Granulocyte-macrophage colony-stimulating factor (GM-CSF) regulates many of the biological activities of human neutrophils. The signaling pathways via which these effects are mediated are not fully understood. We have shown previously that GM-CSF treatment of human neutrophils activates the Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathway and, more specifically, Jak2, STAT3, and STAT5B in neutrophils. GM-CSF also stimulates the activity of the phosphatidylinositol 3-kinase (PI3-kinase) in a tyrosine kinase-dependent manner. Here we report that pretreating the cells with a Jak2 inhibitor (AG-490) abolishes tyrosine phosphorylation of the p85 subunit of PI3-kinase induced by GM-CSF. Furthermore, p85 was found to associate with Jak2, but not with Lyn, in stimulated cells in situ and with its autophosphorylated form in vitro; however, Jak2 did not bind to either of the two Src homology 2 (SH2) domains of the p85 subunit of PI3-kinase. Although STAT5B bound to the carboxy-terminal SH2 domain of p85, it was absent from the complex containing PI3-kinase and Jak2. These results suggest that stimulation of the activity of PI3-kinase induced by GM-CSF is mediated by Jak2 and that the association between Jak2 and p85 depends on an adaptor protein yet to be identified.

Neutrophils are the most numerous of the white blood cells. They play, among others, a critical role in the nonspecific immune response. Several cytokines regulate the differentiation as well as the activity of neutrophils (1–4). Among these is granulocyte-macrophage colony-stimulating factor (GM-CSF), a glycoprotein released by several cell types which supervises the maturation process of granulocyte and monocyte progenitors (5–7). In addition, several of the biological activities of mature neutrophils are also regulated by GM-CSF, which acts mainly by priming these cells and making them more responsive to secondary stimuli. For example, superoxide production (7–9) and phagocytosis (10) are both enhanced in neutrophils pretreated with GM-CSF as is the activation of several signaling pathways including the mobilization of calcium (11), the activation of phospholipase D (12, 13), and the stimulation of tyrosine phosphorylation (14–16). In addition, a number of direct effects of GM-CSF on neutrophils have been reported. These include the stimulated synthesis of interleukin (IL)-1 and IL-1 receptor antagonist (IL-1Ra) (10), increases in the surface expression of adhesion molecules of the β2 integrin family (17), in the number as well as the affinity of fMLP receptors (7, 10), and induction of cytosolic alkalization and tyrosine phosphorylation (14, 18). Although most of these activities have been well documented, the signaling mechanisms that underlay them are still poorly understood.

The GM-CSF receptor is made up of two subunits, termed α and β (19). The α subunit binds GM-CSF with low affinity, and only in presence of the β subunit high affinity binding achieved (20). Because of its short cytoplasmic tail, the α subunit was originally believed to play little role in signaling (19, 21). This, however, is being challenged by recent reports suggesting its involvement in regulating cell growth (22, 23). The β subunit, on the other hand, has a long intracytoplasmic tail and plays a major role in transmitting the signal inside the cell (24). Neither subunit has intrinsic enzymatic activity, nor are they known to link to G proteins directly (19). Signaling by the GM-CSF receptor depends on the activation of the tyrosine phosphorylation pathways mediated by the stimulation of a number of cytosolic protein tyrosine kinases (25, 26). In neutrophils, we and others have shown that three tyrosine kinases, Lyn (26–28), Fes (29, 30), and Jak2 (29, 31), are activated upon stimulation with GM-CSF. Each of these kinases is involved in the signaling pathways associated with various cytokines (25, 32–34). For example, Jak2 will associate with and phosphorylate the β subunit of the GM-CSF receptor upon ligand binding in TF-1 cells (25).

The Jak family functions upstream of a family of transcription factors called STATs (signal transducers and activators of transcription) (35, 36). Eight different STATs have been identified so far (STAT1 α and β, STAT2–4, STAT5A and B, and STAT6). The STATs are activated by tyrosine phosphorylation (37) and in some cases by an additional serine phosphorylation (38). The phosphorylated STATs will form homo- as well as heterodimers and migrate to the nucleus where they bind specific DNA motifs (35, 36, 39). Which members of the Jak and STAT family are activated varies greatly among different agonists and for the same agonist but in different cell systems (29, 40–43). In neutrophils, we have shown that GM-CSF treatment induces the selective activation of Jak2, STAT3, and STAT5B (44).

Another effector system activated by GM-CSF involves phosphatidylinositol 3-kinase (PI3-kinase) (26). This enzyme phos-
phosphorylates the third hydroxyl group of the inositol ring. In vitro, it uses phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns-4-P), and phosphatidylinositol 4,5-diphosphate (PtdIns-4,5-P2) as substrates leading to the production of PtdIns-3-P, PtdIns-3,4-P2, and PtdIns-3,4,5-P3, respectively (45–47). In vivo, however, only the levels of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 are modulated by PI3-kinase upon stimulation, whereas the levels of PtdIns-3-P remain unchanged (47). PI3-kinase has been implicated in regulating cell proliferation (48), protein secretion (49), and membrane ruffling (50) in various cell systems. In addition, the products catalyzed by the activation of PI3-kinase, namely PtdIns-3,4-P2 and PtdIns-3,4,5-P3, serve as cofactors for several members of the calcium-insensitive protein kinase C family (51, 52) as well as other enzymes such as protein kinase B (53–55). Several members of the PI3-kinase family have been identified so far. The α, β, and δ are made up of a dimer containing a 85 regulatory subunit and a 110 catalytic subunit (56–58). PI3-kinase γ is made up of a 110 catalytic subunit and a 101 regulatory subunit apparently activated by G-protein-linked receptors (31, 59). We have shown that GM-CSF treatment significantly activated PI3-kinase α in human neutrophils and that this activation is mediated by tyrosine kinases (31). However, the identity of the kinases involved remains elusive.

The aim of this report was to examine the potential interrelationships between the Jak/STAT and PI3-kinase pathways during the stimulation of human neutrophils by GM-CSF. The results presented show that inhibition of the tyrosine kinase activity of Jak2 abolished the GM-CSF-induced tyrosine phosphorylation of the p85 subunit of PI3-kinase associated with it. In addition, the p85 subunit was found to associate with Jak2 upon stimulation with GM-CSF in situ as well as with auto-phosphorylated Jak2 in vitro. Finally, STAT5 but not STAT3 associated with the carboxyl SH2 domain of p85 in cellular lysates of cells treated with GM-CSF.

**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 Amersham Pharmacia Biotech. The monoclonal antiphosphotyrosine antibody UB 05-321, the agarose-conjugated antiphosphotyrosine antibodies, the polyclonal anti-Jak2 (06-255) and anti-PI3-kinase (06-195) antibodies as well as purified Jak2 (14-134) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). The GST fusion proteins carrying the SH2 domains of the p85 subunit of PI3-kinase from Upstate Biotechnology Inc. (Lake Placid, NY). Polyclonal antibodies (06-195) antibodies as well as purified Jak2 (14-134) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Polyclonal antibodies to Lyn (sc-15), STAT3 (sc-482), and STAT5B (sc-853) were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, Ca). The GST fusion proteins carrying the SH2 domains of the p85 subunit of PI3-kinase were a generous gift from Dr. Tony Pawson (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada). The enhanced chemiluminescence (ECL) Western blotting system was obtained from Amersham Pharmacia Biotech.

**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, neutrophils were purified under native conditions as described above. The migration solution contained 2-propanol and 2M acetic acid at a 2:1 v/v ratio. The phosphorylated lipids were revealed by autoradiography.

**Cell Stimulation and Lysis**—Neutrophil suspensions (1 ml of 40 × 106 cells/ml) were either stimulated with 4 μg/ml GM-CSF or treated with the vehicle alone and the diluted (0.01%) bovine serum albumin used for the indicated periods of time at 37°C. For lysates prepared under reducing conditions (60), 500 μl of the cell suspensions was added to equal amounts of denaturing buffer A containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 2 mM Na3VO4, 20 mM NaFOP4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μM pepstatin, 1 μM phenylmethylsulfonyl fluoride, 1% SDS, and 0.6% β-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 G-10/sample was suspended in 10 ml of buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 137 mM NaCl, 10 mM MgCl2. The assay to measure the activity of PI3-kinase was conducted as described previously (61). Briefly, PI3-kinase was immunoprecipitated by adding 4 μg of anti-p85 antibodies to 900 μl of non-denatured precleared lysates and left at 4°C for 2 h. The antibodies were collected after a 1-h incubation at 4°C with protein A-Sepharose. The immunoprecipitates were washed once with each of the following: lysis buffer, LiCl buffer (0.5 mM LiCl, 20 mM Hepes, pH 7.4), 1% Nonidet P-40, and LiCl buffer. The enzyme (10 μg) was washed with phosphate-buffered saline and incubated with 1 ml of cellular lysates taken from untreated neutrophils that were prepared under native conditions as described above. The mixture was left at 4°C for 2 h before being washed twice with LiCl buffer (0.5 mM LiCl, 20 mM Hepes, pH 7.4).

**In Vitro Measurement of PI3-Kinase Activity—Neutrophils (4 × 106 cells/ml) were treated with 200 μM AG-490 or diluent (dimethyl sulfoxide). The immunoprecipitates were washed with LiCl buffer containing 500 μl of kinase buffer (0.5 mM MgCl2, 5 mM MnCl2, 25 mM NaCl, and 0.1 mM Na3VO4 for 40 min at 30°C with or without 1 mM ATP. The enzyme (10 μg) was washed with phosphate-buffered saline and incubated with 1 ml of cellular lysates taken from untreated neutrophils that were prepared under native conditions as described above. The mixture was left at 4°C for 2 h before being washed twice with LiCl buffer (0.5 mM LiCl, 20 mM Hepes, pH 7.4).

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cells, and the protein bands were revealed using the ECL Western blotting detection system following the manufacturer's directions. To ensure the presence of equal amounts of immunoprecipitated proteins under each condition, the membranes were routinely rebotted with the respective immunoprecipitating antibody. Reprobing was conducted as follows. The polyvinylidene difluoride membranes were treated with TBS buffer containing 1% H2O2 for 5 min at room temperature. The membranes were washed extensively with TBS buffer containing no H2O2 and were then blotted with the immunoprecipitating antibodies as described above.

**RESULTS**

The first experiment was designed to examine the effect of AG-490, a reportedly specific Jak2 inhibitor (62), on the pattern of tyrosine phosphorylation of cellular proteins induced by GM-CSF in human neutrophils. Cells at 4 x 10^6/ml were incubated with 200 μM AG-490 or its diluent (dimethyl sulfoxide) for 90 min at 37 °C. The cells were then suspended in Hanks’ balanced salt solution at 40 °C for 90 min at 37 °C. The cells were then incubated with GM-CSF or diluent (0.01% BSA) for 10 min at 37 °C and subsequently added an equal volume of boiling sample buffer.

Cellular proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and immunoblotted with antiphosphotyrosine antibodies. As shown in Fig. 1, GM-CSF induced tyrosine phosphorylation of a number of proteins in the 150-, 140-, 120-, 90-, 80-, 70-, 55-, and 40-kDa region (63, 64). Treating cells with AG-490 led to a decrease in the level of tyrosine phosphorylation of most of these proteins especially those with molecular masses in the 140-, 120-, 90-, 80-, and 40-kDa ranges (indicated by arrows). AG-490 was used at 200 μM, a concentration that provided the optimal inhibition of Jak2 within 90 min of preincubation without affecting the survival of the cells as evidenced from testing by trypan blue (data not shown). In addition, neutrophils pretreated with AG-490 were responsive to treatment with IL-8 and fMLP, two agonists that utilize G-protein-linked receptors (data not shown). The inhibitory effects of AG-490 were time- and concentration-dependent. Shorter incubation times (30–60 min) or lower concentrations (50, 100, 150 μM) inhibited progressively less the tyrosine phosphorylation stimulated by GM-CSF (data not shown).

We have shown previously that the stimulation of human neutrophils by GM-CSF induced tyrosine phosphorylation of the p85 subunit and activation of PI3-kinase (31). We examined next the effect of AG-490 on these two parameters. Cells were incubated with AG-490 as described above and treated with 4 nM GM-CSF or diluent for 15 min at 37 °C. The cells were then lysed under denaturing conditions in buffers containing SDS and β-mercaptoethanol as described under “Experimental Procedures.” After the removal of the denaturing agents, the lysates were incubated with agarose-conjugated antiphosphotyrosine antibodies. The immunoprecipitates were divided into two parts, separated by SDS-polyacrylamide gel electrophoresis, and immunoblotted with anti-p85 or anti-Jak2 antibodies. As illustrated in Fig. 2A, GM-CSF increased the level of tyrosine phosphorylation of Jak2 (first two lanes from left). This increase was reduced significantly (75.1%) in cells pretreated with AG-490 (p = 0.0017, n = 4) (third and fourth lanes). As reported previously (31), GM-CSF also increased the tyrosine phosphorylation of p85 (Fig. 2B, first two lanes), an effect that was abolished in cells treated with the Jak2 inhibitor (p = 0.0051, n = 4) (third and fourth lanes). The measurement of the effect of Jak2 inhibition on the level of activity of PI3-kinase induced by GM-CSF in neutrophils followed. Cells were prepared as described above; after stimulation with GM-CSF they
were lysed under native conditions. PI3-kinase was immuno-precipitated using specific antibodies for p85, and in vitro kinase assays were conducted using phosphatidylinositol crude extracts as substrate as described under “Experimental Procedures.” Immunoprecipitation was conducted using anti-Lyn (first two lanes) and anti-Jak2 (third and fourth lanes) antibodies. The membrane was immunoblotted with anti-p85 antibodies (n = 4). Western blot; lpp, immunoprecipitation.

The influence of the tyrosine phosphorylation of Jak2 on its association with tyrosine-phosphorylated Jak2. Cells were lysed under native conditions as described under “Experimental Procedures.” Immunoprecipitation was conducted using anti-p85 antibodies (that p85 recognizes and associates with Jak2 but mostly if the anti-Jak2 antibodies. The results presented in Fig. 4 showed that p85 recognizes and associates with Jak2 but mostly if the latter has been allowed to autophosphorylate in vitro (p = 0.231, n = 5). Subsequent immunoblotting with anti-Jak2 antibodies showed the presence of equal Jak2 loading in both conditions.

The next set of experiments was aimed at identifying the role that the SH2 domains of the p85 subunit of PI3-kinase might play in its association with tyrosine-phosphorylated Jak2. Cells were suspended at 40 × 10^6/ml and stimulated with 4 nM GM-CSF for 15 min at 37 °C. They were then lysed under denaturing conditions, and the lysates were incubated with GST fusion proteins of the amino- or the carboxyl-terminal SH2 domains of the p85 subunit of PI3-kinase. Immunoblotting with anti-Jak2 antibodies did not reveal any association of the kinase with either GST fusion proteins, even after stimulation with GM-CSF (Fig. 5A).

Because it is possible that Jak2 and PI3-kinase are linked by another protein and because we have shown that GM-CSF activates two members of the STAT family, namely, STAT3 and STAT5B, we tested for the presence of these STATs in the Jak2-PI3-kinase complexes as well as for their potential interactions with the SH2 domains of p85. Immunoblotting the membranes containing the p85/Jak2 complex did not reveal the presence of either STAT3 or STAT5B (data not shown). We then prepared denatured cellular lysates and incubated them with GST fusion proteins of the SH2 domains of p85. Immunoprobing these precipitates with anti-STAT3- or anti-STAT5B-specific antibodies showed that neither protein was associated with the NH2-terminal SH2 domain. On the other hand, STAT3 was detected in the COOH-terminal SH2 domain precipitates in small amounts that were not affected by GM-CSF treatment (Fig. 5B), whereas STAT5B that was bound to the COOH-terminal SH2 domain of p85 in control cells showed an increase upon treatment of cells with GM-CSF (Fig. 5C).

DISCUSSION

GM-CSF plays key roles in regulating the biological activities of neutrophils (5, 8, 10–12). The signaling pathways involved in mediating these effects are not fully identified or understood. The GM-CSF receptor is made up of two subunits, α and β (19), neither of which has an intrinsic kinase domain or is linked to a G-protein (19). Instead, the receptor acts by associating with and activating a number of cytosolic tyrosine kinases including Lyn (26, 27), Fes (30, 65), and Jak2 (29, 31), and another protein and because we have shown that GM-CSF activates two members of the STAT family, namely, STAT3 and STAT5B, we tested for the presence of these STATs in the Jak2-PI3-kinase complexes as well as for their potential interactions with the SH2 domains of p85. Immunoblotting the membranes containing the p85/Jak2 complex did not reveal the presence of either STAT3 or STAT5B (data not shown). We then prepared denatured cellular lysates and incubated them with GST fusion proteins of the amino- or the carboxyl-terminal SH2 domains of the p85 subunit of PI3-kinase. Immunoblotting with anti-Jak2 antibodies did not reveal any association of the kinase with either GST fusion proteins, even after stimulation with GM-CSF (Fig. 5A).

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The membrane was immunoblotted with anti-Jak2 antibodies. Panel B, membrane was immunoblotted with anti-STAT3 antibodies. Panel C, membrane was immunoblotted with anti-STAT5B antibodies. The first set of experiments reported in this manuscript involved a specific Jak2 inhibitor, AG-490 (62), which blocks the kinetic activity of Jak2 and its ability to transphosphorylate upon dimerization of the GM-CSF receptor (68). The use of this inhibitor allowed the examination of the role of Jak2 in the stimulation by GM-CSF of the overall level of tyrosine phosphorylation of human neutrophils and of that of the p85 subunit of PI3-kinase in particular. GM-CSF, as shown previously, induced the tyrosine phosphorylation of a number of cellular proteins. The identities of those proteins are not fully known; however, the band at 120 kDa includes Jak2 (31, 44) and Cbl (69), those at 90 kDa the STATs (29, 44), those at 80 kDa contain the p85 subunit of PI3-kinase (31), and the 40 kDa bands include members of the mitogen-activated protein kinases (70–72). The proteins at 140, 120, 90, 80, and 40 kDa and reduced that of Jak2. These results underline the wide scope of the involvement of Jak2 in GM-CSF signaling. AG-490 also completely abolished the tyrosine phosphorylation of p85 normally seen upon stimulation of neutrophils with GM-CSF. This result suggests that Jak2 is involved, directly or indirectly, in the tyrosine phosphorylation of p85, a crucial step in the activation of PI3-kinase.

The next set of experiments was aimed at studying further the relationship between Jak2 and PI3-kinase. Using a coimmunoprecipitation protocol, the formation of a complex between p85 and Jak2 was observed upon stimulation with GM-CSF. This interaction was selective to Jak2 because p85 was absent from Lyn immunoprecipitates, a tyrosine kinase that has been suspected of playing a role in PI3-kinase activation (26). This observation was strengthened further by the experiments that made use of purified Jak2. The results obtained showed that when Jak2 was allowed to autophosphorylate in vitro before being incubated with cellular lysates, it recognized and bound the p85 subunit of PI3-kinase. This association was significantly less when Jak2 was not phosphorylated. Thus the phosphorylation state of Jak2 is crucial to its stimulation association with PI3-kinase.

The p85 subunit of PI3-kinase contains two SH2 domains that mediate the interactions with tyrosine-phosphorylated proteins (73–75). GST fusion proteins carrying the NH2-terminal SH2 or the COOH-terminal SH2 domains of p85 were incubated with denatured cellular lysates. Surprisingly, no Jak2 was detected with either of these two domains. These results suggest several interpretations. First, the association between Jak2 and p85 may be mediated by another, as yet unidentified, protein. Alternatively, the severe degradation problems encountered with neutrophil lysates (60), which dictated the use of harsh denaturing lysis protocol, may have altered the required tertiary confirmation of Jak2. Finally, the interaction between Jak2 and PI3-kinase may depend on sequences other than the SH2 domains of PI3-kinase. It is worth mentioning that no Jak2 was associated with the SH3 domain of p85 in stimulated neutrophils (data not shown).

The Jak tyrosine kinases function upstream of the STAT family of transcription factors (35, 36). A recent report showed that STAT3 associates with p85 and links it to the interferon-γ receptor (76). Because GM-CSF activates STAT3 and STAT5B in neutrophils (44), the possibility that either of them might act in linking Jak2 to PI3-kinase was also examined. Neither of the two STATs was recovered in the complex formed between Jak2 and PI3-kinase (data not shown). However, STAT5B did associate with the COOH-terminal SH2 domain of p85 in cells treated with GM-CSF. The apparent discrepancy between these two sets of results might be for a number of reasons. One possibility is that the binding between the COOH-terminal SH2 domain and STAT5B observed in vitro does not reflect what goes on in situ. Another possibility is related to the high proteolytic potential of neutrophil lysates. STAT5 was degraded rapidly even in the presence of the mixture of protease inhibitors used (see “Experimental Procedures”) when lysis was conducted under nonnondenaturing conditions. Thus it is possible that STAT5B is normally present in the complex forming between Jak2 and PI3-kinase, but because of heavy degradation it goes undetected in immunoblots performed on precipitates derived from undenatured lysates. As they stand, however, the present results do not support the hypothesis that one of the STATs acts as a bridge linking p85 to Jak2. As mentioned above, this function might be performed by an anchor protein whose identity is presently unknown.

In conclusion, our results show that Jak2 is involved in the tyrosine phosphorylation of the p85 subunit of PI3-kinase and

**FIG. 5.** Neutrophils were suspended at 40 × 10^6/ml before stimulation with 4 nM GM-CSF. Denatured cellular lysates were prepared as described under “Experimental Procedures” and were incubated with GST fusion proteins carrying the amino-terminal and the carboxy-terminal SH2 domains of the p85 subunit of PI3-kinase. Panel A, membrane was immunoblotted with anti-Jak2 antibodies. Panel B, membrane was immunoblotted with anti-STAT3 antibodies. Panel C, membrane was immunoblotted with anti-STAT5B antibodies (n = 5). Wb, Western blot.
that an association between the two kinases takes place which is subsequent to tyrosine phosphorylation of Jak2 (and thus presumably to its activation). The association appears to be mediated by an intermediary protein other than STAT3 or STAT5β whose identity is presently unknown.

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