Requirement of Src Kinase Lyn for Induction of DNA Synthesis by Granulocyte Colony-stimulating Factor*

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Treatment of cells with granulocyte colony-stimulating factor (G-CSF) leads to tyrosine phosphorylation of cellular proteins. G-CSF stimulates both the activation of protein tyrosine kinases Lyn, Jak1, and Jak2 and the association of these enzymes with the G-CSF receptor. Wild-type, lyn-deficient, and syk-deficient chicken B lymphocyte cell lines were transfected with the human G-CSF receptor, and stable transfectants were studied. G-CSF-dependent tyrosyl phosphorylation of Jak1 and Jak2 occurred in all three cell lines. Wild-type and syk-deficient transfectants responded to G-CSF in a dose-responsive fashion with increased thymidine incorporation, but none of the clones of lyn-deficient transfectants did. Ectopic expression of Lyn, but not that of c-Src, in the lyn-deficient cells restored their mitogenic responsiveness to G-CSF. Ectopic expression in wild-type cells of the kinase-inactive form of Lyn, but not of the kinase-inactive form of Jak2, inhibited thymidine incorporation in response to G-CSF. These studies show that the absence of Lyn results in the loss of mitogenic signaling in the G-CSF signaling pathway and that activation of Jak1 or Jak2 is not sufficient to cause mitogenesis.

Granulocyte colony-stimulating factor (G-CSF)4 critically directs the proliferation and differentiation of granulocytic precursors (1, 2). Its receptor belongs to the cytokine receptor superfamily and more closely resembles the receptor complex for interleukin-6 (3–6). The earliest biochemical changes upon the engagement of the G-CSF receptor involve protein tyrosine phosphorylation (7–10). Because none of the cytokine receptors contain tyrosine kinase catalytic domains, changes in tyrosyl phosphopeptides must occur through recruitment of protein tyrosine kinases (PTK) (11). Members of the Jak family of PTKs are activated by a variety of cytokines and interferons and are considered to be the primary PTKs that mediate hematopoietic cell signaling (12, 13). Another major family of cytosolic protein tyrosine kinases is related to the Src proto-oncogene product, which is required for mitogenesis by receptor protein tyrosine kinases (14–16).

Redundant signaling molecules may obscure hierarchical or sequential cascades that may mediate ligand-specific responses. G-CSF stimulates three distinct classes of PTKs: the Src-like kinase Lyn (17), the tandem Src homology domain 2 containing kinase Syk (18), and Jak1 (19–20). Lyn, Syk, and Jak1 co-immunoprecipitate with the receptor, but the physiological roles of these PTKs are not defined. Therefore, we used gene targeting and expression of kinase-inactive forms to assign specific functions to candidate PTKs involved in G-CSF signaling.

EXPERIMENTAL PROCEDURES

Cells—By classic gene targeting through homologous recombination, we generated lyn-deficient and syk-deficient lines from a chicken B cell line (DT40) (21). None of the other Src-related PTKs are detected by immunoblotting in this cell line. The parental line (DT-40) and derived lyn-deficient (D33-3) and syk-deficient (S1-10) cells were maintained in RPMI 1640 supplemented with 200 μM glutamine, 50 μM 2-mercaptoethanol, 10% fetal bovine serum, and 1% chicken serum in a 5% CO₂-humidified atmosphere.

Expression Vectors and Transfections—The cDNA for the class I human G-CSFR (a gift from ImmuneX, Seattle, WA) was cloned into the pApuro vector (21), the expression of which is driven by the chicken actin promoter and confers puromycin resistance. The pApuro-hGCSFR construct was electroporated (Bio-Rad Gene Pulser with 550 V and 25 μF settings) into the wild-type, lyn-deficient, and syk-deficient cell lines. Stable transfectants, denoted by the suffix GR, were selected for by treatment with puromycin after limiting dilution. The cDNA for the kinase-inactive form of Lyn (LynK275R) was obtained by site-directed mutagenesis and sequenced. Lack of kinase activity was confirmed by in vitro kinase assay. The cDNA for the kinase-inactive form of Jak2 (Jak2ΔVIII) was obtained from Dr. Don Wojchowski (Pennsylvania State University) and has been described elsewhere (22). The cDNA for LynK275R or Jak2ΔVIII was ligated into the pCDNA3 vector (Invitrogen, Carlsbad, CA). Wild-type cells were electroporated and assayed for G-CSF-induced tyrosyl phosphorylation of the G-CSF receptor.

Receptor Binding Analysis—Stable transfectants were selected for resistance to puromycin. Individual clones were established by limiting dilution. Cells (10⁴) from the three different clones were suspended in 1 ml of RPMI 1640 medium supplemented with chicken serum (CS) (1%) at 4 °C and incubated with trace amounts of ³¹P-labeled G-CSF (75,000 cpn/sample) in the presence of increasing concentrations of unlabeled G-CSF for 2 h on ice. Cell bound G-CSF was separated from free material by centrifugation (2 min, 1500 rpm, 4 °C) and followed by...
three washes with 1 ml of medium. All incubations and washes were performed at 4 °C to minimize internalization of the radiolabeled ligand. Bound 125I-G-CSF was determined by gamma counting. Nonspecific binding was determined in the presence of 1 μM G-CSF and subtracted from the data. The data were fit to a single set of binding sites using Scatchard analysis (Prism, Graphpad Software).

**Immunoprecipitations and Immunoblotting**—Approximately 105 cells, starved of FCS and CS overnight, were stimulated with human G-CSF (Amen, Thousand Oaks, CA) (100 ng/ml) for 10 min at 37 °C. Cells were lysed in 10 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1% Nonidet P-40, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 30 min at 4 °C. Debris were removed by centrifugation, and the lysates were analyzed by SDS-polyacrylamide gel electrophoresis followed by transfer onto Immobilon (Millipore, Bedford, MA). After being blocked with bovine serum albumin (2%) solution, the membrane was blotted with the anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology), and proteins were detected by enhanced chemiluminescence (Amersham). For the control experiments, the blotting was done with Lyn monoclonal antibody (Transduction Laboratories, Lexington, KY) and protein G-agarose (Sigma). Anti-phosphotyrosine blotting was done with monoclonal antibody 4G10. Anti-Lyn blotting was done with Lyn monoclonal antibody (Transduction Laboratories, Lexington, KY).

**Immunoprecipitated Tyrosine Kinase Assays**—Approximately 5 × 106 cells, starved overnight of FCS and CS, were stimulated with indicated concentrations of G-CSF. Immune complex kinases were done, as described (4). Rabbit polyclonal antibody against Lyn was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against Syk was described elsewhere (21). To verify that there was increased catalytic activity, acid-denatured enolase was added as an exogenous substrate in some of the experiments. Enolase (Boehringer Mannheim) was treated with 2 mM acetic acid for 30 min on ice. 10 μg/ml of acid-denatured enolase was then added to the kinase reaction buffer. In the immune complex kinase assays for detection of Lyn’s association with the G-CSFR, rabbit polyclonal antibody against the human G-CSFR (Santa Cruz Biotechnology) was used. Autoradiographs were analyzed with ImageQuanNT software on a Molecular Dynamics personal densitometer SI/PhosphorImager.

**Tritiated Thymidine Incorporation Analysis**—Cells were washed in sterile phosphate-buffered saline and resuspended in medium without serum. After an overnight incubation in serum-free medium, cell viability was determined to be >85%, and the cells were resuspended in serum-free medium at 105 cells/ml in 96-well plates. The cells were stimulated with the indicated concentrations of G-CSF and incubated for 20 h in 37 °C and 5% CO2. The cells were then pulsed for 4 h with 1 μCi/well [3H]thymidine (NEC Life Science Products). Cells were harvested onto filter discs, dried, and measured by scintillation. Results are represented as the mean ± standard deviation. For the ectopic expression of Lyn, 105 D33-3GR cells were washed and electroporated with or without 1 μg of pcDNA3-Lyn and resuspended in growth medium. After 24 h, the cells were washed and resuspended in RPMI without serum. Cells were stimulated with G-CSF and pulsed with tritiated thymidine for 6 h before being harvested. Results are expressed as the mean ± standard deviation.

**RESULTS**

**G-CSF Receptor Binding Characteristics**—Stable transfectants following electroporation were selected by growth in puromycin. Levels of G-CSF expression were determined by binding studies done on independent DT40GR (wild-type), D33-3GR (lyn-deficient), and S1-10GR (syk-deficient) clones. Cells were incubated with human 125I-labeled G-CSF, and the binding data were analyzed (Fig. 1). The DT40GR, D33-3GR, and S1-10GR lines expressed 130,000, 37,000, and 9,800 receptors/cell, respectively. Normal myeloid-derived cells expressed ~500 receptors/cell. To measure Kd, 2.6 nM, 0.5 nM, and 0.07 nM, respectively, corresponded closely with the Kd = ~0.3 nM of the high affinity myeloid cell receptor (24). Nontransfected DT40 cells did not demonstrate any binding of radiolabeled G-CSF.

**Reconstitution of Tyrosine Kinase Signaling**—In nontransfected cells, G-CSF stimulation failed to induce changes in phosphotyrosine content. Protein immunoblotting with antibody to phosphotyrosine done on lysates (1% Nonidet P-40), obtained from transfectants stimulated with G-CSF, demonstrated a prominent tyrosyl phosphoprotein of 135 kDa (Fig. 2A). The human G-CSFR also migrates with an apparent size at ~140 kDa, so the prominent 135-kDa band probably represents the tyrosyl phosphorylated receptor. Immunoprecipitation of proteins with antibody to the human G-CSFR, followed by blotting with antibody to phosphotyrosine, demonstrated tyrosyl phosphorylation of the receptor in wild-type cells (Fig. 2B). G-CSF-induced tyrosyl phosphorylation of its cognate receptor also occurred in lyn-deficient and syk-deficient cells (data not shown). In the phosphotyrosine blot of lysates (Fig. 2A), a doublet with a molecular mass of ~57 kDa was seen in wild-type and syk-deficient cells. This tyrosyl-phosphorylated doublet may correspond to p53/p56Lyn, because no such doublet was seen in the lyn-deficient cells. In lysates prepared from wild-type cells, immunoprecipitation of proteins with antibody to the human G-CSFR followed by a kinase assay demonstrated activity that corresponds to Lyn (Fig. 2C), which is similar to that reported in neutrophils (17). To verify that the activity was Lyn, lysates were cleared of Lyn by immunoprecipitation with antibody directed against Lyn. Immune complex kinase assay on lysates, first depleted of Lyn and then subjected to immunoprecipitation with antibodies against Lyn of G-CSFR, did not demonstrate Lyn activity (lanes 4 and 5), which suggests that the kinase activity seen was due to Lyn (lanes 1 and 2). Kinase activity of lysates which were subjected to Lyn immunoprecipitation (lane 4) was recovered in the Lyn immunoprecipitates (lane 6). Blotting of proteins immunoprecipitated with antibody to the human G-CSFR demonstrated Lyn, also the blotting of proteins immunoprecipitated with

![Fig. 1. Scatchard analysis of G-CSF binding to wild-type, lyn-deficient and syk-deficient cell lines stably transfected with the human G-CSFR.](image)
antibody to Lyn demonstrated the human G-CSFR (data not shown).

Lyn or Syk immune complex kinase assays were done on wild-type, lyn-deficient, and syk-deficient cell lines. Densitometric analysis (ImageQuaNT, Molecular Dynamics) showed a 4.4-fold increase in phosphorylation of Lyn in G-CSF-stimulated wild-type cells and a 2.2-fold increase in phosphorylation of Lyn in syk-deficient cells (Fig. 2D). To confirm that autophosphorylation of Lyn correlated with its activation, we added acid-denatured enolase as an exogenous substrate to the kinase reaction. For instance, in syk-deficient cells, G-CSF stimulation resulted in a 2-fold increase in enolase phosphorylation (data not shown). Autophosphorylated Lyn does not appear in the immune complex kinase assay on lyn-deficient cells, as would be expected. Syk activity did not increase appreciably in G-CSF-stimulated wild-type and lyn-deficient cell lines, and it was not present in the syk-deficient cells (Fig. 2E). The lack of increased Syk activity in nondifferentiated, proliferating cell
Lyn Requirement for DNA Synthesis

requires a nonspecific Src kinase, we transfected cells with an expression construct containing src and tested for tritiated thymidine incorporation. Even though Src was expressed in cells, as confirmed by in vitro kinase assay, no G-CSF-dependent increase in thymidine incorporation occurred (data not shown). The cDNA for either kinase-inactive Lyn or kinase-inactive Jak2 was transfected into the wild-type cells that expressed the G-CSFR, and stable transfectants were selected by growth in neomycin. Stable transfectants were analyzed for Lyn kinase activity by immune complex kinase assay (Fig. 5A), and Jak2 kinase activity was assayed by anti-phosphotyrosine blotting of Jak2 immunoprecipitates (Fig. 5B). In both cases, the kinase-inactive form inhibited endogenous activity, and in the case of Lyn it blocked the phosphorylation of acid-denatured enolase. The wild-type cell line with the kinase-inactive Lyn showed an inhibition of G-CSF-induced tritiated thymidine incorporation, but the wild-type cell line with the kinase-inactive Jak2 showed no effect (Fig. 5C).

DISCUSSION

The data presented here show that G-CSF signaling can be reconstituted in B cells. The loss of mitogenic signaling as measured by tritiated thymidine incorporation in lyn-deficient cells suggests a critical role for Lyn in signaling by G-CSF. Stimulation with G-CSF of wild-type or syk-deficient cells showed a −2.2–4.4-fold increase in Lyn’s kinase activity. A 2-fold increase in Src’s activity is associated with ligand-induced or mitogenic-associated events (25–27). A role for Jak1 and Jak2 in nonmitogenic pathways or in the mitogenic pathway remains, but their ligand-dependent activation is not sufficient for G1/S phase progression. A 135-kDa protein appeared in all three lines and corresponds to the tyrosine-phosphorylated G-CSFR. Because phosphorylation of the 135-kDa protein was evident in the lyn-deficient cells, it appears that the receptor may be phosphorylated by a PTK other than Lyn or that redundant PTKs phosphorylate the receptor, and tyrosyl phosphorylation of the receptor is not sufficient to initiate a proliferative response. This conclusion is consistent with the mutational analyses of the receptor, which demonstrated that the C-terminal region that contains the phosphorylated tyrosine(s) is not required for proliferation (28). The data suggest that Lyn and the G-CSF receptor interact in specific ways. G-CSF or the GM-CSF/interleukin-3/interleukin-5 receptors form a macromolecular complex with Lyn (29, 30). The interaction of cytokine and growth factor receptors with intracellular kinases is considered as strong evidence of the involvement of specific kinases in the signaling pathways associated to the receptors (11). A recent crystallographic analysis of Src demonstrates a polyproline helix II structure between the Src homology domain 2 and the kinase domains and that the Src homology domain 3 domain can bind to this polyproline helix II structure (31, 32). It is, therefore, very interesting that the proximal domain of the G-CSF receptor, which is critical for proliferative signaling, contains a PXTPX sequence found in a polyproline helix II structure. A yeast two-hybrid analysis showed an interaction between the unique and Src homology domain 3 regions of Lyn and the cytoplasmic domain of the closely related erythropoietin receptor (33). Whereas the functional requirement for Lyn in mediating G-CSF’s induction of DNA synthesis is similar to that for Src kinases in mediating the platelet-derived growth factor or CSF-1’s induction of DNA synthesis (14–16), the structural mechanism of G-CSFR and Lyn association is probably different. In conclusion, the functional data presented here and the direct interaction of the G-CSF receptor with Lyn strongly suggests that the Lyn requirement for G-CSF signaling is not due to a parallel underlying process but to a direct involvement of Lyn in the G-CSF signaling pathway.

FIG. 3. Analysis of the Jak pathway in wild-type, lyn-deficient, and syk-deficient cells. Proteins from wild-type, lyn−/− or syk−/− cells were immunoprecipitated with antibody to Jak1 (Upstate Biotechnology) (A) or Jak2 (Upstate Biotechnology) (B). Proteins were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with antibody to phosphotyrosine. Proteins were visualized with enhanced chemiluminescence.

lines differs from the pronounced increase in Syk activity following G-CSF treatment in differentiated, nonproliferating neutrophils (17).

Jak Activation—We examined whether the activation of either Jak1 or Jak2 was affected by G-CSF in the transfected cell lines. Jak1 (19) or Jak2 (9) are activated in myeloid cells treated with G-CSF. Treatment of all three cell lines resulted in ligand-dependent tyrosyl phosphorylation of Jak1 and Jak2 (Fig. 3, A and B). Jak3 could not be detected by immunoblotting in these B cell lines.

G-CSF-Induced DNA Synthesis Studies—As shown, G-CSF-associated biochemical events were reconstituted in the B cell lines. Next, we sought to determine whether physiological responses to G-CSF could occur. Wild-type, lyn-deficient, and syk-deficient clones were cultured in the absence of fetal calf serum and chicken serum for 24 h and then stimulated with G-CSF and pulsed with tritiated thymidine. G-CSF-induced tritiated thymidine incorporation occurred in the wild-type and syk-deficient transfectants. Maximal effects of G-CSF was observed at 100–1000 ng/ml. These effects were of the same magnitude as those detected with fetal calf serum and chicken serum for 24 h and then stimulated with G-CSF. The cDNA for either kinase-inactive Lyn or kinase-inactive Jak2 was transfected into the wild-type cells that expressed the G-CSFR, and stable transfectants were selected by growth in neomycin. Stable transfectants were analyzed for Lyn kinase activity by immune complex kinase assay (Fig. 5A), and Jak2 kinase activity was assayed by anti-phosphotyrosine blotting of Jak2 immunoprecipitates (Fig. 5B). In both cases, the kinase-inactive form inhibited endogenous activity, and in the case of Lyn it blocked the phosphorylation of acid-denatured enolase. The wild-type cell line with the kinase-inactive Lyn showed an inhibition of G-CSF-induced tritiated thymidine incorporation, but the wild-type cell line with the kinase-inactive Jak2 showed no effect (Fig. 5C).
Phosphotyrosine-generated signal diversification of PI 3-kinase activity, tyrosine phosphorylation of Shc, and mitogen-activated protein kinase activity is defective in lyn-deficient cells. Studies of Syk kinase activity in these B cells differ from our report of Syk’s activity and co-precipitation with the G-CSF receptor in neutrophils (17). Baseline Syk activity is present in the B cells, whereas it is absent in neutrophils. Syk activity following G-CSF treatment is transient but pronounced in neutrophils. The presence of Syk activity in the lyn-deficient cells following stimulation is supported by evidence for Src-independent mechanisms for Syk (34, 35). Based on these studies, we conclude that Syk does not play a pivotal role in G-CSF-induced G1/S phase progression, although it may play a role in an accessory function such as G-CSF-enhancement of phagocytosis (1).

A Lyn knockout mouse strain has been developed from embryonic cell gene targeting, which demonstrates multiple defects in B cell and mast cell function (36, 37). Besides Lyn, myeloid cells contain the Src-related Hck or Fgr. The redundancy of Src-related PTK that occurs in myeloid cells might compensate for loss of a particular Src-related PTK. Although Lyn is found predominantly in both B cells and myeloid cells, B cells, such as those that we used in somatic cell targeting, do not contain the myeloid-specific Hck or Fgr (38). Thus, the problem of redundancy is avoided in our experimental system. All of the intracellular events, including Ras-GTP loading, Shc tyrosine phosphorylation, mitogen-activated protein kinase activation, and STAT3 and STAT5 gel shift activities observed in G-CSF-treated dividing cells have been reconstituted in this B cell system. Here, we have shown that a Src kinase is critical for the mitogenic signaling of a cytokine receptor and that

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2 M. J. Boyer, G. Romero, J. Gomez-Cambronero, S. Xu, P. Dombrosky-Perlan, T. Kurosaki, and S. J. Corey, manuscript submitted.

3 J. Xu, S. Zuo, and S. J. Corey, unpublished observations.
activation of Jak is not sufficient for this critical downstream event.

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