Mechanisms of STAT Protein Activation by Oncogenic KIT Mutants in Neoplastic Mast Cells

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Mutations in the c-kit gene occur in the vast majority of mastocytosis. In adult patients as well as in the cell line derived from mast cell neoplasms, the mutations occur almost exclusively at amino acid 816 within the kinase domain of KIT. Among the downstream effectors of KIT signaling, STAT3 and STAT5 have been shown to be critical for cell proliferation elicited by the KIT-Asp816 mutant protein. However, little is known about the mechanisms of activation of STAT proteins. In this study, we identify and clarify the contribution of various STAT kinases in two widely used neoplastic mast cell lines, P815 and HMC-1. We show that STAT1, -3, and -5 proteins are activated downstream of the KIT-Asp816 mutant. All three STAT proteins are located in the nucleus and are phosphorylated on serine residues. KIT-Asp816 mutant can directly phosphorylate STATs on the activation-specific tyrosine residues in vitro. However, within cells, SRC family kinases and JAKs diversely contribute to tyrosine phosphorylation of STAT proteins downstream of the KIT mutant. Using a panel of inhibitors, we provide evidence for the implication or exclusion of serine/threonine kinases as responsible for serine phosphorylation of STAT1, -3, and -5 in the two cell lines. Finally, we show that only STAT5 is transcriptionally active in these cells. This suggests that the contribution of STAT1 and STAT3 downstream of KIT mutant is independent of their transcription factor function.

The growth factor stem cell factor (SCF) and its receptor KIT play a pivotal role in the development of hematopoietic cells, germ cells, interstitial cells of Cajal, and melanoblasts. Perturbations of either SCF or KIT physiological functions contribute to human neoplasms related to these cells such as mastocytosis, acute myeloid leukemia, germ cell tumors, gastrointestinal stromal tumors, and melanoma. The most common dysfunctions of the SCF/KIT pathway are the mutations in the c-kit gene that affect residues or domains involved in the inactive to active conformation transition, thus leading to the constitutive activation of the receptor (2). Among them, mutations in the juxtamembrane domain are found in the majority of gastrointestinal stromal tumors (3) and in some melanomas (4), whereas substitution of aspartic acid 816 (Asp814 in the mouse) is found in about 80% of mastocytosis patients (5, 6) and in some cases of acute myeloid leukemias (7, 8), melanomas, and testicular germ cell tumors (9–11).

KIT signals through the recruitment of proteins on its intracellular docking sites thereby inducing the formation of a large multiprotein signaling complex. Known signaling pathways activated by wild-type (WT) KIT include PI3K/AKT, RAS-ERK, the SRC family kinases (SFK), phospholipase Cγ, and JAK/STAT pathways (1, 12). Signal transducers and activators of transcription (STAT) proteins are latent cytoplasmic transcription factors that transduce the effects of a broad range of hormones, cytokines, and growth factors on target gene expression. There are seven mammalian STAT proteins (STAT1–4, -5A, -5B, and -6) that all share the same arrangement of functional motifs. STAT proteins become activated upon tyrosine phosphorylation and are subsequently translocated into the nucleus where they act on target gene promoters (13).

Constitutive activation of STAT proteins has been demonstrated in various leukemias (14, 15). In normal signaling, STAT activation is rapid and transient. In contrast, aberrant permanent STAT activation has been associated with malignant progression in both solid tumors and blood malignancies (14, 15). This activation is associated with the persistent activity of oncogenic protein-tyrosine kinase and has been shown to be directly linked to cellular transformation. For example, STAT3 activation directly participates in the transformation by the SRC oncoprotein (16), and STAT5 activation is essential for the transformation by BCR-Abl oncogenic fusion protein (17).

STAT3 and STAT5 have been shown to be essential for cell proliferation in the context of KIT-Asp816 gain-of-function mutant, in cell lines, or in activated mastocytes from patients with systemic mastocytosis (18–20). However, the pathways linking the KIT receptor to STAT phosphorylation and acti-
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EXPERIMENTAL PROCEDURES

**Cells**—P815 and FMA3 are mouse mastocytoma cell lines carrying endogenous activating KIT-D814Y mutation and juxtamembrane deletion, respectively. The human mast cell leukemia cell line HMC-1 carrying the two point mutations V560G and D816V (HMC1.2) was kindly provided by Dr. J. H. Butterfield (21). TF1 KIT-D816V and MO7e KIT-D816V cells were derived by retroviral infections of parental TF-1 and MO7e cells, two human cytokine-dependent hematopoietic cell lines. Cell populations were selected by cell sorting and growth in the absence of GM-CSF. TF1 KIT-D816V and MO7e KIT-D816V cell lines were verified by sequencing of c-kit cDNAs. They are both KIT-dependent cell lines as controlled by siRNA and by using inhibitors of KIT. All cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). All reagents were from Invitrogen. Primary cultures of bone marrow-derived mast cells (BMMC) from wild-type mice were prepared and maintained in Opti-MEM medium with 10% FBS, 2 mM glutamine, 50 μM β-mercaptoethanol, and 1% conditioned medium from baby hamster kidney cell cultures that express murine IL3. SCF stimulations were done with 250 ng/ml murine SCF (PeproTech). BMMC derived from transgenic mice expressing KIT-D816V were grown in BMMC media without exogenous IL-3 (22). COS-7 cells were grown in DMEM with 10% FBS, 1 mM sodium pyruvate.

**Antibodies and Inhibitors**—Anti-AKT, anti-phospho-AKT (Ser473), anti-KIT, anti-phospho-KIT (Tyr719), anti-phospho-STAT1 (Tyr701), anti-phospho-STAT3 (Tyr705), anti-phospho-STAT5 (Tyr694), anti-p38, anti-GSK3 rabbit polyclonal antibodies, and anti-phospho-GSK3 (Ser9) rabbit monoclonal antibody were from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-active pan-ERK (Thr202/Tyr204) and anti-phospho-LYN (Tyr396) antibodies were from Promega (Madison, WI). Anti-phospho-LYN (Tyr396) rabbit monoclonal antibody was from Epitomics (Burlingame, CA). Anti-KIT mouse monoclonal antibody, anti-ERK2, anti-STAT1, anti-STAT3, anti-STAT5, and anti-VAV rabbit polyclonal antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-active pan-ERK (Thr202/Tyr204) and anti-p38 (Thr180/Tyr182) rabbit polyclonal antibodies were from Promega (Madison, WI). Anti-phospho-LYN (Tyr396) rabbit monoclonal antibody was from Epitomics (Burlingame, CA). Anti-KIT mouse monoclonal antibody (MAB332) was from R&D Systems. Anti-phospho-IgG mouse monoclonal (clone 4G10), anti-phospho-STAT1 (Ser727), and anti-phospho-STAT3A/B (Ser265/271) rabbit antiserum, anti-JAK3 rabbit antiserum recognizing murine JAK3, and anti-human JAK3 C terminus were from Upstate, Millipore. Anti-phospho-STAT3 (Ser727) mouse monoclonal antibody (MAB3705) was from Chemicon International. Anti-tubulin mouse monoclonal was from Sigma.

Dasatinib (BMS-354825) was purchased from Sequoia Research Product. JAK inhibitor I (pan-JAK inhibitor), SB203580 (p38 MAPK inhibitor), SU6656 (SFK inhibitor), and U0126 (MEK1/2 inhibitor) were purchased from Calbiochem (Merck). Bisindolylmaleimide, H-89, KN-93, and PKG inhibitor were from the “serine/threonine kinases inhibitor set” (Calbiochem). SB216763 (GSK3 inhibitor) was purchased from Sigma.

**Immunoprecipitation, Peptide Pulldown, and Western Blotting**—Before SCF stimulation, BMMCs were starved in Opti-MEM, 0.5% FCS, and 1% bovine serum albumin (BSA) for 4 h. Prior to chemical treatment, P815 and HMC-1 cells were starved in RPMI 1640 medium with 0.5% FCS for 3 h.

Cells were lysed at 4 °C in ice-cold “HNTG” lysis buffer (50 mM Hepes (pH 7), 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 1% Triton X-100, 10% glycerol, 0.2% NaF, 1 mM orthovanadate, protease inhibitors mixture (Complete Tablets; Roche Diagnostics)). Insoluble material was removed by centrifugation at 15,000 × g for 30 min at 4 °C. Protein concentrations in the remaining soluble cell lysate fraction (SCL) were assessed with the protein assay from Bio-Rad. Immunoprecipitation were performed with 1 mg of protein lysates, 1 μg of antibody, and 10 μl (bed volume) of protein A-Sepharose beads (Amersham Biosciences).

For peptide pulldown experiments, 15-mer peptides corresponding to amino acids 563–577 of human KIT INGNNYVIDPTQLP (Eurogentec) including phosphorylated or unphosphorylated Tyr569/Tyr579 were coupled to N-hydroxysuccinimide-activated Sepharose beads (Amersham Biosciences) and incubated with cell lysates for 2 h at 4 °C.

Lysates, immunoprecipitates, or affinity complexes were resolved by SDS-PAGE and transferred onto PVDF membrane (Immobilon-P; Millipore) followed by Western blotting with specific antibodies. Signals were revealed using West Pico chemiluminescent substrate (Pierce).

**Vectors, siRNA, and Transfections**—The vectors used for COS-7 transfection are pcDNA3-KIT-WT and pcDNA3-KIT-D816V. The following vectors were used for the production of STAT fusion proteins used in *in vitro* kinase assays: pGEX-STAT1 (a gift from Drs. Ali and Sayeski (23)); pGEX-STAT3 (a gift from Drs. Cao and Cheh Peng (24)); and pGEX-STAT5A (amino acids 494–793) (a gift from Dr. Ikuta (25)). pGEX-STAT5A-Y694F was obtained by site-directed mutagenesis using the QuikChange kit (Stratagene) according to the manufacturer’s instructions. The following vectors were used for STAT luciferase reporter assay: pGAS-Luc (Stratagene); p4×TSV-Luc containing the core region of the LIF-response element of the rat a2M gene (a gift from F. Gouilleux and G. M. Hocke (26)); and pBCAS-Luc (a gift from W. Leonard (27)) to monitor STAT1, -3, and -5 activities, respectively. pRL-TK expressing Renilla luciferase was used as control in the transfection experiments (Promega). Luciferase activities in the figures are expressed as arbitrary units relative to control Renilla luciferase.

COS-7 cells were transfected with FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer’s protocol. HMC-1 and P815 cells were transfected by electroporation with an Xcell pulser (Bio-Rad). 8 × 10⁶ cells in 500 μl of RPMI 1640 medium were shocked in 4-mm electroporation cuvettes at 250 V and 950 microfarads for HMC-1 cells and 280 V and 950 microfarads for P815 cells. jak3, src, and lyn siRNAs were from Dharmacon (ON-TARGET plus SMART-pool). c-kit siRNA was described previously (32). For
siRNA knockdown, 0.4 nmol of siRNA was used for each electroporation.

In Vitro Kinase Assay—Purified KIT-D816V and SRC kinases are a gift from L. Gros (AB Science). They were obtained from a baculovirus expression system and purified as described previously (28). Vectors expressing GST-STAT fusion proteins were transformed in the Rosetta™ Escherichia coli strain (Merck). Fusion proteins were purified and eluted by GST-glutathione affinity chromatography (glutathione-Sepharose 4 Fast Flow, GE Healthcare).

In vitro kinase assays were performed with the indicated quantity of enzymes and substrates in the presence of an excess of ATP (250 μM). Reactions were carried out in kinase buffer (50 mM Hepes (pH 7.8), 10 mM MgCl₂, 2 mM MnCl₂, Brij 0,01%) at room temperature and stopped after 1 h by adding 50 mM EDTA. GST-STAT substrates were then collected in wells coated with anti-GST antibodies (GST-Tag Antibody Plate, Novagen), and binding was allowed for 1 h followed by three washes in PBS, 0.05% Tween. Substrate phosphorylation was then revealed by detecting the binding of the primary anti-phosphotyrosine-Stat1/3/5 antibodies (Cell Signaling Technology, used at a 1:200 dilution) and incubation with tetramethylbenzidine (substrate reagent, R&D Systems, Minneapolis, MN). The final reaction product was quantified by spectrophotometry at 370 nm.

STAT Luciferase Reporter Assay—COS-7 cells were transfected with a plasmid containing WT KIT or KIT-D816V, a STAT Firefly luciferase reporter plasmid, and a control Renilla luciferase plasmid in a 1:1:0.2 ratio using FuGENE 6 according to the protocol described above. The total amount of plasmid DNA used was 3.3 μg to transfec 3 × 10⁵ cells. Each transfection was done in triplicate. Luciferase activity was determined 24 h after transfection.

HMC-1 and P815 were transfected by electroporation as described above. The STAT reporter plasmid and control plasmid ratio used was 10:1, and the total amount of transfected DNA was 5.5 μg. Immediately after electroporation, each cuvette was split in two, and cells were grown for 6 h with 1 μM dasatinib or with an equivalent volume of DMSO as a control.

The luciferase activity was determined using a Dual-Luciferase™ reporter assay kit (Promega). Each measure was done in triplicate. In all figures, specific STAT activity was represented as the ratio of Firefly luciferase over Renilla luciferase activity for 10 μg of cell lysates proteins.

Cellular Fractionation—HMC-1 cells were treated with hypotonic buffer (25 mM Hepes, 5 mM KCl, 0.5 mM MgCl₂, 1 mM DTT, 0.5% Nonidet P-40, protease inhibitor mixture). Cytoplasmic and nuclei fractions were separated by centrifugation for 1 min at 1200 × g. Pelleted nuclei were suspended in hypertonic buffer (25 mM Hepes, 350 mM NaCl, 10% sucrose, 1 mM DTT, 0.5% Nonidet P-40, protease inhibitor mixture), and cleared protein extracts were obtained following 1 h of incubation at 4 °C and centrifugation at 16,000 × g for 10 min.
although AKT and ERK pathways were down-regulated as in the context of wild-type KIT stimulation, showing the distinctive feature of STAT proteins activation (Fig. 1D, last lane).

Thus, under KIT gain-of-function mutants, the STAT pathway was not down-modulated as upon WT KIT stimulation, resulting in permanent tyrosine phosphorylation of STAT1, -3, and -5.

**Contribution of SFKs and JAKs to STAT Tyrosine Phosphorylation**

To identify the STAT kinases downstream of KIT in neoplastic mast cells, we used a candidate approach. The kinases responsible for the tyrosine phosphorylation of STATs include the cytosolic kinases of the Janus (JAK) or SRC family (SFK). Phosphorylation STAT proteins can also occur through direct phosphorylation by an upstream receptor tyrosine kinase as described for FGFR3 (35), PDGFR (36, 37), or FLT3 (38).

First, we asked whether JAKs were involved in STATs activation. Members of JAK family kinases have been previously shown to be phosphorylated in the HMC-1 cell line (Fig. 2A) (39). In our experiments, JAK3 is the main JAK activated in P815 and HMC-1, and its phosphorylation is dependent on KIT catalytic activity (data not shown). The treatment of P815 cells with JAK inhibitor I, a potent inhibitor of JAK1, -2, and -3 and Tyk2, resulted in inhibition of JAK3 autophosphorylation (Fig. 2A, upper panels) but had no influence on STAT1, -3, and -5 tyrosine phosphorylation (Fig. 2A, lower panels). The same result was obtained following JAK3 expression knockdown using RNA interference in P815 cells (supplementary Fig. 2). These results suggest that STATs phosphorylation is independent of JAKs activity in P815. In HMC-1 cells, a reduction of phosphorylation of STAT1 and STAT5 was observed, showing a contribution of JAKs to activation of STAT proteins (Fig. 2B). Unlike in P815, JAK inhibition in HMC-1 resulted in decreased SFK Tyr416 phosphorylation.

Second, to determine the implication of SFK, we conducted the same experiments using the selective inhibitor SU6656. As shown on Fig. 2C, the treatment of both P815 and HMC-1 cells resulted in the total disappearance of phospho-STAT1 and a partial reduction of phospho-STAT3. Phospho-STAT5 was not affected by SFK inhibition in both cells. These results suggest an implication of SFK in STAT1 and STAT3 phosphorylation but not in the activation of STAT5.

To further demonstrate the implication of SFK, we silenced two members of this family, SRC and LYN, which are activated in the HMC-1 cell line. Reduction of LYN expression using specific siRNA did not diminish the phosphorylation of

**FIGURE 1. Constitutive activation of STAT1, -3, and -5 by oncogenic KIT.** A, constitutive activation of STAT1, -3, and -5 by KIT gain-of-function mutants. SCLs from different mastocyte cell lines (FMA3, P815, and HMC-1) were analyzed for STAT1, -3, and -5 tyrosine phosphorylation by Western blotting using specific anti-phosphotyrosine antibodies as indicated. B, STAT phosphorylation is dependent on KIT-D816V activity. Starved P815 and HMC-1 cells were treated with dasatinib (1 μM) for 2 h. SCLs were analyzed by Western blotting using specific antibodies directed against each phosphotyrosine of STAT. Membranes were also probed with anti-tubulin as a control. C, STAT phosphorylation is dependent on KIT-D816V expression. Control or c-kit siRNAs were transfected in HMC-1 cells, and lysates were analyzed as in B, except that ERK2 was used as loading control. D, ectopic expression of KIT-D816V induces STAT tyrosine phosphorylation. STAT protein phosphorylation in parental and KIT-D816V stably transfected TF-1, and MO7e cells were investigated as in A. Membranes were also probed with anti-ERK2 as a loading control. E, KIT-dependent STAT tyrosine phosphorylation in transfected cells. TF-1-KIT-D816V and MO7e-KIT-D816V cells were transfected with control or c-kit siRNAs, and phosphorylation of STAT proteins was analyzed.

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**FIGURE 1. Constitutive activation of STAT1, -3, and -5 by oncogenic KIT.** A, constitutive activation of STAT1, -3, and -5 by KIT gain-of-function mutants. SCLs from different mastocyte cell lines (FMA3, P815, and HMC-1) were analyzed for STAT1, -3, and -5 tyrosine phosphorylation by Western blotting using specific anti-phosphotyrosine antibodies as indicated. B, STAT phosphorylation is dependent on KIT-D816V activity. Starved P815 and HMC-1 cells were treated with dasatinib (1 μM) for 2 h. SCLs were analyzed by Western blotting using specific antibodies directed against each phosphotyrosine of STAT. Membranes were also probed with anti-tubulin as a control. C, STAT phosphorylation is dependent on KIT-D816V expression. Control or c-kit siRNAs were transfected in HMC-1 cells, and lysates were analyzed as in B, except that ERK2 was used as loading control. D, ectopic expression of KIT-D816V induces STAT tyrosine phosphorylation. STAT protein phosphorylation in parental and KIT-D816V stably transfected TF-1, and MO7e cells were investigated as in A. Membranes were also probed with anti-ERK2 as a loading control. E, KIT-dependent STAT tyrosine phosphorylation in transfected cells. TF-1-KIT-D816V and MO7e-KIT-D816V cells were transfected with control or c-kit siRNAs, and phosphorylation of STAT proteins was analyzed.
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STAT proteins (Fig. 1D, right panels). On the other hand, reduction of SRC expression abolished STAT1 tyrosine phosphorylation (Fig. 1D, left panels). This result strongly supports the implication of SRC as the STAT kinase for STAT1 in HMC-1.

KIT Phosphorylates STAT1, -3, and -5 In Vitro—We next sought to determine whether KIT-D816V could directly phosphorylate STAT proteins as demonstrated for some other receptor tyrosine kinases. We used an in vitro kinase assay coupled with an ELISA detection system to assess STAT1, -3, and -5 phosphorylation. In vitro kinase assays were performed using purified KIT-D816V and GST-STAT fusion proteins as substrates. Kinase assays were performed in excess of ATP (250 μM) and substrates (400 nM) and increasing concentrations of enzyme. Substrate phosphorylation was revealed using specific STAT anti-phosphotyrosine antibodies. As seen in Fig. 3A (upper panel), the tyrosine phosphorylation of all three STATs increased with KIT-D816V protein concentration. As a control, the tyrosine mutant GST-STAT5-Y694F did not show phosphorylation in this assay or in control Western blots (Fig. 3B). Thus, KIT-D816V phosphorylated all three STATs in vitro at the conserved tyrosine residue.

Intrinsic Kinase Properties of KIT-D816V and SRC May Account for STAT Substrate Selectivity—Having shown that KIT-D816V and SFK differentially contribute to tyrosine phosphorylation of STATs in cells, we asked whether this could be explained by the intrinsic catalytic properties of the kinases toward STAT substrates. To address this question, we performed kinase assays to determine SRC and KIT-D816V kinetic parameters toward the different STAT substrates. Enzyme titration assays were performed in excess of substrates to settle the appropriate amount of recombinant enzyme to use in the assays. As seen on Fig. 3A (lower panel), GST-STAT phosphorylation displayed a linear increase followed by saturation as the concentration of SRC was raised. For KIT-D816V, saturation was reached for STAT3 but remained linear for STAT1 and STAT5. For each enzyme, the quantity of KIT and SRC enzymes was chosen in the linear part of the curves, i.e. 1 μg for both enzymes. The phosphorylation rates of the different GST-STAT proteins by SRC and KIT-D816V were then compared using the set enzyme quantity, in 1 h with increasing concentration of STAT substrates. As seen on Fig. 3C, phosphorylation of each STAT fusion proteins increased with substrate concentration, and the reactions followed Michaelis-Menten kinetics. For STAT3 phosphorylation, KIT and SRC kinases showed overlapping kinetics indicating similar enzyme kinetics parameters (V_{max} and K_{m}). Regarding STAT1 phosphorylation, SRC kinase showed higher V_{max} and lower K_{m} values than KIT, which indicated that SRC is more efficient than KIT for STAT1 tyrosine phosphorylation. Finally, STAT5 phosphorylation kinetics show very different enzymatic parameters with lower K_{m} values for KIT but higher V_{max} values for SRC; therefore, the comparison of SRC and KIT efficiencies to phosphorylate STAT5 in vitro was not conclusive. In conclusion, the enzymatic parameters may account for the contribution of SRC in STAT1 phosphorylation and for the cooperative effect of SRC and KIT on STAT3 phosphorylation observed in cells.

Endogenous Interactions of KIT-D816V with STAT1, -3, and -5—Having shown that KIT-Asp816 mutant can phosphorylate STAT proteins in vitro, we asked whether STAT1, -3, and -5 were part of the KIT-D816V receptor signaling complex. KIT-D816V was immunoprecipitated from HMC-1 cells, and co-precipitated proteins were probed for STATs by Western blotting using specific antibodies. As seen on Fig. 4A
KIT-D816V co-immunoprecipitated with STAT1, -3, and -5 (also seen on supplemental Fig. 4, A and B). Furthermore, these interactions were dependent on KIT-D816V catalytic activity as they were lost or severely diminished when the kinase activity of the receptor was abolished with dasatinib treatment. Interestingly, SFKs were also part of this complex (Fig. 4A, right panel).

The di-tyrosine motif Tyr$_{589}$–Tyr$_{591}$ within the juxtamembrane domain of FLT3-ITD has been involved in STAT5 activation (40). Furthermore, the homologous motif in KIT is thought to recruit SFK (41). We assumed that this docking site on KIT, consisting of tyrosines 568 and 570, could also be implicated in STAT binding. We performed affinity pulldown assays with synthetic peptides containing the di-tyrosine motif either in a nonphosphorylated form or in one of the three possible phosphorylated states, i.e. phosphotyrosine 568 alone (Tyr(P)$_{568}$), phosphotyrosine 570 alone (Tyr(P)$_{570}$), or both 568–570 tyrosines phosphorylated (Tyr(P)$_{568}$–Tyr(P)$_{570}$).
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A, co-immunoprecipitation of endogenous proteins in HMC-1 cells. HMC-1 cells were starved overnight and subsequently treated or not for 2 h with dasatinib. SCLs were subjected to immunoprecipitation (IP) with anti-KIT antibody. Proteins from immunocomplexes were resolved by SDS-PAGE and probed with anti-STAT antibodies (left panel) or with anti-activated SFK antibodies (Y396-LYN and Y1416-SRC) (right panel). B, peptide pulldown assays. HMC-1 SCL was used for affinity pulldown assay with 2 nmol of either nonphosphorylated peptide (Y568Y570) or mono-phosphorylated peptides (pY568 and pY570) or bi-phosphorylated peptide (pY568pY570). The binding of STAT1, -3, and -5 was revealed with specific antibodies. Another SH2-containing protein, VAV, was analyzed as a control. SCL was used as positive control.

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FIGURE 4. Interaction between KIT-D816V and STATs in HMC-1 cells. A, co-immunoprecipitation of endogenous proteins in HMC-1 cells. HMC-1 cells were starved overnight and subsequently treated or not for 2 h with dasatinib. SCLs were subjected to immunoprecipitation (IP) with anti-KIT antibody. Proteins from immunocomplexes were resolved by SDS-PAGE and probed with anti-STAT antibodies (left panel) or with anti-activated SFK antibodies (Y396-LYN and Y1416-SRC) (right panel). B, peptide pulldown assays. HMC-1 SCL was used for affinity pulldown assay with 2 nmol of either nonphosphorylated peptide (Y568Y570) or mono-phosphorylated peptides (pY568 and pY570) or bi-phosphorylated peptide (pY568pY570). The binding of STAT1, -3, and -5 was revealed with specific antibodies. Another SH2-containing protein, VAV, was analyzed as a control. SCL was used as positive control.

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seen Fig. 4B, STAT1, -3, and -5 bound to the phosphorylated peptides. The interaction was phosphorylation dependent as shown by the nonphosphorylated peptide (Tyr568, Tyr560). As a control, STAT proteins did not bind a control tyrosine phosphorylated peptide derived from the sequence of CD28 (supplemental Fig. 5). These results suggest that STAT proteins are part of the KIT receptor signaling complex and that the recruitment of STAT proteins occur in the juxtamembrane di-tyrosine motif Tyr568, Tyr570.

**STAT5 Is Transcriptionally Active in the Neoplastic Mast Cells, whereas STAT1 and STAT3 Are Not**—STAT tyrosine phosphorylation is required for their translocation to the nucleus and thus for their activation as transcription factors. We used a luciferase-based reporter assay to test STAT transcriptional activity downstream of KIT-D816V. Cells were transfected with reporter plasmids expressing Firefly luciferase under the control of promoters containing STAT1, -3, or -5-responsive elements, together with a control plasmid encoding Renilla luciferase driven by the ubiquitous thymidine kinase promoter. To validate the assay, the c-kit mutant cDNA and the reporter constructs were first transfected in COS-7 cells. Transient transfection of KIT-D816V in COS cells leads to robust STAT protein phosphorylation (data not shown). As seen in Fig. 5A, KIT-D816V ectopic expression in COS-7 cells led to the activation of all three STAT reporter constructs. This experiment indicated that KIT-D816V can activate STAT1, -3, and -5 transcriptional activity.

We then addressed whether STAT proteins were transcriptionally active in the mastocyte cell lines. The same experiment as above was performed in P815 (Fig. 5B) and HMC-1 (Fig. 5C) cells. As shown in Fig. 5, B and C, STAT5 reporter was active in both cell types, and this activity was dependent on KIT-D816V kinase activity as revealed by dasatinib treatment. By contrast, we could not detect any transcriptional activity of either STAT1 or STAT3 reporters in those cells. In conclusion, KIT-D816V can activate STAT1, -3, and -5, but only STAT5 is transcriptionally active in P815 and HMC-1 mast cells.
Serine Phosphorylation and Nuclear Localization of STATs in Neoplastic Mast Cell Lines—Additional serine phosphorylation in the C-terminal transactivation domain of STAT proteins is necessary for full transcriptional activity (42). Because STAT1 and STAT3 were not active, we asked whether they were phosphorylated on the specific serine residues. As shown in Fig. 6A, STAT1, -3, and -5 are phosphorylated on their conserved serines 727, 727, and 726/731, respectively, both in P815 and HMC-1 cells. Furthermore, the inhibition of KIT-D816V activity led to the disappearance of phospho-serine STAT3 and STAT5 in both cells showing that these phosphorylations are KIT-dependent. By contrast, STAT1 serine phosphorylation is basal and independent of KIT catalytic activity.

To further delineate the pathways responsible for STATs serine phosphorylation, we applied a systematic approach using chemical inhibitors. The MAPK family kinases were targeted either with U0126 to inhibit MEK1/2 and as a consequence the ERK pathway or with SB203580 to inhibit the p38 pathway. We also used KN-93 to inhibit CaMKII, bisindolylmaleimide, H-89, and protein kinase G inhibitor to target PKC, PKA, and PKG, respectively, and SB216763 to inhibit GSK3. All these inhibitors were used at the minimal dose required to inhibit the respective kinases (43). As shown on Fig. 6B, treatment of both P815 and HMC-1 with U0126 resulted in the inhibition of STAT3 serine phosphorylation, with no effect on STAT1 and STAT5. A reduction of STAT5 serine phosphorylation was observed with treatment of bisindolylmaleimide (2 μM) for 30 min as shown in Fig. 6C. The cytoplasmic and nuclear localization of STATs was analyzed by Western blotting both with phosphotyrosine-specific antibodies and antibodies directed against total proteins.
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maleimide (Fig. 6C). Thus, ERK and PKC pathways are likely to be involved in STAT3 and STAT5 serine phosphorylation, respectively, downstream of KIT-D816V. The other inhibitors had no effect on STAT serine phosphorylation (supplemental Fig. 3).

As all three STATs were phosphorylated on tyrosine and serine residues, but only STAT5 was transcriptionally active, we finally checked their nuclear localization in HMC-1 cells. We performed cellular fractionation, and as shown in Fig. 6D, all STATs were found both in the cytoplasm and nucleus. Furthermore, we observed an enrichment of the tyrosine-phosphorylated forms in the nuclear fraction compared with the cytoplasmic one, with phosphotyrosine STAT5 only detectable in the nuclear fraction. Thus, neither serine phosphorylation nor nuclear localization could account for the observed absence of transcriptional activity of STAT1 and STAT3.

**DISCUSSION**

We show here that KIT is responsible for the permanent phosphorylation of all three STAT proteins STAT1, -3, and -5 in P815 and HMC-1 cell lines with diverse contributions of SFK and JAKs depending on the STAT protein and on the cell line. We also report the involvement of ERK MAPks and PKC for serine phosphorylation of STAT3 and STAT5, respectively, downstream of KIT in the two cell lines. Importantly, only STAT5 showed transcriptional activity both in P815 and HMC-1 cells. Overall, similar results and conclusions were reached for both murine P815 and human HMC-1 cells. However, a crucial difference was pointed out regarding the implication of JAKs restricted to HMC-1 cells.

We had previously reported the constitutive tyrosine phosphorylation of all three STAT proteins STAT1, -3, and -5 by KIT juxtamembrane mutants, i.e. regulatory type mutants mostly found in gastrointestinal stromal tumors (29). In neoplastic mast cells, the most common KIT alteration is the kinase domain substitution Asp816Val by a valine residue. This mutant is also found in germ cell tumors (9–11) and in acute myeloid leukemias (7, 8). Previous studies have reported the activation of STAT1 or STAT3 by KIT-D816V in transfected cell lines (18, 30, 44); however, very few have addressed the activation of STAT in neoplastic cells. A noticeable exception is a recent description of STAT5 activation and STAT5 requirement for cell proliferation in neoplastic mast cell lines (29). In neoplastic mastocytoma P815, SFK are the main STAT1 tyrosine kinase, although SFK and KIT cooperate to phosphorylate STAT3, and KIT is the main candidate as STAT5 kinase. In human mast cell leukaemia cell line HMC-1, we reached the same conclusion for the contribution of SFK, but in these cells the activations of SFK are in part dependent on JAKs. As mentioned above, the contribution of JAKs upstream of SFK is the main difference we found between P815 and HMC-1.

Our kinase assays suggest that KIT is a STAT1, -3, and -5 kinase in vitro. This was previously shown by Deberry et al. (51) for STAT1. We have shown for the first time a physical interaction of KIT with all three STAT endogenous proteins in HMC-1 cells, which suggest that STAT proteins could also be KIT substrates within cells as well. However, the suspected site of interaction, a di-tyrosine motif in KIT juxtamembrane region, is a docking site involved in the recruitment of many signaling proteins, including docking molecules such as SHP2 (52) as well as SFK (41). Therefore, this site is involved in the formation of a multiprotein signaling receptor complex that includes other potential STAT kinases. Interestingly, the homologous site in the closely related receptors FLT3 and PDGFR was implicated in STAT5 activation by the oncogenic mutants FLT3-ITD and TEL-PDGFRβ, respectively (40, 53).

Unlike in the context of KIT mutant receptors, in the case of FLT3-ITD, only STAT5 is activated, but as for KIT the phosphorylation of STAT5 is thought to be independent of SFK and JAKs. Therefore, there are both similarities and differ-
ences in the activation of STAT proteins by these related mutant receptors.

In our experiments, JAK3 but not JAK2 is activated downstream of KIT in the two mast cell lines (Fig. 2). It is of interest to note that JAK1 and JAK3 have been implicated in the phosphorylation of STAT proteins in the closely related HMC-1.1 cell line that was derived from the original HMC-1 (39). Although not obtained with the same cells, these results support a model implicating JAKs in the phosphorylation of STAT proteins in HMC-1.

There are nine members in the SRC family kinase, many of which are expressed in mast cells. The inhibitors used here do not discriminate among family members. LYN is one candidate as it is activated downstream of KIT in both cell lines. We knocked down LYN expression by RNA interference in HMC-1 and found no differences in STATs' phosphorylation status, excluding LYN as the major STAT kinase. We found, however, that SRC is essential for STAT1 tyrosine phosphorylation. It remains to be determined which SFK member could act as a STAT3 kinase in the neoplastic mast cell.

In addition to tyrosine phosphorylation, serine phosphorylation is required for the full transcription activation of STAT proteins (42). The conserved serine is located in the transactivation domain of STAT proteins and embedded in a PMSP motif, a potential consensus site for MAPKs. In P815 and HMC-1 cells, all three STAT proteins were phosphorylated at the conserved serine residue. STAT1 serine phosphorylation was constitutive in these cells independently of KIT activation, whereas STAT3 and STAT5 serine phosphorylation was dependent on KIT kinase activity. In line with several other studies, which concluded that MAPKs are bona fide STAT3 serine kinases (54), our data suggest that the MEK-ERK1/2 pathway is involved in STAT3 serine phosphorylation both in P815 and HMC-1. For STAT5, our screen using chemical inhibitors pointed to kinases of the PKC family in both cell lines. PKCs have already been proposed as STAT kinases for STAT1 in type I interferon signaling and STAT3 in Insulin signaling context (55, 56).

In the original paradigm of the STAT signaling pathway, STAT proteins were described as direct signaling effectors connecting membrane receptors to transcription. Indeed, the activation of STAT proteins results in the expression of genes that control critical cellular functions, including cell proliferation, survival, differentiation, and development (13). However, recent studies on STAT protein point to more complex functions for some STAT proteins. For instance, a function of STAT3 in the mitochondria was recently discovered, in which STAT3 promoted oxidative phosphorylation and increased transformation by oncogenic Ras independently of its transcription activity (57, 58). Therefore, STAT3 could contribute to KIT-Asp816 oncogenicity independently of its role as transcription factor.

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