THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 24, Issue of June 11, pp. 16965–16972, 1999

Printed in U.S.A.

STAT Protein Recruitment and Activation in c-Kit Deletion Mutants*

(Received for publication, November 12, 1998, and in revised form, March 19, 1999)

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Stem cell factor (SCF) and its tyrosine kinase receptor, c-Kit, play a crucial role in regulating migration and proliferation of melanoblasts, germ cells, and hemopoietic cell progenitors by activating a number of intracellular signaling molecules. Here we report that SCF stimulation of myeloid cells or fibroblasts ectopically expressing c-Kit induces physical association with and tyrosine phosphorylation of three signal transducers and activators of transcription (STATs) as follows: STAT1α, STAT5A, and STAT5B. Other STAT proteins are not recruited upon SCF stimulation. Recruitment of STATs leads to their dimerization, nuclear translocation, and binding to specific promoter-responsive elements. Whereas STAT1α, possibly in the form of homodimers, binds to the *sis*-inducible DNA element, STAT5 proteins, either as STAT5A/STAT5B or STAT5/STAT1α heterodimers, bind to the prolactin-inducible element of the β-casein promoter. The tyrosine kinase activity of Kit appears essential for STAT activation since a kinase-defective mutant lacking a kinase insert domain was inactive in STAT signaling. However, another mutant that lacked the carboxyl-terminal region retained STAT1α activation and nuclear translocation but was unable to fully activate STAT5 proteins, although it mediated their transient phosphorylation. These results indicate that different intracellular domains of c-Kit are involved in activation of the various STAT proteins.

The stem cell factor (SCF)[1] has been implicated in a number of important developmental processes (1–3) and is a potent co-stimulating protein that acts synergistically with hemopoietic colony-stimulating factors, such as granulocyte-macrophage colony-stimulating factor and interleukin-3 (IL-3), to promote proliferation of early hemopoietic progenitors (1). SCF effects are mediated by the presence, on the surface of its target cells, of the transmembrane receptor c-Kit. SCF receptor belongs to the tyrosine kinase receptor family, and in addition to the presence of an intrinsic enzymatic activity it shares with other members of the family, such as platelet-derived growth factor receptor (PDGFR) and the colony-stimulating factor-1 (CSF-1) receptor, the presence of an interkinase domain and consensus sequences for recognition of Src homology 2 domains (SH2) (4–7). SCF binding to its receptor leads to dimerization, transphosphorylation, and increased binding of SH2-containing proteins such as phospholipase C-γ, phosphatidylinositol 3-kinase, Syp, and JAK2 (8–13).

The JAKs are non-transmembrane protein tyrosine kinases that are rapidly tyrosine-phosphorylated upon ligand binding and play a critical signaling function downstream of cytokine receptors (14). One such function of JAKs is the activation of STAT proteins (signal transducers and activators of transcription), latent cytoplasmic proteins that undergo rapid tyrosine phosphorylation following cytokine stimulation (15–17). Phosphorylation on a tyrosine immediately distal to the SH2 domain induces their homo- or heterodimerization through phosphotyrosine-SH2 interactions. As a consequence they acquire DNA binding activity, translocate into the nucleus, bind to specific promoter elements, and control the expression of target genes. So far seven different STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6) have been cloned (14–17). STAT1 has been originally identified as a component of an interferon-activated transcriptional factor that upon tyrosine phosphorylation and homodimerization binds to the GAS sequences (18). STAT1 can also be activated by EGF (19) and PDGF (20) to bind to the serum-inducible element (SIE) of c-Fos as homodimers or heterodimers with another STAT member defined as STAT3 (19). A third STAT implicated in the receptor tyrosine kinase signaling pathway, such as EGF (21), ErbB-1 (22), or PDGF (23), is STAT5. STAT5 was originally described as a prolactin-responsive transcription factor in the mammary gland epithelium (24), and subsequently two different but highly homologous STAT5 proteins were isolated and defined as STAT5A and STAT5B (25). These transcriptional factors recognize a specific palindromic sequence that is found in the β-casein promoter (24) and also in a number of promoters of genes other than mammary genes (25), suggesting that STAT5 may play a role other than the induction of milk proteins. Consistent with this hypothesis is the observation that a dominant negative variant of STAT5 was able to inhibit IL-3-mediated cell proliferation (26), and a mutation of tyrosine residues of the IL-2 receptor β chain, mediating STAT5 docking and activation, strongly reduced cell proliferation (27). Moreover, a potential role of STAT5 in regulating the response of bone marrow-derived mast cells to SCF has been reported (28).

In the present study we investigated the pattern of STAT

* This work was supported by the Associazione Italiana per la Ricerca sul Cancro, by Consiglio Nazionale delle Ricerche, and by Progetto Finalizzato A.C.R.O. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: SCF, stem cell factor; IL-3, interleukin-3; STATs, s signal transducers and activators of transcription; SIE, serum-inducible element; PIE, prolactin-inducible element; ΔKI, a c-Kit mutant lacking the kinase insert domain; ΔCT, a carboxyl-terminal deletion mutant of c-Kit; HRS, NIH-3T3 fibroblasts transfected and selected for overexpression of the human c-Kit cDNA; MO7e, human growth factor-dependent megakaryoblastic cell line; Pipes, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; GH, growth hormone; PDGFR, platelet-derived growth factor receptor; CSF, colony-stimulating factor-1; SH2, Src homology 2; EGF, epidermal growth factor.

protein activation in response to SCF stimulation in growth factor-dependent MO7e cell line and in transfected cells expressing the wild type receptor or two different c-Kit mutants. We found that SCF was able to induce tyrosine phosphorylation of STAT1α, STAT5A, and STAT5B in cells expressing the wild type receptor and in cells expressing a carboxyl-terminally deleted mutant (ΔCT). However, other STAT proteins, *e.g.* STAT2, STAT3, STAT4, and STAT6, which are present in the human myeloid cell line MO7e, underwent no activation in response to SCF. Consistent with a role in regulation of gene expression, the modified STAT1α, STAT5A, and STAT5B acquired the ability to undergo dimerization and bind to specific DNA elements. Analysis of two mutants of c-Kit attributed a role to the carboxyl-terminal region in activation of STAT5 proteins, whereas STAT1α activation appears to be independent of this receptor domain.

EXPERIMENTAL PROCEDURES

Materials—Iscove's modified Dulbecco's medium (Life Technologies Inc.) supplemented with 5% bovine calf serum (HyClone, Logan, UT) was the culture medium used throughout. RPMI 1640 medium (Life Technologies, Inc.) was from HyClone (Logan, UT). SCF was gift from Dr. S. Gillis (Immunex, Seattle, WA). Recombinant human IL-3 was kindly provided by Sandoz Forschungsinstitut, Austria. The protein A-Sepharose was purchased from Sigma. Nitrocellulose filters, horseradish peroxidase-conjugated protein A, molecular weight markers, [α-^{32}P]dCTP, and chemiluminescence reagent (ECL) were from Amersham Pharmacia Biotech. Poly(dI·dC):poly(dI·dC) was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

Cells—MO7e cells, maintained in Iscove's modified Dulbecco's medium supplemented with 5% bovine calf serum and IL-3 (5 ng/ml), were washed twice, serum- and growth factor-starved in RPMI 1640 medium overnight, and incubated for 10 min at 37 °C without or with IL-3 (10 ng/ml) (29) or SCF (20 ng/ml) (30). NIH-3T3 fibroblasts transfected and selected for overexpression of the human c-KIT cDNA (HRS), NIH-3T3 fibroblasts transfected with a c-Kit mutant lacking the kinase insert domain (ΔKI), and a carboxyl-terminal deletion mutant of c-Kit (ΔCT) were cultured as described previously (9).

Antisera—Polyclonal antibodies to p91 were produced in rabbits by injection with a synthetic peptide corresponding to the sequences of 84–96 residues of human transcription factor ISGR-3 (GenBank™ accession number M97935), RKSKRNLQDNFQEDC (31). The 4G10 and PY20 anti-phosphotyrosine antibodies were obtained from Upstate Biotechnology, Lake Placid, NY, and from Affinity Research Products Ltd, Nottingham, UK, respectively. Anti-STAT5A (L-20), anti-STAT5B (G-2), and anti-STAT5B (C-17), antisera raised against the carboxyl terminus of STAT5 proteins, anti-STAT5B (N-20), raised against the amino terminus of STAT5B, and anti-STAT2, anti-STAT3, anti-STAT4, and anti-STAT6 antisera were purchased from Santa Cruz Biotechnology, Heidelberg, Germany. Anti-c-Kit antibodies (K44, Ab212, and anti-Kit-X) were obtained as described previously (9, 32).

Western Blot Analysis and Immunoprecipitation Studies—The MO7e cells, HRS, ΔCT-, and ΔKI-expressing cells, serum- and growth factor-starved for 18 h at 37 °C, were supplemented for 4 h with phosphate-buffered saline (30% v/v), sodium orthovanadate 0.2 mmol/liter, EDTA 1 mmol/liter, and then incubated without or with SCF (20 ng/ml) or IL-3 (10 ng/ml) at 37 °C for the indicated times. The cells were then extracted with cold DIM buffer (50 mmol/liter Pipes, pH 6.8, 100 mmol/liter NaCl, 5 mmol/liter $MgCl_2$, 300 mmol/liter sucrose, 5 mmol/liter EGTA, 2 mmol/liter sodium orthovanadate) plus 1% Triton X-100 and a mixture of protease inhibitors (1 mmol/liter phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.15 units/ml aprotinin, 1 μg/ml pepstatin A) for 20 min at 4 °C, and centrifuged at 15,000 $\times$ *g* for 20 min. The clarified supernatants were precleaned for 1 h with 50 μl of protein A-Sepharose (3 mg/sample). The protein concentration of cell lysates was determined by the Bradford's technique, and the protein content of the samples was normalized to 250 mg/samples by appropriate dilution with the cold DIM buffer. The samples were then adsorbed by antisera coupled to protein A-Sepharose. Bound proteins were washed several times in DIM buffer and eluted in boiling Laemmli buffer. Thirty μl of eluted proteins were subjected to 8% SDS-PAGE. Proteins were then transferred electrophoretically to nitrocellulose; the filters were incubated with blocking solution (10% low fat milk or bovine serum albumin in 20 mmol/liter Tris-HCl, pH 7.6, and 17 mmol/liter NaCl) for 1 h. Antisera were then added at the same solution, and the incubation was carried out for 2 h at room temperature. For detection, the filters were washed three times (10 min each wash) with phosphate-buffered saline, 0.05% Tween 20 and reacted for 1 h at room temperature with horseradish peroxidase-conjugated protein A. The enzyme was removed by washing as above. The filters were reacted for 1 min with a chemiluminescence reagent (ECL) and exposed to an autoradiography film for 1–15 min. To reprobe, nitrocellulose filters were first stripped of antibody by 62 mmol/liter Tris-HCl, pH 6.7, 2% SDS, 100 mmol/liter β_2-mercaptoethanol.

Preparation of Nuclear Extract and Gel Retardation Assay—Nuclear extracts from untreated and SCF-treated cells were prepared by Nonidet P-40 lysis as described by Sadowski and Gilman (33). The oligonucleotides used, corresponding to the prolactin-inducible element (PIE) of the β-casein promoter, were G GGG GGA CTT CTT GGA ATT AAG GGA and G GGG TCC CTT AAT TCC AAG AAG TCC (24) and corresponding to the serum-inducible element of c-Fos (SIE) were G GGG CAT TTC CCG TAA ATC and G GGG GAT TTA CGG GAA ATG (34). The annealed oligonucleotide was labeled by filling in the over-hanging ends with Klenow fragment in the presence of [α-^{32}P]dCTP. Gel retardation reactions were performed in 13 mmol/liter Hepes, pH 7.6, 80 mmol/liter NaCl, 3 mmol/liter NaF, 3 mmol/liter $NaMoO_4$, 1 mmol/liter dithiothreitol, 0.15 mmol/liter EDTA, 0.15 mmol/liter EGTA, and 8% glycerol (including contribution from the nuclear extract) and contained 75 μg/ml poly(dI·dC):poly(dI·dC) approximately 0.3 ng of radiolabeled probe, and 5–10 μg of protein. Reactions were carried out at room temperature for 40 min and then resolved on 4% polyacrylamide gels containing 0.25$\times$ TBE (1$\times$ TBE is 89 mmol/liter Tris borate, 1 mmol/liter EDTA, pH 8) and 5% glycerol. Gels were run at 4 °C in 0.25$\times$ TBE at 20 V/cm, dried, and autoradiographed. Oligonucleotide competition was performed by preincubating nuclear extracts with the competitor oligonucleotide (50 fold excess) and poly(dI·dC):poly(dI·dC) for 30 min at room temperature before the addition of labeled probe. Gel mobility shift assays were done with nuclear extract that had been reacted for 1 h at 4 °C with the indicated antibodies.

RESULTS

c-Kit Expression and Tyrosine Phosphorylation in Human Myeloid Cells Expressing Wild Type or c-Kit Mutants—To evaluate SCF-induced signaling we used the following cell types: the myeloid growth factor MO7e cell line that survives and proliferates in response to different growth factor, including SCF (30), NIH-3T3 fibroblasts transfected and selected for overexpression of the human c-Kit cDNA (HRS) (9), and two previously described mutants of c-Kit (9) as follows: NIH-3T3 fibroblasts transfected with a c-Kit mutant lacking the kinase insert domain (ΔKI), and a carboxyl-terminal deletion mutant of c-Kit (ΔCT). Three antibodies to c-Kit were used for immunoprecipitation or Western blotting. These are the anti-Kit-X antiserum raised against the extracellular domain of c-Kit, the Ab212 rabbit antiserum directed to the most carboxyl-terminal 14-amino acid-long sequence of c-Kit, and monoclonal antibody K44 recognizing the second immunoglobulin-like domain of the human c-KIT. As is evident from Fig. 1*A*, anti-Kit-X was able to detect the full-length receptor in the MO7e, HRS, and ΔCT cells, as well as the lower molecular weight mutant of c-Kit protein in the ΔKI cells. By contrast, the Ab212 antiserum was unable to detect the c-Kit mutant lacking the carboxyl terminus (Fig. 1*A*). When the four cell lines were stimulated with SCF and c-Kit proteins analyzed for tyrosine phosphorylation, we observed increased phosphorylation of the wild type and ΔCT c-Kit proteins (Fig. 1*B*). However, no SCF-induced tyrosine phosphorylation of the ΔKI mutant was detectable, in agreement with a previous report that characterized this mutant as a catalytically inactive form of c-Kit (9).

STAT1α Tyrosine Phosphorylation and Coupling to c-Kit in SCF-treated Cells—It has been previously shown that STAT1α, as well as STAT3, becomes tyrosine-phosphorylated upon activation of certain growth factor receptors (14–17). To evaluate the ability of SCF to induce STAT1α phosphorylation on tyrosine residues, we treated cells with SCF, immunoprecipitated STAT1α, and analyzed its state of phosphorylation by using the

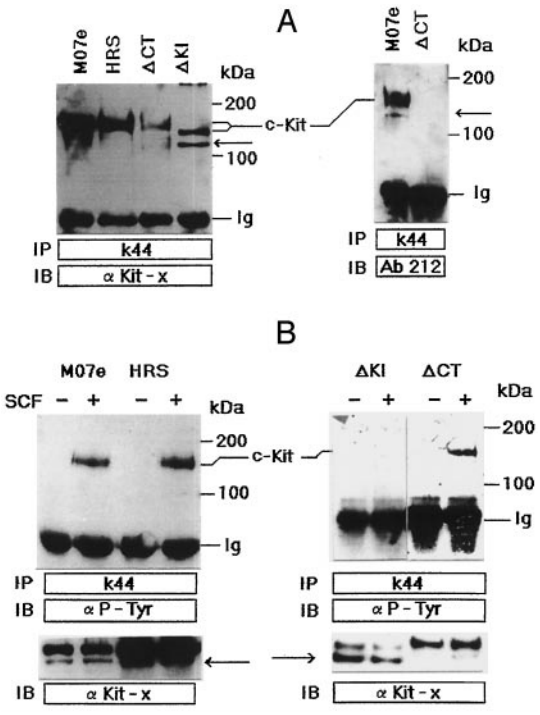

FIG. 1. **Expression and tyrosine phosphorylation of wild type and mutant Kit proteins.** *A,* expression of wild type and mutant Kit proteins. Cells were lysed and Kit proteins immunoprecipitated with an anti-Kit antibody (*K44*). The immunocomplexes were subjected to SDS-PAGE and immunoblotted with the anti-Kit-X. MO7e and ΔCT cell lysates were also immunoprecipitated with the K44 antibody and immunoblotted with the *Ab 212* antiserum (*right panel*). *B,* SCF-mediated tyrosine phosphorylation of Kit proteins. Cell lysates prepared from unstimulated (−) or SCF-stimulated (+) cell lines were subjected to immunoprecipitation (*IP*) with K44 antibody. Proteins were electrophoretically transferred to nitrocellulose filters which were then immunoblotted (*IB*) with an anti-phosphotyrosine (*P-Tyr*) antibody and reprobed with the anti-Kit-x antiserum. Note that the ΔKI mutant undergoes no tyrosine phosphorylation in response to SCF. The positions of the various Kit proteins are indicated. The *arrows* correspond to the less glycosylated Kit precursor.

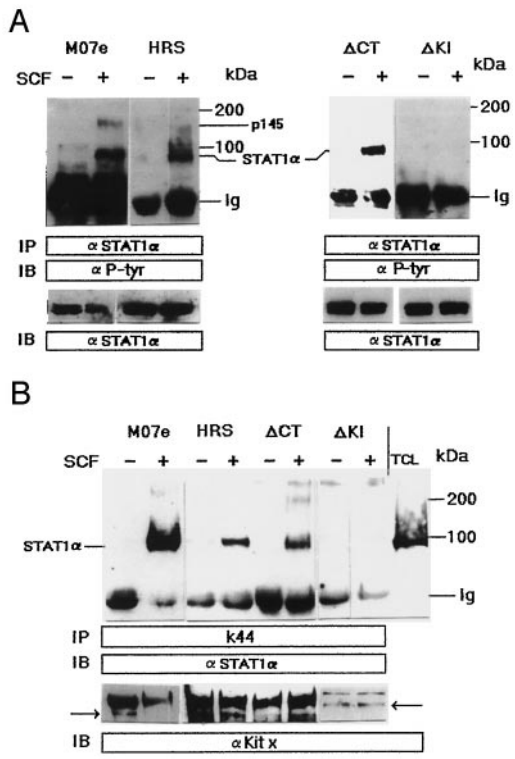

FIG. 2. **SCF-induced tyrosine phosphorylation of STAT1α and physical association with Kit.** *A,* tyrosine phosphorylation of STAT1α. Unstimulated (−) and SCF-stimulated (+) cells were lysed, and cell extracts were subjected to immunoprecipitation (*IP*) with an anti-STAT1α antiserum. Following SDS-PAGE proteins were electrophoretically transferred to nitrocellulose filter, and the filter was immunoblotted (*IB*) with an anti-phosphotyrosine antibody (*upper panel*) and reprobed with an anti-STAT1α antiserum (*lower panel*). The location of the STAT1α protein is indicated. *B,* STAT1α physically associates with the activated c-Kit receptor. The indicated cell lines were lysed, and their whole extracts were subjected to immunoprecipitation with the K44 antibody. Proteins were electrophoretically transferred to nitrocellulose filter, and the filter was immunoblotted with an anti-STAT1α antiserum (*upper panels*), stripped, and reprobed with the anti-Kit-X antiserum (*lower panel*). MO7e total cell lysate (*TCL*) was used to indicate the location of STAT1α. The positions of p145 protein and STAT1α are indicated.

anti-phosphotyrosine antibody. The results presented in Fig. 2*A* demonstrate that SCF was able to induce tyrosine phosphorylation of STAT1α in MO7e, HRS, and ΔCT cells but not in the ΔKI-expressing cells, consistent with the defect of c-Kit in these cells. Moreover, kinetic analysis of STAT1α phosphorylation in MO7e cells revealed that its tyrosine phosphorylation occurred already after 5 min of SCF stimulation, but it disappeared after 15 min of ligand stimulation (data not shown). Importantly, a 145-kilodalton protein corresponding to the full-length c-Kit was detectably co-immunoprecipitated with STAT1α from lysates of SCF-stimulated MO7e and HRS cells, and in the ΔCT mutant was also detectable upon long film exposure, but no association with the ΔKI mutant was evident (Fig. 2*A*). Nevertheless, physical association of the ΔCT mutant, as well as the wild type c-Kit, with STAT1α was evident from a reciprocal co-immunoprecipitation assay in which anti-c-Kit immunoprecipitates were analyzed by using an anti-STAT1α antibody (Fig. 2*B*). In conclusion, both wild type and a carboxyl-terminally deleted c-Kit proteins are able to increase tyrosine phosphorylation of and physically associate with the STAT1α protein, but the kinase-defective ΔKI mutant is inactive in STAT1α coupling.

SCF Induces the Formation of a Protein Complex Containing STAT1α on the SIE—It is known that STATs can bind to a specific DNA element in the c-Fos promoter, located distally to the serum response element (SRE) and known as the c-*sis*-inducible element (SIE) (19). SIE binds to STATs other than STAT1 and STAT3 rather weakly, but it displays high affinity for homo- and heterodimers of STAT1 and STAT3 (19). Therefore, we addressed the ability of the SCF-activated STAT1α to bind to the SIE. To this end we incubated nuclear extract from SCF-treated cells with a radiolabeled SIE probe and analyzed complex formation by using non-denaturing gel electrophoresis. The results presented in Fig. 3*A* indicate that SCF stimulation induced SIE binding activity in MO7e cells but not in cells expressing a kinase-defective ΔKI mutant of c-Kit. That STAT1α is present in the SCF-induced DNA-protein complex was evident from a supershift analysis in which an antibody to STAT1α specifically caused a mobility shift of the SIE-containing complex (Fig. 3*B*). Similarly, a carboxyl-terminal deletion mutant of c-Kit was able to mediate formation of an SIE·STAT1α complex (Fig. 3*B, right panel*), implying that the distal part of the intracellular domain of c-Kit is not essential for coupling to and activation of STAT1α.

STAT5A and STAT5B Become Tyrosine-phosphorylated upon SCF Stimulation and Form a Stable Complex with Kit, Possibly through Its Carboxyl-terminal Region—To extend the analysis of the interaction between Kit and STAT proteins, we tested tyrosine phosphorylation of and physical association

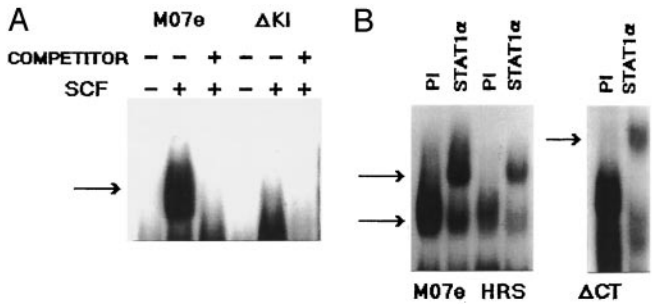

FIG. 3. **SCF-induced formation of the SIE complex in wild type Kit- and ΔCT-expressing cells but not in ΔKI-expressing cells.** *A,* SIE complex formation. Nuclear extracts were prepared from the indicated untreated (−) or SCF-treated (+) cells in the presence or in the absence of unlabeled oligonucleotide (*competitor*). *B,* the SCF-induced SIE-binding complex is antigenically related to STAT1α. Nuclear extracts from SCF-treated cells were preincubated with a preimmune serum (*PI*) or with an anti-STAT1α antiserum. The DNA-protein complexes were then resolved by nondenaturing polyacrylamide gel electrophoresis. The SCF-induced complexes and the supershifted species are indicated by *arrows* (*lower and upper arrows,* respectively).

with other members of the STAT family. Although STAT2, STAT3, STAT4, and STAT6 are expressed in MO7e cells, we were unable to detect their interaction with a ligand-activated c-Kit (data not shown). However, two other STAT proteins, STAT5A and STAT5B, were found to be coupled to c-Kit upon ligand binding. These STAT5 proteins were originally reported to undergo activation in response to prolactin in the mammary gland (24), but subsequently a large body of evidence supporting STAT5 activation in response to many cytokines and growth factors has accumulated (14–17). For example, stimulation of MO7e cells with IL-3 resulted in phosphorylation of STAT5A and STAT5B (Fig. 4, *A* and *B*). Likewise SCF-mediated tyrosine phosphorylation of STAT5A and STAT5B was detectable in both MO7e and HRS cells (Fig. 4, *A* and *B*). Similarly analysis of cells expressing a carboxyl-terminally deleted mutant of c-Kit (ΔCT), by immunoblotting with anti-phosphotyrosine antibody, revealed that this mutant retained the ability to phosphorylate both proteins (Fig. 4*C*). However, in experiments that are not presented, no tyrosine phosphorylation of these proteins was detectable in fibroblasts expressing the kinase-defective ΔKI mutant of c-Kit. Moreover, in lysates from IL-3- and SCF-stimulated cells, additional phosphorylated bands of unknown tyrosine-phosphorylated substrates were also detected (Fig. 4, *A* and *B*). Kinetic analysis of STAT5 protein activation in ΔCT-expressing cells (Fig. 4*C*) revealed that their tyrosine phosphorylation was rapid and transient, occurring already after 1 min of SCF stimulation and disappearing by 10 min of ligand stimulation. By contrast, a less transient (still present after 15 min of ligand stimulation) tyrosine phosphorylation of STAT5A (Fig. 4*D*) and STAT5B (data not shown) was detectable in SCF-stimulated MO7e cells.

It has been shown that upon ligand stimulation the activated STAT proteins tightly interact, through their SH2 domains, with specific phosphotyrosine residues of activated receptors (16). To evaluate the ability of the activated wild type and a carboxyl-terminally deleted c-Kit to form a stable complex with STAT5 proteins, we performed co-immunoprecipitation experiments whose results are shown in Fig. 5, *A* and *B*. Evidently, STAT5A and STAT5B could be immunoprecipitated with the wild type form of c-Kit from cell extracts of SCF-stimulated MO7e cells (Fig. 5, *A* and *B*). However, despite the ability of the ΔCT mutant to mediate tyrosine phosphorylation of STAT5 proteins, our co-immunoprecipitation experiments failed to detect complex formation between STAT5 proteins and this mutant form of c-Kit (Fig. 5, *C–E*).

The Carboxyl-terminally Deleted c-Kit Shows an Impaired PIE Complex Formation in Response to SCF—STAT5 proteins can interact with the β-casein promoter region known as the prolactin-inducible element (PIE) (25). Therefore, to evaluate the ability of the activated STAT5 protein to bind to the PIE, nuclear extract from SCF-stimulated MO7e, HRS, and ΔCT cells were incubated with a radiolabeled PIE probe, and complex formation was analyzed by non-denaturing gel electrophoresis. The results shown in Fig. 6*A* indicate that SCF stimulation leads to the formation of a PIE complex in MO7e and in HRS cells but not in cells expressing the carboxyl-terminally deleted c-Kit. That the SCF-induced DNA-protein complex contains both STAT5 proteins was evident from supershift experiments in which antibodies to STAT5A and STAT5B were able to induce a mobility shift of the PIE complex (Fig. 6*B*).

SCF Stimulation Leads to the Formation of Different STAT Dimers—Receptor activation leads to the recruitment of STAT monomers which then homo- or heterodimerize and migrate to the nucleus to activate gene transcription (14–17). To extend the analysis of STAT protein activation upon SCF stimulation, we evaluate STAT dimer formation in MO7e and in ΔCT cells. Co-immunoprecipitation experiments, whose results are shown in Fig. 7, were performed using non-cross-reacting antisera to STAT5A and STAT5B. Heterodimer formation between STAT5A and STAT5B was clearly detectable in MO7e cells. However, despite the ability of the ΔCT receptor mutant to induce tyrosine phosphorylation of STAT5, reciprocal co-immunoprecipitation assays were unable to detect dimer formation between STAT5A and STAT5B in SCF-stimulated ΔCT cells (Fig. 7, *right panel*).

It has been reported that upon activation of CSF-1 and PDGF receptors tyrosine phosphorylation of STAT5 and STAT3 proteins can interact to form heterodimers (35). Thus, we addressed the ability of SCF-activated STAT1α and STAT5 proteins to form stable complex. To this end we performed reciprocal co-immunoprecipitation experiments in MO7e and in ΔCT-expressing cells. As is evident from the anti-STAT1α immunoblot presented in Fig. 8*A*, both STAT5 proteins can heterodimerize with STAT1α in SCF-stimulated MO7e cells. By contrast, analysis of cells expressing a carboxyl-terminally deleted mutant of c-Kit (Fig. 8*B*) detected no STAT1α/STAT5 heterodimers. The DNA sequence specificity of the STAT1α/STAT5 heterodimeric complexes in MO7e cells was evaluated by electrophoretic mobility shift assay, using PIE and SIE elements as probes. As shown in Fig. 8*C*, the apparent mobility of SCF-mediated PIE complex could be altered by preincubating nuclear extracts with an antiserum to STAT1α. By contrast, no supershifted species was detectable upon adding either an antiserum to STAT5A or an antiserum to STAT5B to SCF-treated nuclear extracts incubated with the SIE probe. In conclusion, the PIE element binds to STAT1α/STAT5 complex, but the SIE element cannot bind to such heterodimer in SCF-stimulated cells.

DISCUSSION

In the present study we investigated the ability of SCF to induce physical association with and tyrosine phosphorylation of STAT proteins. Wild type and two different deletion mutants of c-Kit were examined. We found that, among different STAT proteins, only STAT1α, STAT5A, and STAT5B were activated in response to SCF stimulation both in human myeloid MO7e cells and in c-Kit-transfected fibroblasts. Moreover, the results obtained with a mutant, lacking the kinase insert domain, demonstrated that ligand stimulation failed to induce STAT tyrosine phosphorylation and physical association with the mutated c-Kit. In the cytokine receptor family ligand stimulation induces rapid activation of JAK proteins leading to STAT ty-

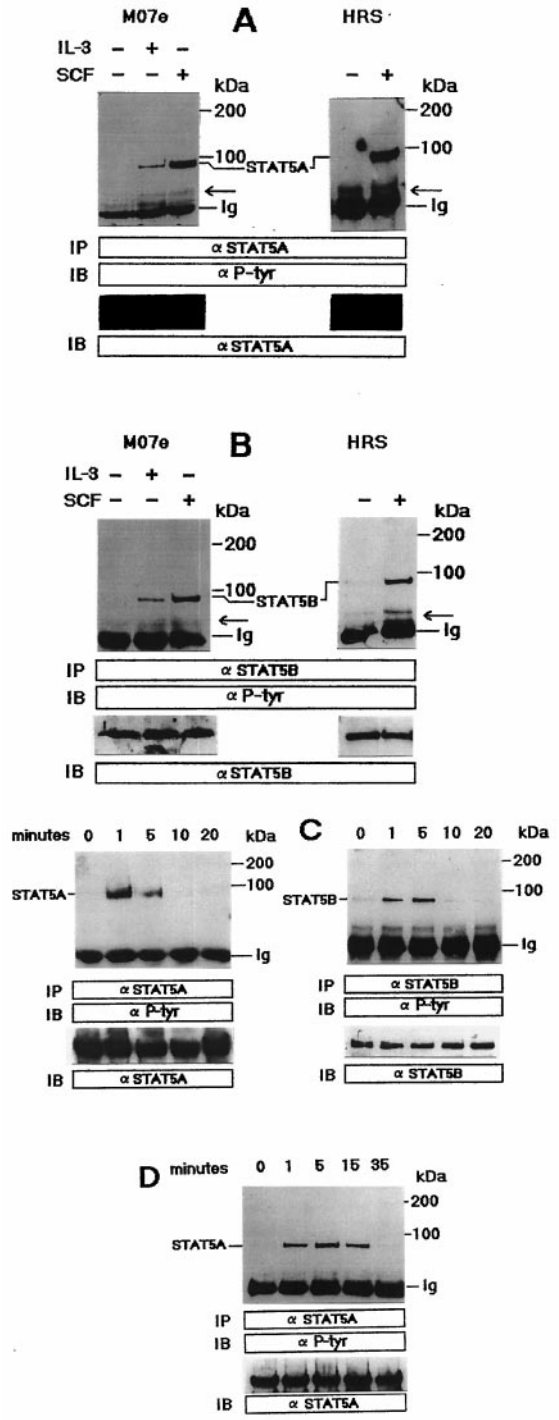

FIG. 4. **Wild type and carboxyl-terminally deleted Kit proteins mediate SCF-induced tyrosine phosphorylation of STAT5A and STAT5B.** *A* and *B,* STAT5A and STAT5B phosphorylation by wild type Kit. The indicated cells were either unstimulated (−) or stimulated (+) with IL-3 or SCF. Cell lysates were prepared and subjected to immunoprecipitation (*IP*) with an anti-STAT5A (*A*) or an anti-STAT5B (*B*) antiserum. *C,* STAT5A and STAT5B phosphorylation by a carboxyl-terminally deleted Kit protein. ΔCT-expressing cells were stimulated with SCF for the indicated time intervals. Cell lysates were prepared and subjected to immunoprecipitation with an anti-STAT5A or an anti-STAT5B antiserum. *D,* kinetics of STAT5A phosphorylation in MO7e cells. MO7e were incubated in the absence or in the presence of SCF for the indicated times, lysed, and immunoprecipitated with an anti-STAT5A antiserum. Proteins were electrophoretically transferred to nitrocellulose filters, and the filters were immunoblotted (*IB*) with an anti-phosphotyrosine (*P-tyr*) antibody (*upper panels*) and reprobed with the indicated antiserum (*lower panels*). The positions of STAT5A and STAT5B proteins were indicated. The positions of unknown tyrosine-phosphorylated substrate are indicated by the *arrows*.

rosine phosphorylation (14–17). By contrast, in tyrosine kinase receptors, the role of JAK proteins in STAT signaling is not yet clearly defined. Consistent with a JAK-independent pathway, a catalytically inactive form of PDGFR is unable to stimulate STAT1 and STAT3 activation in response to PDGF stimulation (36). Likewise, our finding that the ΔKI mutant of c-Kit, which displayed reduction in autophosphorylation *in vitro* (9), cannot signal to STAT (Fig. 2) or to JAK2 upon SCF treatment (data not shown), supports the possibility that STAT proteins may represent direct substrates of receptor tyrosine kinases, and that, indeed, a full functional enzymatic activity of the receptor is required for coupling to and activate STATs. Consistent with this hypothesis are the observations that STAT1 can directly interact with EGF (37) and PDGF (20) receptors and that these receptors can phosphorylate STAT1 *in vitro* (20, 38). Moreover, the absence of any JAKs does not affect the ability of EGF or PDGF to phosphorylate STAT1 (20, 36, 39). Furthermore, the non-receptor tyrosine kinase, Src, is associated with STAT3 *in vivo* and *in vitro,* and it can phosphorylate STAT3 *in vitro* (40, 41). However, our observation does not rule out the possibility that the kinase insert domain, which is known to selectively interact with the p85 regulatory subunit of phosphatidylinositol 3-kinase (9), also contains STAT-binding sites. It has been recently shown that activation of STAT5 by PDGF is dependent on phosphorylation sites in PDGFR β juxtamembrane and kinase insert domains (23). Comparing the sequences surrounding receptor's tyrosine residues mediating STAT docking and activation, it appears that a consensus YVDP or YLDP is required for binding of the STAT5 SH2 domain (20, 42). A similar consensus sequence does not surround tyrosine residue 721 and 730 contained in the kinase insert domain of c-Kit (4, 9), suggesting that reduction of the catalytic activity of the receptor, rather than loss of tyrosine residues, may account for the inability of the ΔKI mutant of c-Kit to interact with and activate STAT proteins.

Tight binding of phosphotyrosine residues of the receptor to the SH2 domain of STAT proteins seems to play a dominant role in STAT signaling (16). Requirement of intracellular phosphotyrosine residues in mediating STAT5 docking and activation has been reported for the GH receptor (43). Similarly, PRL-induced activation of STAT5 was abolished in fibroblasts expressing PRL receptor mutants lacking all intracellular tyrosines (44). Our results demonstrate that following SCF stimulation, STAT1α and STAT5 proteins, phosphorylated on tyrosine, physically associate with the full-length but not with the kinase-defective receptor indicating that a catalytically active receptor, able to provide phosphorylated docking site for STAT, is also required in the tyrosine kinase receptor family. Furthermore, evidence for alternative mechanisms of STAT receptor interaction has been reported for GH. In particular, GH receptor mutants devoid of all cytoplasmic sites for tyrosine phosphorylation can nonetheless support STAT1 and STAT3 activation when GH is provided (45). In contrast, STAT5 activation relies on the presence of different tyrosine-phosphorylated residues in the cytoplasmic tail of the GH receptor (46), indicating that a redundancy in tyrosine residue requirement may be employed in GH-mediated STAT5 signal (46). Likewise, three different tyrosine-phosphorylated residues are required for maximal STAT5 activation in response to PRL (44), suggesting that full STAT5-mediated gene transcription depends on several tyrosine-phosphorylated docking sites. Therefore, the lack of tyrosine residues in the cytoplasmic tail of the ΔCT mutant can explain why the SCF-activated COOH-terminally deleted c-Kit is unable to recruit STAT5s but still retains the ability to transiently phosphorylate these proteins. However, other models can account for our observation as follows: JAK2 itself,

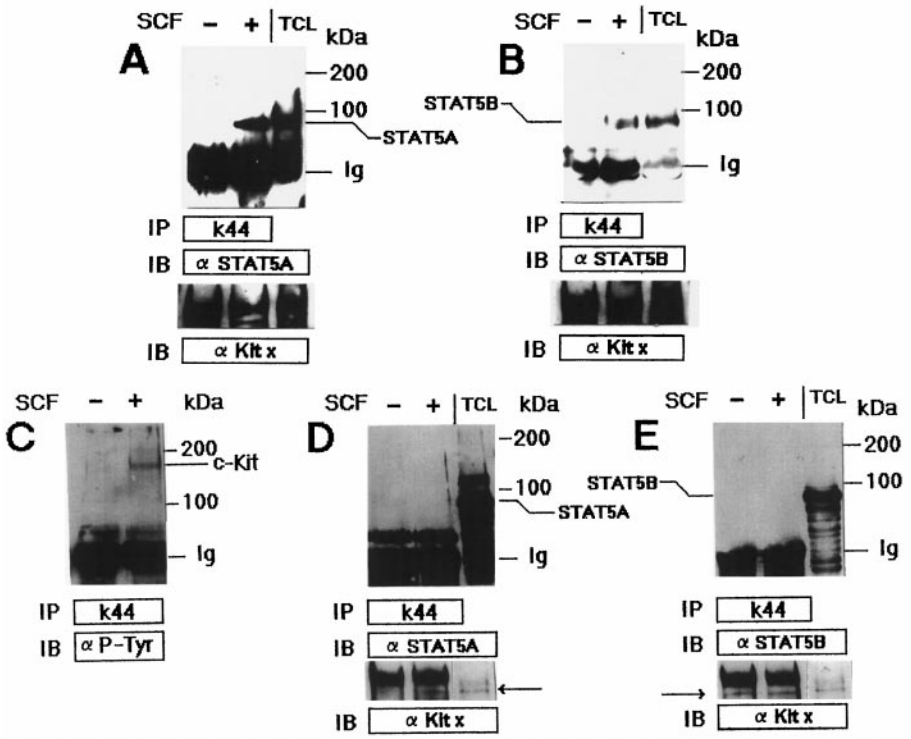

FIG. 5. **STAT5A and STAT5B physically associate with the activated wild type protein but not with the carboxyl-terminally deleted ΔCT mutant.** Unstimulated (−) or SCF-stimulated (+) MO7e (*A* and *B*) and ΔCT-expressing cells (*C–E*) were lysed, and their extracts were subjected to immunoprecipitation (*IP*) with the K44 antibody. The immunoprecipitates were divided into 2 or 3 aliquots that were subjected to SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose filters, and the filters were separately immunoblotted (*IB*) with an anti-STAT5A antiserum (*A* and *D*), an anti-STAT5B antiserum (*B* and *E*), or with the anti-phosphotyrosine (*P-Tyr*) antibody (*C*) as indicated. The *arrows* correspond to the less glycosylated Kit precursor. *TCL* , total cell lysate.

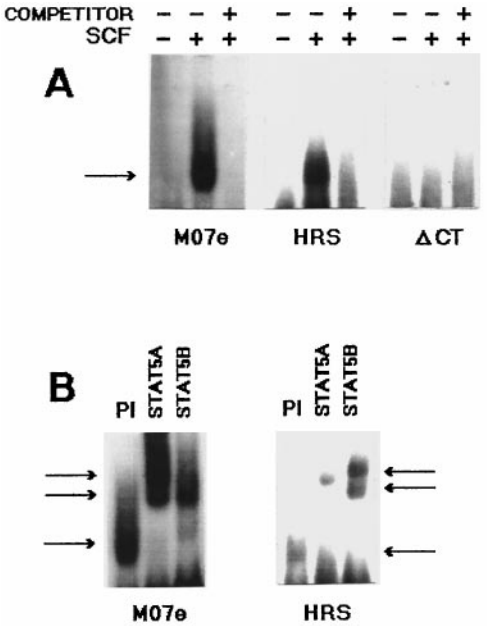

FIG. 6. **SCF induces the formation of a PIE-binding complex in MO7e and HRS cells but not in ΔCT-expressing cells.** *A,* PIE complex formation. Nuclear extracts were prepared from the indicated untreated (−) or SCF-treated (+) cells in the presence or in the absence of unlabeled oligonucleotide (*competitor*). *B,* the SCF-induced PIE-binding complex is antigenically related to STAT5A and STAT5B. Nuclear extracts from SCF-treated MO7e and HRS cells were preincubated with a preimmune serum (*PI*) or with an anti-STAT5A or an anti-STAT5B antiserum. The DNA-protein complexes and the supershifted species (*lower* and *upper arrows,* respectively) were then resolved by nondenaturing polyacrylamide gel electrophoresis.

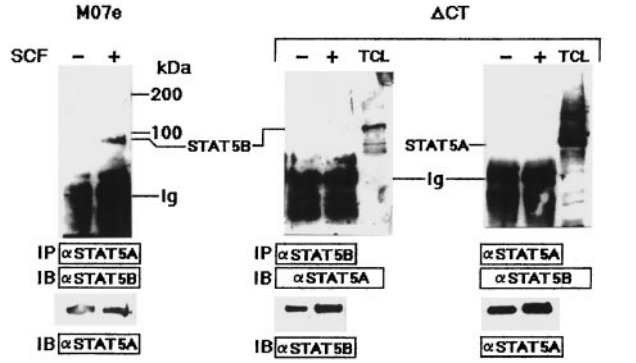

FIG. 7. **SCF induces heterodimerization of STAT5A and STAT5B proteins in MO7e cells.** Cells were unstimulated (−) or stimulated (+) with SCF, lysed, and STAT5 proteins immunoprecipitated (*IP*). Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose filters, and the filters were immunoblotted (*IB*) with the reciprocal anti-STAT5, as indicated (*upper panels*). The filters were then reprobed with the other anti-STAT5 antibody as indicated (*lower panels*). Total cell lysates (*TCL*) from MO7e cells were used to localize the STAT5A and STAT5B proteins.

which is activated upon SCF stimulation (12, 13), may function as a bridge, bringing STAT5 proteins to the receptor through its tyrosine residues (47). We were unable to detect any JAK2-STAT5 interaction (data not shown). However, we cannot rule out the possibility that a weak molecular interaction may account for this result. Alternatively, non-receptor tyrosine kinases, other than JAK2, may be implicated in STAT tyrosine phosphorylation and/or STAT-receptor interaction. Nevertheless, independently of the exact mechanism, the ability of the ΔCT mutant to signal to STAT1α but not to STAT5 proteins suggests that the recruitment of STAT1α and STAT5 depends on different intracellular c-Kit domains. This interpretation is in agreement with the model in which STAT5 but not STAT1 activation requires the box 3 region of the cytoplasmic tail of GH receptor (46).

Accumulating evidence suggests that tyrosine-phosphorylated STATs undergo intermolecular dimerization through their SH2 domain and conserved phosphotyrosine present in the COOH-terminal of the SH2 domain (48). This dimerization is required for subsequent release of STAT from the receptor complex and for translocation to the nucleus for DNA binding (15). Our result demonstrates that in the ΔCT mutant, which fails to recruit the phosphorylated STAT5 proteins, no dimer

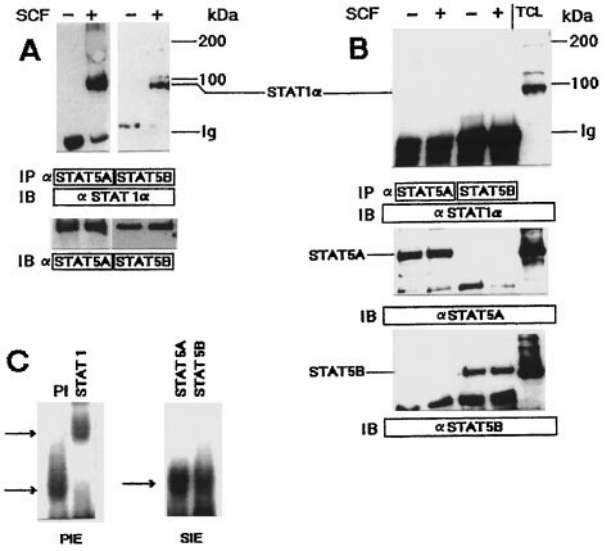

FIG. 8. **The carboxyl-terminal region of c-Kit is essential for STAT5/STAT1α heterodimer formation, which binds specifically to the PIE.** MO7e (*A*) and ΔCT-expressing cells (*B*) were unstimulated (−) or stimulated (+) with SCF, lysed, and the indicated STAT proteins immunoprecipitated (*IP*) with the respective antiserum. Proteins were electrophoretically transferred to nitrocellulose filters that were immunoblotted (*IB*) with an anti-STAT1α antiserum (*upper panels*) and reprobed with an anti-STAT5A or an anti-STAT5B antiserum (*lower panels*). Total cell lysates (*TCL*) from ΔCT-expressing cells were used to indicate the migration of STAT1α. *C,* STAT1α/STAT5 heterodimers bind to PIE but not to the SIE sequence. Nuclear extracts from SCF-treated MO7e cells were preincubated with the indicated antibodies (*PI,* preimmune serum). Then, radiolabeled oligonucleotides corresponding to the PIE or the SIE sequences, as indicated, were added. The formed DNA-protein complexes and the supershifted species are indicated by *arrows* (*lower* and *upper arrows,* respectively).

and PIE complex can be formed, supporting the possibility that, as described previously for STAT1α (49), a specific receptor interaction may also dictate dimerization and DNA binding activity of STAT5 proteins.

It has been reported that STAT5 proteins, besides forming homo- and heterodimers, may also contribute to the formation of heterodimers with other STAT proteins (33). In response to CSF-1 and PDGF stimulation, STAT5/STAT3 heterodimers apparently bind to the c-Fos promoter region, whereas STAT5 homodimers are inactive. On the other hand, binding to the β-casein promoter occurs as homodimers or STAT5A/STAT5B heterodimers (35). Similarly, we found that SCF stimulation leads to the formation of heterodimers containing STAT5A or STAT5B together with STAT1α. The analysis of the DNA binding activity of these heterodimeric complexes revealed that the sequence corresponding to the β-casein promoter region (PIE), but not that corresponding to the c-Fos promoter region (SIE), could be specifically recognized by these STAT5s/STAT1α heterodimeric complexes. Although the biological significance of these different dimers is thus far unclear, the observation that the heterodimeric complexes formed by STAT5 proteins and STAT1α were detected in cells expressing the full-length receptor, but not in ΔCT-expressing cells, supports the possibility also that the rate of dephosphorylation of the already activated molecules is crucial for dimer formation and final transcriptional output from activated STATs.

The results of the present study demonstrate that different regions of c-Kit are involved in STAT1α- and STAT5-receptor interaction and that the COOH-terminal region of c-Kit is essential for full activation of STAT5A and STAT5B but not of STAT1α. Moreover, we demonstrate that upon SCF stimulation STAT1α can also form heterodimers with STAT5A or STAT5B, and the complex formed specifically binds to the PIE sequence. Better understanding of the role played by various activated STATs may provide new insights into the signaling pathways required to achieve divergent biological effects in response to the same growth factor.

REFERENCES

1. Zsebo, K. M., Wypych, J., Mc Niece, I. K., Lu, H. S., Smith, K. A., Karkare, S. B., Sachdev, R. K., Yuschenkoff, V. N., Birkett, N. C., Williams, L. R., Satyagal, V. N., Tung, W., Bosselman, R. A., Mendiaz, E. A., and Langley, K. E. (1990) *Cell* **63,** 195–201
2. Williams, D. E., Eismman, J., Baird, A., Rauch, C., van-Ness, K., March, C. J., Park, L. S., Martin, U., Mochizuki, D. Y., Boswell, H. S., Burgess, G. S., Cosman, D., and Lyman, S. D. (1990) *Cell* **63,** 167–174
3. Huang, E., Nocka, K., Beier, D. R., Chu, T. Y., Buck, J., Lahm, H. W., Wellner, D., Leder, P., and Besmer, P. (1990) *Cell* **63,** 225–233
4. Yarden, Y., Kuang, W. J., Yang-Feng, T., Coussens, L., Munemitsu, S., Dull, T. J., Chen, E., Schlessinger, J., Francke, U., and Ullrich, A. (1987) *EMBO J.* **6,** 3341–3351
5. Qiu, F., Ray, P., Brown, K., Barker, P. E., Jhanwar, S., Ruddle, F. H., and Besmer, P. (1988) *EMBO J.* **7,** 1003–1011
6. Yarden, Y., and Ullrich, A. (1988) *Annu. Rev. Biochem.* **57,** 433–478
7. Yarden, Y., and Schlessinger, J. (1987) *Biochemistry* **26,** 1434–1442
8. Rottapel, R., Reedijk, M., Williams, D. E., Lyman, S. D., Anderson, D. M., Pawson, T., and Bernstein, A. (1991) *Mol. Cell. Biol.* **11,** 3043–3051
9. Lev, S., Givol, D., and Yarden, Y. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 678–682
10. Tauchi, T., Feng, G.-S., Marshall, M. S., Shen, R., Mantel, C., Pawson, T., and Broxmeyer, H. E. (1994) *J. Biol. Chem.* **269,** 25206–25211
11. Yi, T., and Ihle, J. N. (1993) *Mol. Cell. Biol.* **13,** 3350–3358
12. Brizzi, M. F., Zini, M. G., Aronica, M. G., Blechman, J. M., Yarden, Y., and Pegoraro, L. (1994) *J. Biol. Chem.* **269,** 31680–31684
13. Weiler, S. R., Mou, S., De Berry, C. S., Keller, J. R., Ruscetti, F. W., Ferris, D. K., Longo, D. L., and Linnekin, D. (1996) *Blood* **87,** 3688–3693
14. Ihle, J. N., and Kerr, I. M. (1995) *Trends Genet.* **11,** 69–74
15. Leaman, D. W., Leung, S., Li, X., and Stark, G. R. (1996) *FASEB J.* **10,** 1578–1588
16. Darnell, J. E., Jr. (1997) *Science* **277,** 1630–1635
17. O'Shea, J. J. (1997) *Immunity* **7,** 1–11
18. Schindler, C., Fu, X. Y., Improte, T., Aebersold, R., and Darnell, J. E., Jr. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7836–7839
19. Zhong, Z., Wen, Z., and Darnell, J. E., Jr. (1994) *Science* **264,** 95–98
20. Choudhury, G. G., Ghosh-Choudhury, N., and Abboud, H. E. (1998) *J. Clin. Invest.* **101,** 2751–2760
21. Ruff-Jamison, S., Chen, K., and Cohen, S. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 4215–4218
22. Mellitzer, G., Wessely, O., Decker, T., Meinke, A., Hayman, M. J., and Beug, H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 9600–9605
23. Valgeirsdottir, S., Paukku, K., Silvennoinen, O., Heldin, C.-H., and Claesson-Welsh, L. (1998) *Oncogene* **16,** 505–515
24. Wakao, H., Gouilleux, F., and Groner, B. (1994) *EMBO J.* **13,** 2182–2191
25. Mui, A. L.-F., Wakao, H., O'Farrell, A. M., Harada, N., and Miyajima, A. (1995) *EMBO J.* **14,** 1166–1175
26. Mui, A. L.-F., Wakao, H., Kinoshita, T., Kitamura, T., and Miyajima, A. (1995) *EMBO J.* **15,** 2425–2433
27. Friedmann, M. C., Migone, T. S., Russell, S. M., and Leonard, W. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 2077–2082
28. Ryan, J. J., Huang, H., McReynolds, L. J., Shelburne, C., Hu-Li, J., Huff, T. F., and Paul, W. E. (1997) *Exp. Hematol.* **25,** 357–362
29. Avanzi, G. C., Brizzi, M. F., Giannotti, J., Ciarletta, A., Yang, Y., Pegoraro, L., and Clark, S. C.(1990) *J. Cell. Physiol.* **145,** 458–464
30. Miyazawa, K., Hendrie, P. C., Mantel, C., Wood, K., Ashman, L. K., and Broxmeyer, H. E. (1991) *Exp. Hematol.* **19,** 1110–1123
31. Brizzi, M. F., Aronica, M. G., Rosso, A., Bagnara, G. P., Yarden, Y., and Pegoraro, L. (1996) *J. Biol. Chem.* **271,** 3562–3567
32. Blechman, J. M., Lev, S., Brizzi, M. F., Leitner, O., Pegoraro, L., Givol, D., and Yarden, Y. (1993) *J. Biol. Chem.* **268,** 4399–4406
33. Sadowski, H. B., and Gilman, M. Z. (1993) *Nature* **362,** 79–83
34. Shuai, K., Ziemiechki, A., Wilks, A. F., Harpur, A. G., Sadowski, H. B., Gilman, M. Z., and Darnell, J. E., Jr. (1993) *Nature* **366,** 580–583
35. Novak, U., Mui, A., Miyajima, A., and Paradiso, L. (1996) *J. Biol. Chem.* **271,** 18350–18354
36. Vignais, M. L., Sadowski, H. B., Watling, D., Rogers, N. C., and Gilman, M. Z. (1996) *Mol. Cell. Biol.* **16,** 1759–1769
37. Okimoto, T., Kohno, K., Kuwano, M., Gopas, J., Kung, H. F., and Ono, M. (1996) *Oncogene* **12,** 1625–1633
38. Quelle, F. W., Thierfelder, W., Witthuhn, B. A., Tang., B., Cohen, S., and Ihle, J. N. (1995) *J. Biol. Chem.* **270,** 20775–20780
39. Leaman, D. W., Pisharody, S., Flickinger, T. W., Commane, M. A., Schlessinger, J., Kerr, I. M., Levy, D. E., and Stark, G. R. (1996) *Mol. Cell. Biol.* **16,** 369–375
40. Yu, C. L., Meyer, D. J., Campbell, G. S., Larner, A. C., Carter-Su, C., Schwartz, J., and Jove, R. (1995) *Science* **269,** 81–83
41. Garcia, R., Yu, C. L., Hundnall, A., Catlett, R., Nelson, K. L., Smithgall, T., Fijita, D. J., Ethier, S. P., and Jove, R. (1997) *Cell Growth Differ.* **8,** 1267–1276
42. Lebrun, J.-J., Ali, S., Ullrich, A., and Kelly, P. A (1995) *J. Biol. Chem.* **270,** 10664–10670
43. Xu, B. C., Wang, X., Darus, C. J., and Kopchick, J. J. (1996) *J. Biol. Chem.* **271,** 19768–19773

44. Pezet, A., Ferrag, F., Kelly, P. A., and Edery, M, (1997) *J. Biol. Chem.* **272**, 25043–25050
45. Wang, Y.-D., Wong, K., and Wood, W. I. (1995) *J. Biol. Chem.* **270,** 7021–7024
46. Wang, X., Darus, C. J., Xu, B. C., and Kopchick J. J. (1996) *Mol. Endocrinol.* **10,** 1249–1260
47. Fjetani, Y., Hibi, M., Fukada, T., Takahashi, M., Hioshyda, H., Himaguchi, T., Sugiyama, K., Hiamanaka, Y., Nakajima, K., and Hirano, T. (1997) *Oncogene* **14,** 751–761
48. Shuai, K., Horvath, C. M., Huang, L. H. T., Quereshi, S. A., Cowburn, D., and Darnell, J. E., Jr. (1994) *Cell* **76,** 821–828
49. Heim, M. H., Kerr, I. M., Stark, G. R., and Darnell, J. E., Jr. (1995) *Science* **267,** 1347–1349

STAT Protein Recruitment and Activation in c-Kit Deletion Mutants

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J. Biol. Chem. 1999, 274:16965-16972.
doi: 10.1074/jbc.274.24.16965