Granulocyte-Macrophage Colony-stimulating Factor-activated Signaling Pathways in Human Neutrophils

SELECTIVE ACTIVATION OF Jak2, Stat3, AND Stat5B*

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Granulocyte-macrophage colony stimulating factor (GM-CSF) regulates many of the biological functions of human neutrophils. This includes the stimulation of protein synthesis and the tyrosine phosphorylation of various proteins among which is JAK2. The present study was aimed at characterizing in detail the pattern of activation by GM-CSF of the JAK/STAT pathway in human neutrophils. The results obtained show that the stimulation of human neutrophils by GM-CSF specifically led to tyrosine phosphorylation of JAK2 and had no effect on JAK1, JAK3, or TYK2. Furthermore, GM-CSF induced the tyrosine phosphorylation of STAT3 and STAT5 but not of STAT1, STAT2, STAT4, or STAT6. Tyrosine phosphorylation of STAT3 was transient reaching its maximum at 15 min. STAT5 presented a different pattern of tyrosine phosphorylation. The anti-STAT5 antibodies identified two proteins at 94 and 92 kDa. The 94-kDa STAT5 was constitutively tyrosine phosphorylated and showed no change upon GM-CSF stimulation. On the other hand, the 92-kDa STAT5 was tyrosine phosphorylated within 1 min of GM-CSF treatment and this was maintained for at least 30 min. By the use of specific antibodies, it was determined that only STAT5B, and not STAT5A, was tyrosine phosphorylated in GM-CSF-treated neutrophils. Furthermore, GM-CSF treatment induced an increase in the ability of STAT3 and STAT5B, but not STAT5A, to bind DNA probes. The specificity of the pattern of activation of the JAK/STAT pathway suggests that it may be directly linked to the modulation of the functions of mature nondividing, human neutrophils by GM-CSF.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that plays key roles in regulating the growth and differentiation of granulocyte and monocyte progenitors (1–3). Its effects extend to mature human neutrophils by GM-CSF.

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§ The abbreviations used are: GM-CSF, granulocyte-macrophage colony stimulating factor; STAT, signal transducers and activators of transcription; PAGE, polyacrylamide gel electrophoresis.

D activity (8, 9), and arachidonic acid release (10). In addition, GM-CSF exerts a number of direct effects on neutrophils. These include inducing the synthesis of interleukin-1 and interleukin-1Ra (6), increasing the surface expression of adhesion molecules of the β2-integrin family (11), and the number as well as the affinity of FMLP receptors (3, 6), stimulating a cytosolic alkalization (12, 13), activating phosphatidylinositol 3-kinase (14) and increasing the level of tyrosine phosphorylation of a number of proteins including that of some tyrosine kinases (14–16). Although most of these activities are well documented, the intracellular mechanisms by which they are mediated are poorly, or incompletely, understood.

Neutrophils express a low number of a high affinity GM-CSF receptor. The receptor is made up of two subunits, termed α and β (17). The α-subunit, which is unique to the GM-CSF receptor, acts mainly as a binding site for GM-CSF (17, 18). Due to its short cytoplasmic tail, its role in signal transduction is thought to be limited (18). The β-subunit, on the other hand, possesses a large cytoplasmic tail which has been shown to be critical to the mediation of the GM-CSF signal (19). Neither subunit of the GM-CSF receptor possesses intrinsic kinase domains nor is apparently directly linked to a G-protein (17). On the other hand, the transduction of the GM-CSF signal appears to be mediated, at least in part, by cytosolic tyrosine kinases (16, 20). Treatment of human neutrophils with GM-CSF activates several tyrosine kinases including Lyn (16, 21, 22) and Fes (23, 24). In addition, we and others have shown that GM-CSF also activates Jak2 kinase, as evidenced by its increased level of tyrosine phosphorylation (14, 15). Jak2 is a member of the Janus family of tyrosine kinases that also includes Jak1, Jak3, and Tyk2. These kinases are involved in the signaling pathways of several cytokines, the prototype of which is interferon-γ (20, 25–27). For example, Jak2 has been reported to associate with the βc-subunit of the GM-CSF receptor upon stimulation (20, 28). The Jak family functions upstream of a family of transcription factors called STATs (signal transducers and activators of transcription) (29, 30). Eight different members of the STAT family have been identified so far (STAT1α, β, STAT2–4, STAT5α, B, and STAT6). STAT proteins exist in latent cytoplasmic forms which, upon stimulation, become tyrosine phosphorylated and form homo- or heterodimers which translocate to the nucleus and bind specific DNA motifs.

Little has been done to identify the specific combinations of members of the Jak and STAT families activated by GM-CSF in different cell lines in general, and more specifically in neutrophils. The available reports in neutrophils document the tyrosine phosphorylation of Jak2 in GM-CSF-stimulated cells (14, 15). The activation of STAT5 in certain cell lines has been reported (31–33). Additionally, tyrosine phosphorylation of...
STAT1 and STAT3 in human neutrophils upon treatment with GM-CSF has been described (15). The present study was initiated to provide a comprehensive examination of the involvement of the Jak and STAT family members in the mediation of the effects of GM-CSF in human neutrophils. The results show that the stimulation of human neutrophils by GM-CSF leads to a specific activation profile of the Jak/STAT pathway and only results in the tyrosine phosphorylation of Jak2 and of STAT3 and STAT5B.

EXPERIMENTAL PROCEDURES

Materials—GM-CSF was generously provided by the Genetics Institute (Cambridge, MA). Nonidet P-40 was obtained from Sigma. Sephadex G-10, protein A, dextran T-500, and Ficoll-Paque were purchased from Pharmacia Biotech (Dorval, Quebec, Canada). The monoclonal antiphosphotyrosine antibody (UB 05-321) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Polyclonal antibodies to Jak1, Jak2, Jak3, Tyk2, STAT2, STAT3, STAT4, STAT5B, and STAT5 were obtained from Santa Cruz Biotechnology Inc. (Lake Placid, NY). The monoclonal antibodies to STAT1 and STAT5 were purchased from Transduction Laboratories (BioCan Scientific, Mississauga, Ontario, Canada). The Affini-pure rabbit anti-mouse IgG (H+L) antibody was obtained from Jackson Immunoresearch Laboratories (West Grove, PA). The enhanced chemiluminescence (ECL) Western blotting detection system was obtained from Amersham Corp. (Oakville, Ontario, Canada).

Neutrophil Preparation—Blood was collected from healthy adult volunteers into heparinized tubes. The cells were centrifuged for 10 min at 1,000 rpm to remove platelet-rich plasma. After 2% dextran sedimentation of erythrocytes for 30 min, the supernatant was used for subsequent centrifugation under sterile conditions by centrifugation on Ficoll-Paque cushions.

Contaminating erythrocytes were removed by hypotonic lysis and the cells were suspended in magnesium-free Hank’s balanced salt solution containing 1.6 mM CaCl2 at a final count of 4 x 106 cells/ml. The entire procedure was carried out steriley at room temperature (34). The final cell preparation comprised at least 97% neutrophils and less than 0.2% monocytes.

Cell Stimulation and Lysis—Neutrophil suspensions (1 ml of 4 x 106 cells/ml) were either stimulated with the indicated agonists or treated with the same volume of the appropriate diluents for the indicated periods of time at 37 °C. For lysates prepared under reducing conditions, 500 µl of the cell suspensions were added to equal amounts of denaturing buffer A containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 2 mM NaVO4, 20 mM NaF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µM pepstatin, 1 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 1% SDS, and 0.6% 2-mercaptoethanol (final concentrations) preheated to 100 °C and incubated for 10 min. The lysates were centrifuged at 12,000 rpm for 10 min at room temperature. The supernatants were then filtered through Sephadex G-10 columns to remove the denaturing agents. To prepare the columns, 3 g of Sephadex/sample were suspended in 10 ml of buffer containing 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM EGTA, and 137 mM NaCl (final concentrations) for at least 3 h at room temperature with occasional shaking before use. Nonidet P-40 (1%) and bovine serum albumin (0.1 mg/ml) were added to the eluates which were subsequently used for immunoprecipitation. This procedure was described previously in detail (35).

Immunoprecipitation of Tyrosine-phosphorylated Proteins—Lysates (1 ml) obtained as described above were incubated with 5 µg of specific antibodies for 5 h at 4 °C on an oscillating platform. In case of immunoprecipitates of STAT1 and STAT5, this was followed by the addition of 1 µl of Affini-pure rabbit anti-mouse IgG, IgM (H+L) which was added and incubated for 20 min at 4 °C. 20 µg of protein A-Sepharose were then added and left for 1 h at 4 °C. The beads were collected by centrifugation and were washed twice with modified buffer A containing 1% Nonidet P-40 but no SDS or β-mercaptoethanol, and twice with LiCl buffer (LiCl 0.5 M, Hepes 20 mM, pH 7.4). The supernatants were removed carefully, 45 µl of 2 x boiling Laemmli’s buffer (1 x is 62.5 M Tris-HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 8.5% glycerol, 2.5 mM NaVO4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.025% bromphenol blue) was then added and the samples were incubated for 10 min at 100 °C. Immunoprecipitates of STAT1 and STAT5 were divided into equal aliquots before separation on the SDS-PAGE as described below.

Electrophoresis and Immunoblotting—Samples were electrophoresed on 7.5–20% SDS-polyacrylamide gradient gels. Electrophoretic transfer cells ( Hoeffer Scientific Instruments, Canberra Packard, Ontario, Canada) were used to transfer proteins from the gels to Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Western blotting was performed as described previously (34). Briefly, nonspecific sites were blocked using 2% gelatin in TBS-Tween (25 mM Tris-HCl, pH 7.8, 190 mM NaCl, 0.15% Tween 20) for 1 h at 37 °C. The first antibody (black blot) was concentrated sera or protein A-Sepharose-agarose were incubated with 1/1,000 (anti-pY) and 1/100 (anti-STAT5 or STAT1), respectively, in fresh blocking solution. The membranes were washed three times at room temperature in TBS-Tween for a total duration of 30 min, and then incubated with horseradish peroxidase-labeled sheep anti-rabbit IgG (Amersham, Oakville, Canada). The immunoprecipitates concerning STAT1 and STAT5, were divided in two equal aliquots, one was blotted with anti-pY antibodies while the other were blotted with the respective immunoprecipitating antibody. Reprobing was conducted as follows. The polyvinylidene difluoride membrane was treated with TBS buffer containing 1% H2O2 for 5 min at room temperature. This was followed by extensive washing with TBS buffer containing no H2O2. Western blot was conducted as described above but this time the second antibody was a donkey anti-rabbit IgG (Amersham, Oakville, Canada). The immunoprecipitates concerning STAT1 and STAT5, were divided in two equal aliquots, one was blotted with anti-pY antibodies while the other were blotted with the respective immunoprecipitating antibody. This allowed reprobing the membranes with polyclonal antibodies directed against STAT5B.

DNA Affinity Purification—Two DNA probes were synthesized: an acute phase response element probe (GATCCCTTGGGAAATTGCTAAGT) (36) and a FcγR, GAS (GATTTCCCCAGAAAAAAGAAC) (37). The probes were biotinylated using a 3’-terminal transferase (Boehringer Mannheim, Laval, Quebec, Canada) before they were hybridized with their complementary strands. They were subsequently incubated with streptavidin-conjugated agarose for 1 h at 4 °C and washed twice with the blocking buffer as described above but with the following modifications: The samples were treated GM-CSF for 15 min at 37 °C. To test the DNA binding of STAT3, cells were solubilized in KCl whole protein extraction buffer (10 mM Hepes, pH 7.4, 400 mM KCl, 10% glycerol, 2 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 1 mM dithiothreitol, 20 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM diisopropyl fluorophosphate). They were kept at 4 °C for 10 min before centrifugation at 13,000 rpm. The KCl concentration was then diluted to 133 mM (35). To avoid degradation of STAT5, cells were suspended in a saline solution of lysis buffer (0.1% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 1 mM EDTA, 20 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM diisopropyl fluorophosphate). The cells were vortexed for 15 s, kept on ice for 5 min, and centrifuged at 300 × g. The resulting pellets were treated with KCl whole protein extraction buffer as described above. Lysates were preincubated with 10 µg of agarose for 1 h at 4 °C before they were treated with agarose for 2 h at 4 °C with the biotinylated probes. The beads were subsequently washed 4 times with the lysis buffer containing 1% Nonidet P-40 before the addition of boiling sample buffer.

RESULTS

The first set of experiments was designed to identify the members of the Jak family of tyrosine kinases which were activated in human neutrophils treated with GM-CSF. Cells were incubated at 37 °C with GM-CSF (4 nM) or an equal volume of diluent (0.01% bovine serum albumin) for 15 min. They were subsequently lysed by direct transfer to an equal volume of lysis buffer containing SDS and β-mercaptoethanol preheated to 100 °C and boiled for at least 10 min. After removing the denaturing agents as described under “Experimental Procedures,” the different members of the Jak family of tyrosine kinases were immunoprecipitated using specific polyclonal antibodies. The denaturing lysis conditions were necessary for maximal and reproducible phosphorylation of the proteins and their phosphorylation levels. The immunoprecipitates were separated on SDS-PAGE gradient gels and the level of tyrosine phosphorylation of the immunoprecipitated Jak kinases was examined by immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 1 (panel A), and previously
reported (14, 15), GM-CSF induced the tyrosine phosphorylation of Jak2. On the other hand, none of the other members of the Jak family, namely, Jak1, Jak3, or Tyk2 showed any evidence of increases in their level of tyrosine phosphorylation in response to GM-CSF. Similar results were observed using the reverse protocol (i.e. immunoprecipitating with anti-phosphotyrosine antibodies and immunoblotting for the different members of the Jak family) (data not shown). The increase in the level of tyrosine phosphorylation of Jak2 was not seen in lysates prepared under non-denaturing conditions (data not shown). Subsequent blotting with the respective antibodies demonstrated that equal amounts of the kinases were loaded in each pair of lanes (Fig. 1, panel B).

To examine the kinetics of phosphorylation of Jak2 in neutrophils treated with GM-CSF, cells were stimulated for 1, 5, 15, and 30 min before lysis under denaturing conditions as described above. After the removal of the denaturing agents, Jak2 was immunoprecipitated and the level of tyrosine phosphorylation at each time point was examined by immunoblotting with anti-phosphotyrosine antibodies. As illustrated in Fig. 2, increased tyrosine phosphorylation of Jak2 was detected as early as 1 min after stimulation. This phosphorylation reached its maximum within 5 min (lane 3) and declined thereafter. Subsequent blotting with anti-Jak2 antibodies demonstrated that equal amounts of Jak2 immunoprecipitates were loaded in each lane (data not shown).

We next examined which members of the STAT family was tyrosine phosphorylated upon treatment of human neutrophils with GM-CSF. Cells were treated with GM-CSF (4 nM) or equal volumes of diluent for 15 min at 37 °C. This was followed by lysis under denaturing conditions. After the removal of the denaturing agents, immunoprecipitation was conducted as described under “Experimental Procedures” using specific antibodies directed against different members of the STAT proteins. The immunoprecipitates were separated on SDS-PAGE gradient gels and the level of tyrosine phosphorylation of the different STAT proteins were examined by immunoblotting using anti-phosphotyrosine antibodies. As illustrated in Fig. 3 (panel A), treatment of human neutrophils with GM-CSF increased the tyrosine phosphorylation of STAT3 and of the faster mobility isoform of STAT5. STAT1 and the lower mobility isoform of STAT5 constitutively expressed a low level of tyrosine phosphorylation and this remained unchanged upon treatment by GM-CSF. No tyrosine phosphorylation of STAT2, STAT4, or STAT6 was detected in control or in GM-CSF-stimulated cells. Reprobing these membranes with their respective immunoprecipitating antibodies showed the presence of equal amounts of proteins in each pair of lanes (Fig. 3, panel B).

The kinetics of the stimulation of tyrosine phosphorylation of STAT3 and STAT5 were examined next. Cells were stimulated for 1, 5, 15, and 30 min before lysis under denaturing conditions as described above. After the removal of the denaturing agents, STAT3 and STAT5 were immunoprecipitated and their levels of tyrosine phosphorylation examined by immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 4, the tyrosine phosphorylation of STAT3 was evident after 5 min of stimulation and reached its maximum after 15 min of treatment with GM-CSF. After 30 min, the tyrosine phosphorylation of STAT3 was no longer detected. Equal amounts of STAT3 immunoprecipitates were present in each lane (data not shown). A different pattern of tyrosine phosphorylation of STAT5 was observed (Fig. 5). As shown in Fig. 5, panel A, and previously described in other cells (31, 38), two isoforms of STAT5 are present in neutrophils. These, most likely, correspond to STAT5A and STAT5B as the antibodies used in this experiment recognize both STAT5 isoforms. Stimulation of the
cells with GM-CSF led to a time-dependent upward shift of the lower STAT5 band. This decreased mobility is characteristic of phosphorylation. This possibility was directly tested by blotting the immunoprecipitates with anti-phosphotyrosine antibodies, and these data are shown in Fig. 5, panel B. The upper STAT5 band was constitutively tyrosine phosphorylated and this was unaltered by GM-CSF treatment. On the other hand, the lower band was undetectable in unstimulated cells, became tyrosine phosphorylated within 1 min of stimulation, and maintained its level of tyrosine phosphorylation for up to 30 min (the longest time point examined). In addition, the lower STAT5 band detected with the anti-phosphotyrosine antibodies exhibited the exact same shift in migration observed with the anti-STAT5 antibodies (Fig. 5, panel A). Since STAT5 has two isoforms, STAT5A (94 kDa) and STAT5B (92 kDa), we investigated whether the lower band was STAT5B. The membranes were reprobed using specific anti-STAT5B antibodies as described under “Experimental Procedures.” The results presented in Fig. 5, panel C, indeed identified the lower band as STAT5B. This was further confirmed by the following experiment in which immunoprecipitation was conducted using agarose-conjugated anti-phosphotyrosine from control as well as GM-CSF-treated neutrophils as described before. The immunoprecipitates were divided before separation on SDS-PAGE under denaturing conditions. STAT5 was immunoprecipitated and the membrane was blotted with anti-phosphotyrosine antibodies. The data shown are representative of five experiments with identical results. Wb, Western blot; Ipp, immunoprecipitation; pY, phosphotyrosine.

After establishing that STAT3 and STAT5B are tyrosine phosphorylated in GM-CSF-treated neutrophils, the DNA binding ability of these proteins was examined next. Cellular proteins were prepared as described under “Experimental Procedures” and incubated with biotinylated acute phase response element probe (for STAT3) or biotinylated FcγR,GAS (for STAT5) bound to streptavidin-conjugated agarose. The precipitated proteins were separated on gradient gels and identified by immunoblotting with the indicated antibodies. Treatment of neutrophils with GM-CSF induced a significant increase in the ability of STAT3 and STAT5B to bind the DNA probes (Fig. 7, panels A and C, respectively). No increase in STAT5A ability to bind DNA was observed (Fig. 7, panel B).

DISCUSSION

Signal transduction through the GM-CSF receptor, neither subunit of which possesses intrinsic enzymatic activity nor is linked to G proteins, is thought to be mediated by the recruitment of cytosolic tyrosine kinases. Accordingly, one of the prominent effects of GM-CSF in neutrophils is an increase in the level of tyrosine phosphorylation of a number of cellular proteins (16, 34) and the activation of some tyrosine kinases, namely Lyn (16), Fes (23), and Jak2 (28). The latter is of particular importance because it is believed to function upstream of the STAT family of transcription factors and GM-CSF induces the transcription-dependent synthesis of a variety of proteins in human neutrophils. Although a few studies have addressed the subject of the activation of the Jak-STAT pathway by GM-CSF (15), little work has been done concerning this issue in neutrophils (14, 15). The generality of the results concerning the Jak/STAT pathway is further complicated by the apparent differences in GM-CSF signaling between various cell types. For example, GM-CSF has been reported to induce the tyrosine phosphorylation of STAT1 and STAT3 in human neutrophils (15) while only stimulating the tyrosine phosphorylation of STAT5 (both A and B) but not STAT 1, 2, 3, 4, or 6 in OTT1 cells (31). Furthermore, GM-CSF was reported to activate STAT5, but not STAT1, in human monocytes (33). Similar variations between different cell types were observed in the case of stimulation by interleukin-3, a cytokine which shares a common signaling pathway, including a common receptor subunit with GM-CSF (39, 40). In addition, the role that other members of the Janus family of tyrosine kinases may play in GM-CSF signaling had not been addressed as of yet. The present study was therefore designed to identify the mem-

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FIG. 7. Increase in DNA binding of STAT3 and STAT5B after stimulation of neutrophils by GM-CSF. Cells were treated with GM-CSF or diluent for 15 min. The cells were then lysed under non-denaturing conditions. The lysates were incubated with annealed biotinylated acute-phase response element (APRE) (panel A) or FcγR GAS (panels B and C) probes. Immunoblotting was conducted with anti-STAT3 (panel A), anti-STAT5A (panel B), or anti-STAT5B (panel C) antibodies. The data shown are representative of four experiments with identical results. Wb, Western blot.

The data shown are representative of four experiments with identical results. The lysates were incubated with annealed biotinylated acute-phase response element (APRE) (panel A) or FcγR GAS (panels B and C) probes. Immunoblotting was conducted with anti-STAT3 (panel A), anti-STAT5A (panel B), or anti-STAT5B (panel C) antibodies. The data shown are representative of four experiments with identical results. Wb, Western blot.

In conclusion, the results presented in this study show that the Jak-STAT pathway by GM-CSF in human neutrophils is mediated exclusively by Jak2 and involves STAT3 and STAT5B. Furthermore, the pattern and kinetics of the activation of the STATs proteins in human neutrophils by GM-CSF differ from that previously reported in other cell lines, possibly reflecting the specialized, terminally differentiated, nature of these cells.

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