Stat1 associates with c-kit and is activated in response to stem cell factor

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Interaction of stem cell factor (SCF), a haematopoietic growth factor, with the receptor tyrosine kinase c-kit leads to autophosphorylation of c-kit as well as tyrosine phosphorylation of various substrates. Little is known about the role of the JAK/STAT pathway in signal transduction via receptor tyrosine kinases, although this pathway has been well characterized in cytokine receptor signal transduction. We recently found that the *Janus* kinase Jak2 associates with c-kit and that SCF induces rapid and transient phosphorylation of Jak2. Here we present evidence that SCF activates the transcription factor Stat1. Phosphorylated c-kit co-immunoprecipitates with Stat1 within 1 min of SCF stimulation of the human cell line MO7e. Co-precipitation experiments using glutathione S-transferase fusion

proteins indicate that association with c-kit is mediated by the Stat1 SH2 domain. Stat1 is rapidly tyrosine-phosphorylated in response to SCF in MO7e cells, the murine cell line FDCP-1 and normal progenitor cells. SCF-induced phosphorylation of Jak2 and Stat1 was also observed in murine 3T3 fibroblasts stably transfected with full-length human c-kit receptor. Furthermore c-kit directly phosphorylates Stat1 fusion proteins in *in vitro* kinase assays. Electrophoretic mobility-shift assays with nuclear extracts from SCF-stimulated cell lines and normal progenitor cells indicate that activated Stat1 binds the m67 oligonucleotide, a high-affinity SIE promoter sequence. These results demonstrate that Stat1 is activated in response to SCF, and suggest that Stat1 is a component of the SCF signal-transduction pathway.

INTRODUCTION

Growth and differentiation of haematopoietic cells is regulated through the interaction of extracellular ligands with their cognate receptors. Two classes of receptors important in haematopoiesis are the members of the haematopoietin receptor superfamily [1] and members of the receptor tyrosine kinase (RTK) superfamily [2–4]. The JAK/STAT (where JAK is *Janus* kinase and STAT is signal transducer and activator of transcription) pathway has been found to be important in signal transduction initiated by ligands interacting with haematopoietin receptor superfamily members [5–8]. JAKs are constitutively associated with the membrane-proximal region of the cytoplasmic domain of these receptors, and cytokine binding activates receptor-associated JAKs, resulting in phosphorylation of the receptor and cross-phosphorylation of associated JAKs.

Activated JAKs have been proposed to mediate tyrosine phosphorylation of members of the STAT family of latent cytoplasmic transcription factors [9]. STATs are recruited to the receptor supercomplex via interactions between their src homology 2 (SH2) domains and specific phosphotyrosyl groups on the activated receptor [10,11]. These STATs subsequently undergo tyrosine phosphorylation and dimerize via their SH2 domains [12], facilitating their release from the receptor complex. STAT dimers translocate to the nucleus, bind specific DNA promoter sequences, and activate gene expression.

In contrast with stimulus–response coupling of the haematopoietin receptor superfamily members, the role of the JAK/STAT pathway in signal transduction via RTKs is not well characterized. Recent evidence indicates that the RTK ligands EGF, PDGF and CSF-1 activate components of the JAK/STAT pathway in fibroblast cell lines [13–15]. For example, EGF stimulation of cells results in tyrosine phosphorylation of Jak1 [16]. EGF also promotes direct association of Stat1 with the

activated EGF receptor [17]. Furthermore Stat1, Stat3 and Stat5 are tyrosine-phosphorylated in response to EGF, and the DNA-binding activity of these STATs is activated [14,16–22].

Stem cell factor (SCF), a growth factor critical for normal haematopoiesis [23], is the ligand for c-kit, a receptor tyrosine kinase [24,25]. The SCF–c-kit interaction leads to autophosphorylation of c-kit as well as elevated tyrosine phosphorylation of a number of signal-transduction molecules including Tec [26], Raf-1 [27,28], mitogen-activated protein kinases [27–29], SHC [30], phospholipase C γ [28] and p95^{vav} [31,32].

Although little is known about the role of STAT family members in SCF-mediated signal transduction, our laboratory and others have previously reported that Jak2 is associated with c-kit and undergoes tyrosine phosphorylation in response to SCF stimulation [33,34]. In this study we provide evidence for SCF-mediated activation of Stat1 in haematopoietic cell lines, c-kit-transfected fibroblasts and normal progenitor cells. Stimulation of MO7e cells with SCF resulted in rapid association of Stat1 with activated c-kit receptor. This association is mediated by the Stat1 SH2 domain. In addition, Stat1 was rapidly and transiently phosphorylated in response to SCF in human and murine cell lines as well as in normal human progenitor cells. Phosphorylation of Stat1 fusion proteins in *in vitro* kinase assays with c-kit suggests that c-kit is capable of directly phosphorylating Stat1. Also, SCF rapidly induced the formation of DNA-binding complexes that contained Stat1. These results indicate that the SCF–c-kit interaction leads to activation of the JAK/STAT signal-transduction pathway.

MATERIALS AND METHODS

Cells and growth factors

Human megakaryoblastic MO7e cells were maintained in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS), 2 mM L-

Abbreviations used: ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility-shift assay; GST, glutathione S-transferase; JAK, *Janus* kinase; mAb, monoclonal antibody; pAb, polyclonal antibody; RTK, receptor tyrosine kinase; SCF, stem cell factor; SH2, src homology type 2; STAT, signal transducer and activator of transcription; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; CSF-1, colony-stimulating factor 1; FBS, fetal bovine serum.

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glutamine, 1 % penicillin/streptomycin, 10 ng/ml recombinant human granulocyte/macrophage colony-stimulating factor and 100 ng/ml human SCF. Murine FDCP-1 cells were grown in RPMI 1640 with 10 % FBS, 2 mM L-glutamine, 1 % penicillin/streptomycin and 20 ng/ml murine interleukin 3. Murine NIH 3T3 fibroblasts expressing wild-type human c-kit receptor (3T3/c-kit cells; a gift from Axel Ullrich, Max-Planck Institute, Martinsried, Germany) were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % FBS, 2 mM L-glutamine and 1 % penicillin/streptomycin. Fetal bovine serum was obtained from Atlanta Biologicals (Atlanta, GA, U.S.A.). Human SCF, human granulocyte/macrophage colony-stimulating factor and murine interleukin 3 were supplied by Peprotech (Rocky Hill, NJ, U.S.A.). Murine SCF was provided by Kirin (Tokyo, Japan). Normal progenitor cells were isolated from human fetal liver obtained from 16–22-week fetuses after induced abortions (Advanced Bioscience, Palo Alto, CA, U.S.A.). All samples were obtained through protocols consistent with International Research Bureau guidelines and with maternal consent. Single-cell suspensions were made from the tissue, and mononuclear cells were isolated using lymphocyte separation medium (Organon Technika, Durham, NC, U.S.A.).

Immunoprecipitation and immunoblotting

Cells were washed and resuspended in appropriate medium + 1 % FBS, stimulated with SCF, rapidly pelleted at 5000 *g* (10000 rev./min) in Microfuge, and resuspended in lysis buffer as described below. For studies using 3T3/c-kit fibroblasts, cultures were grown to near-confluence and starved for 12–16 h in Dulbecco's modified Eagle's medium + 1 % FBS before stimulation. After SCF stimulation, medium was removed and monolayers were washed once with ice-cold PBS + 1 mM Na₃VO₄. Cells were scraped into ice-cold lysis buffer as described below.

All cells were lysed in either ice-cold lysis buffer (1 % Triton X-100, 50 mM NaCl, 10 mM Tris/HCl, 5 mM EDTA, 5 mM sodium pyrophosphate, 5 mM NaF, 100 μ M Na₃VO₄, 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin and 0.1 % BSA, pH 7.6) or ice-cold HPI lysis buffer (1 % Triton X-100, 50 mM NaCl, 10 mM Tris/HCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 5 mM NaF, 5 mM Na₃VO₄, 25 mM β -glycerophosphate, 1 mg/ml *p*-nitrophenyl phosphate, 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, pH 7.6). Lysates were incubated for 30 min on ice and clarified by centrifugation at 5000 *g* (10000 rev. min) at 4 °C for 5 min. Proteins were immunoprecipitated from clarified lysates at 4 °C for 2 h using the antibodies specified in the text. Protein A–Sepharose was added to capture the antibody–antigen complexes, and immunoprecipitates were incubated for an additional hour at 4 °C. Immunoprecipitates were washed three times with lysis buffer and boiled for 3 min in SDS sample buffer. Proteins were resolved by SDS/PAGE, transferred to Immobilon (Millipore Corp., New Bedford, MA, U.S.A.), and immunoblotted as previously described [35].

Immunoprecipitation of c-kit was performed with anti-c-kit monoclonal antibody (mAb) SR-1 (a gift from Virginia Broudy, University of Washington, Seattle, WA, U.S.A.). Immunoblots were probed for phosphotyrosine with 4G10 mAb from Upstate Biotechnologies (Lake Placid, NY, U.S.A.), for c-kit with polyclonal antibody (pAb) obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) or 3D6 mAb from Boehringer-Mannheim (Indianapolis, IN, U.S.A.), and for Stat1 with ISGF3 pAb [anti-ISGF3 (p91/84) affinity-purified pAb purchased from Transduction Laboratories (Lexington, KY, U.S.A.)]. Immunoblots were visualized by enhanced chemi-

luminescent detection (ECL; DuPont/NEN, Wilmington, DE, U.S.A.). Membranes to be probed a second time were stripped with 15 % H₂O₂ for 30 min at room temperature.

Glutathione S-transferase (GST) fusion proteins

DNA corresponding to the Stat1 SH2 domain (SH2, amino acids 569–700), SH3 and SH2 domains (SH3+SH2, amino acids 506–700), SH2 and C-terminal domains (SH2+C-TERM, amino acids 569–750) and SH3, SH2 and C-terminal domains (SH3+SH2+C-TERM, amino acids 506–750) [36,37] was generated by PCR from a plasmid containing full-length human Stat1 cDNA (a gift from Dr. James Darnell, Rockefeller University, New York, NY, U.S.A.). Primers were designed to generate PCR products with 5' *Bam*HI and 3' *Eco*RI restriction sites. PCR products were gel-purified, double-digested with *Bam*HI and *Eco*RI, and ligated into the *Bam*HI–*Eco*RI sites of the GST fusion protein vector pGEX4T1 (Pharmacia, Piscataway, NJ, U.S.A.). Constructs were transformed into *Escherichia coli* DH5 α and screened for fusion protein expression as described below. DNA sequence was confirmed by dideoxy sequencing of miniprep plasmid DNA using pGEX antisense primers by the Sequenase protocol (Amersham).

Fusion proteins were expressed and purified by a modification of the methods of Smith and Johnson [38] and Frorath et al. [39]. Briefly, overnight cultures of transformed bacteria were diluted 1:10 into Luria broth + 50 μ g/ml ampicillin and grown at 37 °C for 60–90 min. Fusion protein expression was induced by the addition of 1 mM isopropyl β -D-thiogalactoside. Bacteria were grown for an additional 3–4 h and harvested by centrifugation. Bacterial pellets were resuspended in HPI lysis buffer + 1 μ g/ml lysozyme and incubated for 15 min on ice. Lysates were clarified by centrifugation and fusion proteins were purified from lysate supernatants by affinity chromatography using glutathione–Sepharose 4B (Pharmacia). After incubation at 4 °C for 60 min, the glutathione–Sepharose resin was washed three times with 20 vol. of HPI lysis buffer. Fusion proteins were stored bound to resin at 4 °C in HPI lysis buffer.

Co-precipitation assays were performed using equivalent amounts of bound fusion proteins incubated with clarified cell lysates for 3 h at 4 °C. Resin was washed three times with HPI lysis buffer, and co-precipitating proteins were eluted by boiling in SDS sample buffer. Proteins were resolved by SDS/PAGE, transferred to Immobilon, and immunoblotted with 3D6 anti-c-kit monoclonal antibody as previously described.

Kinase assays

Activated c-kit was immunoprecipitated from clarified lysates of SCF-stimulated 3T3/c-kit cells as described above. After being washed in lysis buffer, immunoprecipitates were washed once with ice-cold 25 mM Hepes, pH 7.6. Fusion protein (bound to glutathione–Sepharose) was added to the appropriate tubes and all samples were washed once with ice-cold kinase buffer (25 mM Hepes, 10 mM MnCl₂, pH 7.6). Reactions were started by the addition of 100 μ M ATP, incubated for 15 min on ice, and terminated by boiling in SDS sample buffer. Proteins were resolved by SDS/PAGE, transferred to Immobilon, and immunoblotted for phosphotyrosine as previously described.

Electrophoretic mobility-shift assays (EMSAs)

EMSAs were performed by a modification of the procedure of Ohashi et al. [40]. Cytoplasmic extracts containing 15 μ g of protein or nuclear extracts containing 10 μ g of protein were

mixed with 5 μ g of poly(dI-dC) (Pharmacia) with or without unlabelled competitor oligonucleotides (100-fold excess over labelled probe) and incubated on ice for 15 min. Probe DNA labelled with Klenow fragment and [α - 32 P]dCTP or T4 polynucleotide kinase and [γ - 32 P]ATP was added so the binding reaction mixture had a final volume of 30 μ l and contained a final concentration of 13 mM Hepes (pH 7.9), 65 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA and 8% glycerol. The mixture was then incubated at room temperature for 30–60 min. For supershift studies, antibodies were added to the mixture of extract and poly(dI-dC) and incubated for 1 h at 4°C before addition of labelled probe. DNA–protein complexes were resolved by electrophoresis for 2 h at 225 V in 0.5 $\times$ TBE [45 mM Tris-borate (pH 8.0), 1 mM EDTA] buffer on a non-denaturing 4% polyacrylamide gel containing 5% glycerol. The gels were dried and exposed to Kodak XAR film at –80°C.

The probe was a double-stranded oligodeoxynucleotide corresponding to the high-affinity serum-inducible element (SIE) (m67) sequence (5'-GTCGACATTTCCTGTAATC-3') [41]. Competitors were unlabelled SIE probe, the Ly-6E γ -activated sequence (GAS) (5'-ATATTCCTGTAAAGTG-3') [42], the γ -response region (GRR) sequence (5'-AAGGATGTATTC-CCAGAAAAG-3') [43] and a non-related sequence (NRS) (5'-ACGTACGTACGTACGTACGT-3'). Antibodies used for supershifts included ISGF3 pAb [anti-Stat1 pAb (amino acids 1–194; G16930)], ISGF3 mAb [anti-Stat1 mAb (amino acids 1–194; G16920)] and Stat1 mAb [anti-Stat1 mAb (amino acids 592–731; S21120)], all obtained from Transduction Laboratories. Stat1 mAb*, an anti-Stat1 mAb (amino acids 613–739; sc464), was purchased from Santa Cruz Biotechnology, Inc.

RESULTS

SCF induces association of Stat1 with c-kit

Ligands interacting with haematopoietin superfamily members elicit rapid association of STATs with specific phosphotyrosyl residues on the cytoplasmic tail of the receptor [16,44]. To examine potential c-kit–STAT associations, we employed the SCF-responsive cell line MO7e, a human megakaryoblastic cell line expressing approx. 10 000 SCF-binding sites/cell [45]. The high level of c-kit expression makes these haematopoietic cells ideal for study of c-kit interactions with signal-transduction components.

To address whether SCF activated STAT family members, we first examined whether SCF induced association of c-kit and the STAT family member Stat1. We chose to analyse Stat1 because it has been shown to be activated in signalling pathways involving the RTKs for EGF, PDGF and CSF-1 in fibroblast cell lines [13–19,46]. MO7e cells were stimulated with SCF for various times and lysates were used in immunoprecipitations with Stat1 mAb*. As shown in Figure 1(A), Stat1 immunoprecipitates from SCF-stimulated MO7e cells co-precipitate a phosphotyrosyl protein of 145 kDa, the approximate size of c-kit [47]. The kinetics of this reaction are very rapid, as the 145 kDa species associates with Stat1 within 1 min of SCF stimulation. The association is also prolonged, persisting for at least 10 min after stimulation.

To determine if the 145 kDa protein represented c-kit, the immunoblot was stripped and the top portion reprobed with antibody specific for c-kit. The c-kit immunoblot shown in Figure 1(B) identifies the associated 145 kDa phosphotyrosyl protein as c-kit. Importantly, identical results were obtained when the c-kit immunoblot was performed directly on the Stat1 immunoprecipitate (results not shown). This excludes the possibility that the band in the c-kit immunoblot (Figure 1B) reflects

Figure 1 SCF induces Stat1 association with c-kit

(A) Co-immunoprecipitation of Stat1 with c-kit. MO7e cells were stimulated with 100 ng/ml human SCF for the times indicated. Lysates were immunoprecipitated with Stat1 mAb* or anti-c-kit mAb (SR-1). Proteins were resolved by SDS/PAGE, transferred to Immobilon, and immunoblotted with a mAb specific for phosphotyrosine. Proteins were visualized using ECL. (B) c-kit immunoblot. The top portion of the blot in (A) was stripped and reprobed with pAb specific for c-kit. (C) Stat1 immunoblot. The bottom portion of the blot in (A) was stripped and reprobed with ISGF3 pAb.

a residual band from the phosphotyrosine immunoblot (Figure 1A). To determine if equivalent amounts of Stat1 were present in each precipitate, the bottom portion of the stripped blot was reprobed with antibody specific for Stat1. Figure 1(C) confirms that equivalent amounts were present in each immunoprecipitate. The absence of detectable Stat1 in c-kit immunoprecipitates is probably due to the high expression of c-kit as compared with Stat1. In addition, comparison of the amounts of c-kit detected in Stat1 immunoprecipitates compared with c-kit immunoprecipitates (Figure 1B) indicates that only a small portion of total cellular c-kit associates with Stat1. Further, the associated c-kit may represent a hyperphosphorylated form of the receptor. Examination of the ratio of phosphorylated c-kit (Figure 1A) to total c-kit protein (Figure 1B) reveals that the amount of phosphorylated c-kit is significantly elevated in Stat1 immunoprecipitates as compared with c-kit immunoprecipitates. Also, the 145 kDa phosphotyrosyl species that co-immunoprecipitates with Stat1 has an increased apparent molecular mass relative to total c-kit detected in c-kit immunoprecipitates. Overall, co-immunoprecipitation of c-kit with Stat1 parallels the SCF-induced phosphorylation of c-kit (results not shown). In total, these results strongly suggest that Stat1 associates specifically with the phosphorylated form of the c-kit receptor.

A phosphotyrosyl protein of approx. 100 kDa (p100) was also detected in Stat1 immunoprecipitates from SCF-stimulated cells (Figure 1A). This protein was recognized by anti-Stat1 antibody in immunoblots (Figure 1C), suggesting that it is antigenically related to Stat1. However, Stat1 itself did not appear to be phosphorylated in response to SCF. No phosphotyrosyl species

Figure 2 c-kit associates with the Stat1 SH2 domain

(A) Co-precipitation of c-kit with GST fusion protein containing the Stat1 SH2 domain. Clarified lysates from SCF-stimulated 3T3/c-kit cells were incubated for 3 h at 4 °C with equivalent amounts of fusion protein (Stat1 SH3+SH2+C-TERM or GST) bound to glutathione-Sepharose. Co-precipitated proteins were resolved by SDS/PAGE, transferred to Immobilon and immunoblotted for c-kit with 3D6 mAb. (B) Co-precipitation of c-kit with Stat1 SH2-containing fusion protein. Repeat of experiment in (A) using lysates from SCF-stimulated M07e cells.

Figure 3 Stat1 is phosphorylated in response to SCF

(A) Tyrosine phosphorylation of Stat1 (p91) in M07e cells. Cells were stimulated with 100 ng/ml human SCF for the times indicated. Lysates were immunoprecipitated with ISGF3 pAb. Proteins were resolved by SDS/PAGE, transferred to Immobilon and immunoblotted with mAb specific for phosphotyrosine. IP, immunoprecipitate; IB, immunoblot. (B) Stat1 immunoblot. The immunoblot in (A) was stripped and reprobed with ISGF3 pAb. (C) Kinetics of Stat1 phosphorylation in response to SCF. Relative phosphorylation was normalized for amount of Stat1 by scanning densitometry of (A) and (B).

corresponding to Stat1 was detected (Figure 1A), although both the p91 (Stat1 α) and p84 (Stat1 β) isoforms of Stat1 were immunoprecipitated (Figure 1C).

One explanation for the absence of Stat1 phosphorylation may be related to the antibody used for this study. Stat1 mAb* is

Figure 4 Stat1 is phosphorylated in response to SCF

(A) Tyrosine phosphorylation of Stat1 (p91) in FDCP-1 cells. Cells were stimulated with 100 ng/ml murine SCF for the times indicated. Lysates were immunoprecipitated with ISGF3 pAb. Proteins were resolved by SDS/PAGE, transferred to Immobilon and immunoblotted with a mAb specific for phosphotyrosine. (B) Stat1 immunoblot. The immunoblot in (A) was stripped and reprobed with ISGF3 pAb specific for Stat1. (C) Stat1 phosphorylation in normal progenitor cells. Normal progenitor cells were isolated from human fetal liver using lymphocyte separation medium. Cells were cultured overnight in serum-free medium (QBSF-58; Quality Biologies, Gaithersburg, MD, U.S.A.) before stimulation with 100 ng/ml human SCF for the times indicated. Lysates were immunoprecipitated for Stat1 with ISGF3 pAb. Proteins were separated by SDS/PAGE, transferred to Immobilon and immunoblotted with mAb specific for phosphotyrosine. (D) Stat1 immunoblot. The blot in (C) was stripped and reprobed with ISGF3 pAb.

directed against amino acids 613–739 near the C-terminus of Stat1, a region that includes the single conserved tyrosine residue (Tyr-701) phosphorylated in response to cytokine stimulation [16,44]. It appears likely that this epitope is masked during Stat dimerization. Since dimers form as a consequence of Stat1 phosphorylation, this particular antibody may selectively bind non-phosphorylated Stat1 in both the latent cytoplasmic and receptor-bound states. Similar experiments were performed using an anti-Stat1 pAb antibody (ISGF3 pAb) which does recognize phosphorylated Stat1 (see Figure 3). This antibody also co-immunoprecipitates a 145 kDa phosphotyrosyl protein which co-migrates with c-kit (results not shown).

The Stat1 SH2 domain mediates association with c-kit

Studies on receptor tyrosine kinases other than c-kit have demonstrated direct binding of SH2 domains within STAT proteins to specific phosphotyrosyl residues on activated receptors [17]. To determine whether such an association occurs between c-kit and Stat1, we performed co-precipitation studies using GST

Figure 5 SCF activates the JAK/STAT pathway in 3T3 fibroblasts transfected with c-kit

(A) SCF-induced phosphorylation of c-kit. Murine 3T3 fibroblasts stably transfected with wild-type human c-kit (3T3/c-kit cells) were stimulated with 100 ng/ml human SCF for the times indicated. Lysates were immunoprecipitated with anti-c-kit mAb. Proteins were resolved by SDS/PAGE, transferred to Immobilon and blotted with anti-phosphotyrosine mAb. (B) Jak2 phosphorylation. 3T3/c-kit lysates were immunoprecipitated with anti-Jak2 pAb. Proteins were resolved by SDS/PAGE, transferred to Immobilon and immunoblotted with anti-phosphotyrosine mAb. (C) Stat1 phosphorylation. 3T3/c-kit cell lysates were used in immunoprecipitates with ISGF3 pAb. Proteins were resolved, transferred and immunoblotted with anti-phosphotyrosine mAb as described previously. (D) Stat1 immunoblot. The blot from (C) was stripped and reprobed for Stat1 with ISGF3 pAb. (E) Comparison of kinetics of phosphorylation of Jak2 and Stat1 in response to SCF. Relative phosphorylation was normalized for amount of Stat1 by scanning densitometry of (C) and (D).

fusion proteins containing the SH2 domain of Stat1. The fusion protein containing the Stat1 SH3 and SH2 domains as well as the C-terminal sequence (SH3+SH2+C-TERM) specifically precipitates c-kit from lysates prepared from both SCF-stimulated 3T3/c-kit cells (Figure 2A) and MO7e cells (Figure 2B). c-kit was also specifically precipitated from SCF-stimulated 3T3/c-kit cell lysates by fusion protein containing the SH2 domain only, as well as by fusion proteins SH3+SH2 and SH2+C-TERM (results not shown). These data confirm the association of Stat1 and c-kit and demonstrate that this interaction is mediated by the SH2 domain of Stat1.

Stat1 is phosphorylated in response to SCF

To determine if SCF induced increases in tyrosine phosphorylation of Stat1, we used a different antibody from the monoclonal one used in the co-immunoprecipitations shown in Figure 1. The antibody used in the phosphorylation experiments was ISGF3 pAb, a polyclonal directed against the N-terminal region of Stat1 (amino acids 1–194). Human megakaryoblastic MO7e cells were stimulated with SCF at room temperature for the indicated times and the lysates were used in immunoprecipitations with ISGF3 pAb. Figure 3 demonstrates that Stat1 is tyrosine-phosphorylated in response to SCF in MO7e cells. Note that phosphorylation is limited to Stat1 α (p91), the higher-molecular-mass isoform of Stat1. There was no apparent phosphorylation of Stat1 β (p84, which lacks the C-terminal 38 amino acids) [36] in MO7e cells (Figure 3A), although Stat1 β was detected in these immunoblots (Figure 3B). In addition, we occasionally observed tyrosine phosphorylation of an approx.

75 kDa species antigenically related to Stat1 α / β (results not shown).

The kinetics of Stat1 α phosphorylation are rapid and transient, with the peak of phosphorylation at approx. 5 min after stimulation (Figure 3A). Normalization of Stat1 phosphorylation levels relative to amounts of Stat1 protein precipitated reveals that tyrosine phosphorylation increased 35-fold after 4 min of SCF stimulation (Figure 3C). Similar results were obtained with a SCF-responsive clone of the murine myeloid cell line FDCP-1 (Figures 4A and 4B). In FDCP-1 cells Stat1 tyrosine phosphorylation was also rapid, extremely transient and restricted to the p91 isoform.

These data demonstrate that SCF induced the tyrosine phosphorylation of Stat1 in both human and murine cell lines. To determine if this occurs in normal haematopoietic tissue, we next examined whether SCF induced Stat1 phosphorylation in normal progenitor cells isolated from human fetal liver tissue. Cells were cultured for 16 h in serum-free medium and stimulated with human SCF; lysates were examined for evidence of Stat1 phosphorylation as described above. Increased phosphorylation of Stat1 α was detected within 10 min of SCF stimulation (Figures 4C and 4D). Further, at 20 min after stimulation, Stat1 α phosphorylation remained 4-fold higher than in unstimulated cells. Thus SCF induced increases in tyrosine phosphorylation of Stat1 in normal progenitor cells as well as MO7e and FDCP-1 cell lines.

Further evidence for SCF activation of the JAK/STAT pathway was obtained using 3T3 murine fibroblasts stably transfected with wild-type human c-kit. SCF treatment of these cells induces immediate increases in protein tyrosine phos-

Figure 6 Stat1 is phosphorylated by c-kit *in vitro*

c-kit was immunoprecipitated from clarified lysates of SCF-stimulated 3T3/c-kit cells. Immunoprecipitates were washed in lysis buffer and Stat1 SH2 + C-terminal fusion protein was added to the indicated samples. All samples were transferred to kinase buffer (25 mM Hepes, 10 mM $MnCl_2$, pH 7.6). Reactions were started by addition of 100 μ M ATP, incubated for 15 min on ice, and terminated by boiling in SDS sample buffer. Proteins were resolved by SDS/PAGE, transferred to Immobilon and immunoblotted with anti-phosphotyrosine mAb. The band indicated by the arrowhead is antibody heavy chain.

phorylation as well as increases in proliferation. We first examined tyrosine phosphorylation of c-kit and Jak2, proteins upstream of STATs in the JAK/STAT signalling pathway. In these experiments, 3T3/c-kit cells were grown to near-confluency, then starved for 12–16 h in 1 % FBS/Dulbecco's modified Eagle's medium. Cells were SCF-stimulated, lysed, and used in immunoprecipitations with anti-c-kit mAb SR-1 and anti-Jak2 pAb.

Both c-kit (Figure 5A) and Jak2 (Figure 5B) are tyrosine-phosphorylated in response to SCF stimulation of 3T3/c-kit cells. Figure 5(C) shows that SCF induced an increase in Stat1 α phosphorylation in 3T3/c-kit cells. Interestingly, the kinetics of SCF-induced phosphorylation of Stat1 α is slower in 3T3/c-kit cells relative to the kinetics observed in haematopoietic cell lines (Figures 3 and 4). In 3T3/c-kit cells, SCF-mediated Stat1 phosphorylation peaks at 12–15 min after stimulation compared with 5 min after stimulation for Stat1 tyrosine phosphorylation in MO7e and FDCP-1 cells. In addition, Jak2 phosphorylation precedes Stat1 phosphorylation after SCF stimulation of the 3T3/c-kit cells (Figure 5E).

c-kit directly phosphorylates Stat1 fusion proteins

The association of Stat1 with activated c-kit, as well as the strong correlation between c-kit activation and Stat1 tyrosine phosphorylation, led us to ask whether Stat1 could be directly phosphorylated by c-kit. To answer this question, we employed GST fusion proteins of Stat1 *in vitro* kinase assays with c-kit. Activated c-kit was immunoprecipitated from SCF-stimulated 3T3/c-kit cells and mixed with a fusion protein containing the SH2 domain and C-terminal region of Stat1 (SH2 + C-TERM). This fusion protein contains the unique tyrosine residue (Tyr-701) shown to be phosphorylated in response to stimulation by cytokines [16,44]. Figure 6 shows that, in the presence of ATP, SH2 + C-TERM was specifically tyrosine-phosphorylated in samples containing activated c-kit. These results demonstrate that c-kit is capable of phosphorylating Stat1 directly.

SCF induces Stat1 DNA-binding activity

To determine if stimulation of cells by SCF induces DNA-binding factors that interact with DNA sequences known to be recognized by activated STAT family members, EMSAs were performed using 32 P-labelled probe corresponding to the high-affinity SIE sequence m67. 3T3/c-kit cells (Figure 7A) and normal progenitor cells (Figures 7B and 7C) were stimulated with SCF for the indicated times and used to generate cytosolic

Figure 7 SCF activates DNA-binding complexes that contain Stat1

(A) SCF induces Stat1 DNA-binding activity in 3T3/c-kit fibroblasts. EMSAs were performed using cytosolic extracts from SCF-stimulated 3T3/c-kit cells. Extracts were incubated with radiolabelled m67 oligonucleotide, a high-affinity mutant SIE sequence (5'-GTGACATTCCCGTAAATC-3'), in the presence of 100 $\times$ molar excess of the indicated oligonucleotide. For supershift experiments, extracts were incubated with 1 μ g of the indicated antibody. DNA-protein complexes were resolved by PAGE and visualized by autoradiography. The position of the DNA-binding complex induced by SCF is indicated by an arrow. (B) SCF induces DNA-binding activity in normal progenitor cells. Nuclear extracts from normal progenitor cells isolated from human fetal liver were incubated with radiolabelled m67 oligonucleotide and resolved and visualized as described for (A). (C) Antibodies to Stat1 supershift DNA-binding complexes. Nuclear extracts from normal progenitor cells were incubated with radiolabelled m67 oligonucleotide in the presence of 100 $\times$ molar excess of the indicated oligonucleotide or 1 μ g of the indicated antibody. The position of the DNA-binding complex induced by SCF is indicated by an arrow. Stat1 pAb, anti-Stat1 pAb (a gift from Andrew Larner, NIH, Bethesda, MD, U.S.A.).

and nuclear extracts. No DNA-binding activity was detected in extracts from unstimulated 3T3/c-kit cells (Figure 7A, lane 0). SCF stimulation of both 3T3/c-kit and normal progenitor cells activated a DNA-binding factor as seen by the single mobility-shift complex detected by the SIE probe (Figures 7A and 7B). SCF stimulation also induced DNA-binding activity in MO7e cells (results not shown). The DNA-binding complex detected with the SIE probe was specific, as determined by competitive inhibition with an excess of unlabelled SIE, GAS and GRR probes. Equivalent concentrations of an unrelated oligonucleotide (NRS) had no effect on the SCF-induced DNA-binding complex.

To determine whether the DNA-binding factors induced by

SCF stimulation were related to Stat1 protein, we next examined whether antibodies specific for Stat1 recognized components of the DNA-binding complex induced by SCF. As shown in Figure 7(A), addition of antibodies directed against both the N-terminus (ISGF3 pAb and ISGF3 and mAb) and the C-terminus (Stat1 mAb) of Stat1 resulted in a supershifted SIE-binding complex in extracts from 3T3/c-kit cells. Similar results were obtained in supershift assays using extracts from normal progenitor cells (Figure 7C). In these assays, two different polyclonal antibodies directed against the N-terminus of Stat1 supershifted the DNA-binding complex. Also, Stat1 mAb* inhibited complex-formation, shifting the DNA-binding complex toward a faster migrating form. These data provide strong evidence that Stat1 is present in the DNA-binding complex induced by SCF in both cell lines and normal progenitor cells.

DISCUSSION

JAK and STAT family proteins are critical components of signal-transduction pathways initiated by interferon receptors as well as members of the haematopoietin receptor superfamily. Cytokine binding to these receptors, which lack intrinsic kinase activity, results in activation of JAK kinases, followed by phosphorylation and activation of STAT transcription factors. A number of recent reports suggest that receptor tyrosine kinases also activate components of the JAK/STAT pathway. Although these reports indicate that the RTKs for EGF, PDGF and CSF-1 activate components of the JAK/STAT pathway, the role of the JAK/STAT pathway in signal transduction by these growth factors remains controversial. Interestingly, although two groups have demonstrated that SCF induced activation of the *Janus* family kinase Jak2, little is known about the effects of SCF on STAT family members. We have examined the effects of SCF on the STAT family member Stat1 in haematopoietic cell lines, normal haematopoietic progenitor cells and fibroblasts transfected with c-kit.

In this paper we have presented data indicating that stimulation of the human megakaryoblastic cell line MO7e with SCF results in rapid association of Stat1 with the RTK c-kit (Figure 1). Stat1 association parallels tyrosine phosphorylation and activation of the c-kit receptor, and appears selective for the phosphorylated form of the receptor. This result is consistent with current models of JAK/STAT signal transduction, in which STATs associate with cytokine receptors by binding of the SH2 domain of STAT proteins to specific phosphotyrosyl residues on activated receptors. Indeed, our co-precipitation experiments with GST fusion proteins containing the Stat1 SH2 domain confirm that Stat1 association with c-kit is mediated by the Stat1 SH2 domain (Figure 2). This result is in agreement with previous work with other receptors, which provided evidence for direct interaction between STAT proteins and RTKs [17,48,49].

The specific phosphotyrosyl residues on activated c-kit to which Stat1 binds are yet to be determined. One candidate residue for Stat1 association is Tyr-823, the homologue of the CSF-1 receptor Tyr-809 which mediates CSF-1-induced activation of STAT proteins [46]. To address this question, we have initiated experiments aimed at mapping the Stat1-binding site(s) on the c-kit receptor.

SCF induces rapid and transient tyrosine phosphorylation of Stat1 α (p91) in both human and murine cell lines (Figures 3, 4 and 5). This result is similar to that of Fu and Zhang [17], who reported that Stat1 α , but not Stat1 β (p84), was phosphorylated in response to EGF. Fu and Zhang also detected phosphorylated p75 under mild immunoprecipitation conditions, noting that it did not translocate to the nucleus. The kinetics of Stat1 phos-

phorylation in response to SCF are similar to the kinetics of Stat1 activation by EGF and CSF-1. Fu and Zhang showed that Stat1 is phosphorylated within 2 min of EGF stimulation, with maximal phosphorylation between 8 and 10 min after stimulation. Novak et al. [46] reported activation of Stat1 and Stat3 DNA-binding activity within 5 min of stimulation with CSF-1.

Stat1 phosphorylation was also detected in murine 3T3 fibroblasts stably transfected with wild-type human c-kit (Figure 5). The utility of using cell lines transfected with heterologous receptors in studies of RTK-mediated activation of the JAK/STAT pathway has been demonstrated by studies with the EGF receptor [50,51], CSF-1 receptor [46] and PDGF receptor [15]. One consistent difference between our results with haematopoietic cell lines and 3T3/c-kit cells was the slower and somewhat prolonged phosphorylation of Stat1 in the fibroblast line. This difference in kinetics may reflect variability between haematopoietic and fibroblast cell lines or may represent characteristics unique to the transfected cell model such as retarded recognition of human c-kit receptor by murine JAK/STAT proteins. Another explanation is the potential absence of putative negative regulatory components (such as haematopoietic cell phosphatase) from the fibroblast cells.

Evidence that activated c-kit can directly phosphorylate Stat1 was obtained from *in vitro* kinase assays using the Stat1 fusion protein SH2 + C-TERM as substrate (Figure 6). This particular Stat1 fusion protein includes Tyr-701, the unique tyrosine residue shown to be phosphorylated in response to cytokines. Although the possibility that the Stat1 fusion protein was phosphorylated by a co-immunoprecipitating tyrosine kinase other than c-kit cannot be ruled out, the very high abundance of c-kit relative to any putative co-precipitating kinases suggests that activated c-kit directly phosphorylates Stat1.

Induction of Stat1 DNA-binding activity was confirmed using cytosolic extracts from 3T3/c-kit cells and nuclear extracts from normal progenitor cells (Figure 7). The difference in the extent of supershifting of the DNA-binding complex exhibited by Stat1 antibodies in Figure 7(A) probably reflects a difference in relative affinity for the Stat1 protein. The anti-Stat1 mAb (Stat1 mAb*) which partially disrupted complex-formation in Figure 7(C) is the same antibody that was used in Figure 1 to detect c-kit-Stat1 association. The results with this antibody, which is directed against an epitope lying between amino acids 613 and 739, suggest that it inhibits DNA-binding complex-formation by disrupting Stat1 homodimers. This is consistent with the observation that the epitope recognized by this antibody lies within the region involved in homodimerization.

We have previously demonstrated that Jak2 is constitutively associated with the RTK c-kit [34]. SCF stimulation leads to recruitment of additional Jak2 to the activated receptor complex and induces rapid and transient tyrosine phosphorylation of Jak2. Interestingly, several recent reports [22,50–52] suggest that signal transduction mediated by EGF and PDGF does not require JAK family members for STAT activation in fibroblast cell lines. In contrast, our laboratory has shown that inhibition of Jak2 expression with antisense oligonucleotides leads to partial inhibition of SCF-induced proliferation of FDCP-1 cells, suggesting that Jak2 is important for optimal proliferative responses to SCF in haematopoietic cell lines. Future work will be directed towards addressing the role of Jak2 in activation of Stat1 in SCF-mediated signal-transduction pathways.

The results presented in this paper demonstrate that SCF induces association of Stat1 with c-kit, as well as Stat1 tyrosine phosphorylation and DNA-binding activity. In studies by Meraz et al. [53] and Durbin et al. [54] using Stat1-deficient mice, the

absence of gross abnormalities in haematopoiesis suggests that additional STAT proteins are crucial for SCF-mediated responses or that other STAT family members compensate for Stat1. In this regard, Gotoh et al. [55] recently reported that, although neither Stat3 nor Stat5 were tyrosine-phosphorylated after SCF stimulation of MO7e cells, Stat3 was serine-phosphorylated in response to SCF. In the light of these reports, we are at present examining SCF-induced activation of other members of the STAT family.

The functional significance of Stat1 activation in response to SCF stimulation remains to be established. It is possible that Stat1 activation is merely a by-product of c-kit activation and plays little or no role in biological responses to SCF. However, our previous experiments suggest that Jak2 may play a role in SCF-mediated proliferation. Experiments examining the role of Stat1 activation in cellular responses to SCF are at present being carried out in our laboratory.

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