JAK2 Is Associated With the c-kit Proto-oncogene Product and Is Phosphorylated in Response to Stem Cell Factor

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Stem cell factor (SCF) is a hematopoietic growth factor that interacts with the receptor tyrosine kinase, c-kit. We have found that SCF stimulates rapid and transient tyrosine phosphorylation of JAK2 in human and murine cell lines, as well as in normal human progenitor cells. JAK2 and c-kit were associated in unstimulated cells with further recruitment of JAK2 to the c-kit receptor complex after SCF stimulation.

S T E M C E L L F A C T O R (SCF) also known as steel factor, kit ligand, and mast cell growth factor) is a critical component of normal hematopoiesis. Mutations in either the steel locus encoding SCF or the white spotting locus encoding c-kit, the SCF receptor, result in profound macrocytic anemias in mice (reviewed by Witte). The c-kit proto-oncogene product is a receptor tyrosine kinase and is closely related to the receptors for colony-stimulating factor-1 (CSF-1) and platelet-derived growth factor.

Another family of growth factors with profound effects on hematopoiesis are those interacting with hemopoietin receptor superfamily members. Included among these are erythropoietin (Epo), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-3 (IL-3). An interesting feature of the hemopoietin receptor superfamily is the capacity for ligands to elicit increases in protein tyrosine phosphorylation in the absence of tyrosine kinase activity intrinsic to the receptor. Recently, JAK2 has been found to be activated in response to GM-CSF, IL-3, and Epo. Furthermore, JAK2 is associated with each of these cytokine receptors, and in the case of the Epo receptor, there is a strong correlation between JAK2 activation, association with the receptor, and Epo-induced proliferation. Considered together, these findings have led investigators to postulate that the JAK family may play a critical role in initiating signal transduction pathways through receptors lacking intrinsic tyrosine kinase activity.

Little is known about the role of JAK family members in the signal transduction pathways of growth factors interacting with receptor tyrosine kinases. We have examined the SCF/c-kit signal transduction pathway to determine the potential involvement of the JAK2 protein tyrosine kinase.

MATERIALS AND METHODS

Cells and growth factors. Mo7e cells were maintained in RPMI 1640, 10% fetal calf serum, 2 mmol/L L-glutamine, and 1% penicillin-streptomycin (cell culture media) supplemented with recombinant human GM-CSF (10 ng/mL) and human IL-3 (1 ng/mL). FDCP-1 cells were maintained in cell culture media supplemented with recombinant murine IL-3 (20 ng/mL). Human GM-CSF, SCF, and IL-3 and murine IL-3 were purchased from PeproTech (Rocky Hill, NJ). Human fetal liver was obtained from 13- to 18-week fetuses after induced abortions (Advanced BioScience, Palo Alto, CA). All samples were obtained through protocols consistent with Institutional Review Board guidelines and with maternal consent. Single-cell suspensions were made from tissue and mononuclear cells isolated using lymphocyte separation media.

Immunoprecipitation, electrophoresis, and immunoblotting. Cells were resuspended in RPMI 1640 with 1% fetal calf serum, supplemented with SCF for the time(s) indicated, rapidly pelleted (3,000 rpm for 30 seconds at 4°C), and resuspended in lysis buffer (1% Triton X-100, 50 mmol/L NaCl, 10 mmol/L Tris, 5 mmol/L EDTA, 30 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 100 μmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.1% bovine serum albumin, pH 7.6). Lysates were incubated for a minimum of 30 minutes on ice, frozen, thawed, and centrifuged at 15,000 rpm at 4°C for 15 minutes. Immunoprecipitations of phosphoryrylated proteins were performed on the clarified supernatant with PY20 (ICN, Costa Mesa, CA), a monoclonal antibody recognizing phosphotyrosine. Immunoprecipitations of JAK2 were performed with antiserum specific for JAK2 (UBI, Lake Placid, NY). After a minimum of 2 hours rotating at 4°C, protein A Sepharose was added to capture the antigen-antibody complexes. Immunoprecipitates were washed three times with lysis buffer with bovine serum albumin and three times with lysis buffer without bovine serum albumin. Samples were then eluted from protein A Sepharose with sodium dodecyl sulfate (SDS) sample buffer. Electrophoresis and immunoblotting were performed as previously described. The blots were visualized using the ECL, Western Blotting detection system (Amersham, Arlington Heights, IL).

Oligonucleotide preparation. Unmodified oligonucleotides were synthesized using an Applied Biosystems 394 DNA/RNA synthesizer (Foster City, CA). The sequence of the JAK2 antisense oligomer was 5'-ATG GAG GCA ACC TTC ACA-3', corresponding to the third...
ATG described by Silvennoinen et al,2 and the sequence of the antisense oligomer was 5'-TGT GGA GGT TGC CTC CAT-3'. The sequences of oligomers used for polymerase chain reaction (PCR) amplification of JAK2 cDNA were 5'-GAC ATG ATG described by Silvennoinen et al? and the sequence of the probe was 5'-GAT GAC CttCA GlAAT CAT CTC AAC-3' and 5'-ATC CCC CGG TAC sequences of oligomers used for polymerase chain reaction (PCR) amplification of JAK2 cDNA were 5'-GCT ATC CTC AGA GAC CAA CAA CAA G'-3'. After synthesis, the oligomers were resuspended in HzO.

Reverse transcriptase-PCR analysis. RNA (500 ng) was reverse-transcribed for 1 hour at 37°C using 5 U/mL Moloney murine leukemia virus reverse transcriptase (RT) with 40 μmol/L oligo d(T),, and PCR-amplified using the GeneAmp RNA PCR kit (Perkin Elmer, Branchburg, NJ) as described by the manufacturer. The cycling conditions for actin amplification were as follows: denaturation at 94°C for 2 minutes, then 25 cycles of 94°C for 1 minute, and 72°C for 1 minute, followed by a chase step of 72°C for 7 minutes. Amplification of JAK2 was identical except that 35 cycles were used. PCR products were separated by electrophoresis on 3% Nusieve:Agarose 3:1 (FMC BioProducts, Rockland, ME), denatured, and transferred to nylon membranes as previously described.17 The filter was hybridized with oligomers internal to the PCR fragment that had been end-labeled using [γ-32P] adenosine triphosphate and T4 polynucleotide kinase. The probe for actin recognized a fragment of 701 basepairs, and the JAK2 probe hybridized to a fragment of 741 basepairs.

Treatment of cells with oligonucleotides for RT-PCR. FDCP-1 cells were washed four times and resuspended at 2.5 × 10^5/mL in cell culture media without growth factor. Aliquots of cells were placed into 24-well plates and incubated overnight (37°C 5% CO2) in the presence of sense or antisense oligonucleotides at a 5-μmol/L concentration. The following morning, cells were treated with sense or antisense oligonucleotides (5 μmol/L), incubated for 30 minutes to allow uptake of oligonucleotides, and then stimulated with growth factor. Six hours later, RNA was extracted using RNA STAT-60 (Tel-Test ‘‘B’’ Inc, Friendswood, TX).

Proliferation assays. FDCP-1 cells were washed four times and resuspended at 5 × 10^4/mL to cell culture media without growth factor. Aliquots of cells were placed into 96-well plates and incubated for 5 hours (37°C, 5% CO2) in the presence of sense or antisense oligonucleotides (5 μmol/L). Cells or murine SCF (200 ng/mL) were added to triplicate wells, and the appropriate wells were treated with additional sense or antisense oligonucleotides. The microtiter plates were incubated 72 hours at 37°C with 5% CO2. Each well was pulsed with 1 μCi 3H-thymidine (6.7 Ci/mmol/L; NEN, Boston, MA) for 6 to 8 hours and then harvested (Skatron Semiautomatic Cell Harvester) onto glass-fiber filter paper (Filtermat; Skatron Inc, Sterling, VA). Filter strips were dried and counted in a liquid scintillation counter (Model 1216; LKB, Piscatway, NJ). Similar results were obtained when cells were pretreated with oligonucleotides overnight. Proliferation assays with fetal liver cells were performed as described earlier, with the following exceptions: assays were performed in the serum-free medium, QBSF-58 (Quality Biologics, Rockville, MD), cells were seeded at 10^5/mL, the proliferation assay was pulsed with 1 μCi 3H-thymidine after 96 hours' incubation, and cells were harvested 12 hours later.

RESULTS

Phosphorylation of JAK2 after SCF stimulation. To characterize the effect of SCF on JAK2 phosphorylation, we first used the Mo7e cell line. These cells, of human origin, proliferate in response to SCF and other cytokines. As previously described, stimulation of Mo7e cells with SCF elicits a rapid increase in protein tyrosine phosphorylation (Fig 1A). The 125-kD phosphotyrosyl protein in SCF-treated cells was of particular note due to the size similarity to JAK2. Immu-
n blotting of p125 with JAK2-specific antisera strongly suggested that JAK2 was one of the phosphotyrosyl proteins induced by SCF (Fig 1B). To further address SCF-induced phosphorylation of JAK2, we performed antiphosphotyrosine immunoblotting of JAK2 immunoprecipitates from Mo7e cells stimulated with SCF. SCF-induced increases in JAK2 tyrosine phosphorylation were detectable within 1 minute, peaked by 2 minutes, and were close to baseline by 5 minutes (Fig 2A). The JAK2 immunoblots shown in Fig 2B demonstrate the presence of equivalent amounts of JAK2 in each point of the timecourse.

JAK2 and c-kit are constitutively associated. JAK2 has been found to be associated with a number of cytokine receptors, including receptors for Epo, GM-CSF, and IL-3. Interestingly, Fig 2A demonstrates coimmunoprecipitation of a 145-kD phosphotyrosyl protein with JAK2 after SCF stimulation. Based on the molecular weight of c-kit (~145 kD), we addressed the possibility that p145 was the SCF receptor. Shown in Fig 3 are the results of immunoblotting of control, c-kit, and JAK2 immunoprecipitates with antibody specific for human c-kit. Although c-kit is constitutively associated with JAK2, comparison with c-kit immunoprecipitates suggests that JAK2 is associated with only a fraction of the c-kit molecules in unstimulated cells. We next examined the effect of SCF on the JAK2/c-kit association. Mo7e cells were treated with SCF for 2 minutes, lysed, JAK2-immunoprecipitated, and then immunoblotted with c-kit antisera specific for human c-kit.

Fig 2. (A) SCF-induced tyrosine phosphorylation of JAK2. Mo7e cells were incubated for the indicated times with 100 ng/mL human SCF, lysed, and immunoprecipitated with JAK2 antisera. Proteins were resolved by SDS-PAGE, transferred to Immobilon, and immunoblotted with antibody specific for c-kit. (B) JAK2 immunoblot. The immunoblot from (A) was stripped and reprobed with antisera specific for JAK2.

Fig 3. (A) Constitutive association of JAK2 and c-kit. Mo7e cells were lysed and immunoprecipitated with either control, kit, or JAK2 antibodies. Proteins were resolved by SDS-PAGE, transferred to Immobilon, and immunoblotted with antibody specific for c-kit. (B) Effects of SCF on association of JAK2 and c-kit. Mo7e cells were stimulated with 100 ng/mL SCF, lysed, and immunoprecipitated with JAK2 antisera. Proteins were resolved by SDS-PAGE, transferred to Immobilon, and immunoblotted with c-kit antibody.
body. Figure 3B demonstrates that stimulation with SCF results in a small increase in JAK2/c-kit association over the constitutive level of association. In support of these findings, we have also observed that recombinant JAK2 protein interacts with c-kit in vitro (D.L., S.M., unpublished results, March 1995).

**Phosphorylation of JAK2 in normal human progenitor cells.** To determine if SCF-induced phosphorylation of JAK2 is relevant in normal hematopoietic tissue, we next examined human progenitor cells isolated from fetal liver. Treatment of fetal liver cells with SCF leads to increases in proliferation (Fig 4A). Similar to our observations in a human cell line, SCF treatment of fetal liver cells elicited rapid increases in tyrosine phosphorylation of JAK2, with peak responses noted 1 to 3 minutes after stimulation (Fig 4B). Lastly, reprobing of the blot from Fig 4B demonstrated that equivalent amounts of JAK2 were present in each immunoprecipitate (Fig 4C). These results demonstrate that SCF induced JAK2 phosphorylation in normal progenitor cells and in cell lines.

**The role of JAK2 in murine SCF signal transduction.** The phosphorylation of JAK2 in response to SCF, as well as its association with c-kit, suggested that JAK2 may play a role in the SCF/c-kit signal transduction pathway. To address this possibility, we examined the effects of JAK2 antisense oligonucleotides on SCF-induced proliferation. To date, only the murine JAK2 sequence has been published. We therefore examined the effect of SCF on phosphorylation of JAK2 in a murine cell line to determine if these cells would provide an adequate model for antisense studies. Shown in Fig 5A are the results of an experiment using the murine cell line, FDCP-1. These data demonstrate that murine SCF induced tyrosine phosphorylation of JAK2 in a manner comparable to that observed in human cells. One difference noted from our studies of the human cell line was the absence of phosphorylated c-kit in JAK2 immunoprecipitates. It is likely that the 10-fold lower expression of c-kit in FDCP-1 cells relative to Mo7e cells makes detection of the c-kit/JAK2 association more difficult. Nevertheless, phosphorylation of JAK2 in response to SCF suggested that antisense studies using murine cells were feasible.

We next examined the effect of JAK2 sense and antisense oligonucleotides on expression of JAK2 mRNA using RT-PCR. Treatment of FDCP-1 cells with JAK2 antisense oligonucleotides resulted in dramatic inhibition of JAK2 mRNA expression, whereas treatment with comparable concentrations of sense oligonucleotides had minimal effect on JAK2 expression (Fig 5B). In contrast, neither antisense nor sense oligonucleotides altered expression of β-actin. The effects of JAK2 antisense oligonucleotides on SCF-induced proliferation were next examined. A 46% decrease in SCF-induced proliferation was observed in the presence of 0.6 μmol/L antisense oligonucleotides as compared with the corresponding concentration of sense oligonucleotides (Fig 5C). A comparison of the dose-response curve for antisense and sense oligonucleotides is shown in Fig 5D. A partial inhibition of SCF-induced proliferation is evident at concentrations as low as 0.15 and 0.3 μmol/L antisense oligonucleotide. In total, these data suggest that the capacity of cells treated with JAK2 antisense oligonucleotides to respond to SCF is partially inhibited, and that this inhibition is related to a decrease in expression of JAK2.

**DISCUSSION**

Our results demonstrate that JAK2 plays a significant role in the SCF signal transduction pathway. In both murine and human cell lines, as well as normal progenitor cells, SCF induces rapid tyrosine phosphorylation of JAK2 (Figs 1, 2,
4, and 5). Furthermore, in the human cell line, Mo7e, some portion of JAK2 and c-kit exists in a preformed complex and SCF interaction with c-kit further recruits JAK2 to the receptor complex (Fig 3). Lastly, inhibition of JAK2 expression with antisense oligonucleotides leads to partial inhibition of SCF-induced proliferation (Fig 5). This could be attributed to several factors. First, complete abrogation of JAK2 protein expression over the entire time frame of the proliferation assay is likely to be difficult to achieve. Second, the JAK2 pathway may serve as only one component of the SCF signal transduction pathway. Previous studies on the hemopoietin receptor superfamily have shown that a dominant negative form of JAK2 completely inhibits Epo-induced proliferation, whereas IL-3–mediated responses are inhibited between 36% and 70%.21 Lastly, SCF also induces weak phosphorylation of JAK1 in Mo7e cells (data not shown). Thus, elimination of both JAK1 and JAK2 may be required for complete inhibition of SCF-induced proliferation.

The role of JAK2 in SCF signal transduction is controversial. Our data, although in agreement with one recent report, are in marked contrast to others.22,23 Tang et al22 reported no changes in tyrosine phosphorylation of JAK2 after a 5-minute stimulation with SCF. Our data suggest that the rapid and transient nature of SCF-induced JAK2 phosphorylation is a likely explanation for these conflicting observations. In contrast to the slower, more protracted phosphorylation of JAK2 in response to GM-CSF or IL-3, SCF-induced phosphorylation of JAK2 peaks 2 to 3 minutes after stimulation and is often at basal levels after a 5-minute stimulation.9,10 (data not shown). In many experiments, we have observed a window of JAK2 phosphorylation as small as 30 to 60 seconds. In addition, we have found that phosphorylation of JAK2 is more dramatic and more prolonged in Mo7e cells maintained in GM-CSF and IL-3. It appears that culturing cells in GM-CSF and SCF results in downregulation of c-kit/JAK2 complexes.
In recent years, much has been learned about the role of JAK family members in the signal transduction of hematopoietic growth factors that interact with receptors in the hemopoietin receptor superfamily. Less is known about the role of JAK family members in the signal transduction pathways of receptor tyrosine kinases. Although one report has demonstrated that EGF stimulated tyrosine phosphorylation of Jak1, the role of Jak1 in the EGF signal transduction pathway remains unclear. More indirectly, tyrosine phosphorylation of Stat1 has been observed in response to platelet-derived growth factor, EGF, and CSF-1. Interestingly, each of these ligands interacts with a receptor tyrosine kinase. In the case of EGF, ligand stimulation results in association of Stat3. These findings, in conjunction with our data, suggest that Jak2 (and potentially other JAK family members) may play a more critical role in the signal transduction pathways of receptor tyrosine kinases than previously thought.

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