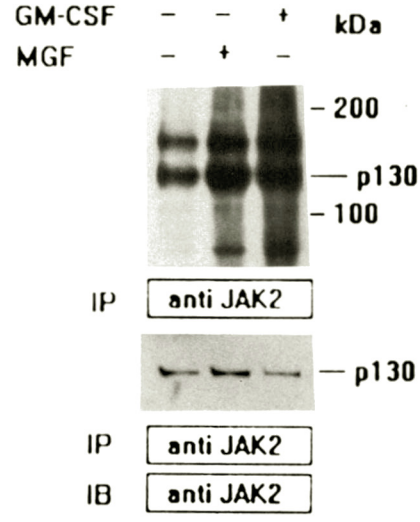

FIG. 5. ***In vitro* JAK2 kinase activity following GM-CSF and MGF stimulation.** Anti-JAK2 immunoprecipitates from unstimulated (*minus sign*) and GM-CSF- and MGF-stimulated (25 ng/ml each for 10 min) (*plus sign*) M-07e cells were washed and divided in two aliquots. The first one was resuspended in the kinase assay buffer containing [γ-^{32}P]ATP for 30 min at room temperature, washed, and eluted with sample buffer for SDS-PAGE, separated on 8% gel, and detected by autoradiography (*upper panel*). The second one was separated on 8% gel and probed with anti-JAK2 (*lower panel*). The p130 JAK2 protein is indicated. *IP*, immunoprecipitated; *IB*, immunoblotted.

be activated upon ligand binding, both in tyrosine kinase receptors, such as epidermal growth factor receptor (47, 48), and in non-tyrosine kinase receptors, such as interferon receptors (49, 50). Moreover it has been suggested that at least two components of the STAT transcriptional complex are substrates of JAK family members (51–53). Overall, the results reported here implicate JAK2 as an important signal-transducing molecule shared not only by IL-3 and GM-CSF but also by MGF in both proliferation and/or functional activation of myeloid cells. However, whether JAK2 represents one of the upstream proteins involved in Ras activation or, as recently proposed (54), an alternative pathway to transcriptional activation via STAT proteins, remains to be established.

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