Steel Factor Stimulates the Tyrosine Phosphorylation of the Proto-oncogene Product, p95\textsuperscript{vav}, in Human Hemopoietic Cells*

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Steel factor (SF) (also called stem cell factor, mast cell growth factor, or c-kit ligand) is a recently cloned hemopoietic growth factor that is produced by bone marrow stromal cells, fibroblasts, and hepatocytes. In both mouse and man it acts synergistically with several colony stimulating factors, including interleukin-3 (IL-3) and granulocyte macrophage-colony stimulating factor (GM-CSF), to induce the proliferation and differentiation of primitive hemopoietic precursor cells. In order to study its mechanism of action and to explore the molecular basis for its synergistic activity we have examined the proteins that become tyrosine phosphorylated in response to SF, IL-3, and GM-CSF. We report herein that SF, but not IL-3 or GM-CSF, dramatically stimulates the tyrosine phosphorylation of the product of the recently discovered proto-oncogene, \textit{vav}, in two SF-responsive human cell lines, M07E and TF-1. Although phosphorylation is very rapid, reaching maximal levels within 2 min at 37 \degree C, co-immunoprecipitation studies suggest that c-kit may either not associate directly with p95\textsuperscript{vav} or bind to it with very low affinity. Nonetheless, our data suggest that c-kit may utilize p95\textsuperscript{vav} to mediate downstream signaling in hemopoietic cells.

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The abbreviations used are: SF, steel factor; mAb, monoclonal antibody; DMEM, Dulbecco’s modified Eagle’s medium; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; CAPS, 3-(cyclohexylamino)propanesulfonic acid; IL-3, interleukin-3; GM-CSF, granulocyte-macrophage colony stimulating factor; β-ME, 2-mercaptoethanol; FCS, fetal calf serum; BSA, bovine serum albumin.

effects by binding to the product of the c-kit proto-oncogene (1–3, 7–11) (reviewed in Ref. 12). In the mouse, both SF and c-kit have been shown to play key roles in hemopoiesis; mutations in either the SF gene, which maps to the steel (Sl) locus on chromosome 10, or the c-kit gene, allelic with the Dominant White Spotting (W) locus on chromosome 5, lead to severe anemia as well as to mast cell deficiency, sterility and defects in pigmentation (13–15). In Sl mutants the anemia is due to defects in the c-kit ligand, which is normally produced in both a soluble and a membrane bound form by stromal cells in the bone marrow. In W mutants, anemia is the result of defective c-kit receptors on the surface of hemopoietic stem cells.

The c-kit receptor belongs to a family of tyrosine kinase receptors that includes the receptors for platelet-derived growth factor and macrophage colony stimulating factor (16). Recent mechanism of action studies with normal mouse mast cells and with human M07E cells suggest that SF induces c-kit to autophosphorylate on tyrosine residues (17, 18). Then, at least in mouse mast cells, tyrosine phosphorylated c-kit appears to bind phosphatidylinositol 3' kinase and phospholipase C-γ1 (17). In M07E cells, SF has also been shown to induce the tyrosine phosphorylation of mitogen-activated protein kinase and the serine phosphorylation of Raf-1 kinase (18). In this study we have further examined the proteins that become tyrosine phosphorylated in human M07E and TF-1 cells and demonstrate that one of these proteins is the product of the recently cloned proto-oncogene \textit{vav}. In these SF-responsive cell lines, SF is substantially more effective than IL-3 or GM-CSF in stimulating the tyrosine phosphorylation of this putative signal transduction intermediate, and this may be responsible in some extent for the synergism observed between SF and these two cytokines.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant human SF was generously provided by Dr. Kristina Zeebo, Amgen, Thousand Oaks, CA. The anti-human c-kit mAb, YB5.88, which was raised against the blast cells from a patient with acute myeloid leukemia (19), was kindly donated by Dr. Leonie Ashman, University of Adelaide, Australia. The anti-phosphotyrosine mAb, 4G10, generated against phosphotyrosine and shown to be highly specific for phosphotyrosine residues (20), was generously provided by Dr. Brian Druker, Dana-Farber Cancer Institute, Boston, MA. Two anti-p95\textsuperscript{vav} rabbit antisera were used in this study; one was generated against a synthetic peptide corresponding to the second nuclear localization signal of uau, i.e. residues 528–541 of the human p85\textsuperscript{vav} protein (21) and the other against the cysteine-rich region of p95\textsuperscript{vav} (22). Recombinant human IL-3 and human GM-CSF were purified, using Sephadex G75 and reversed phase high performance liquid chromatography, from concentrated serum free supernatants of COS cells transfected with expression vectors containing the human IL-3 cDNA, kindly provided by Genetics Institute, and the human GM-CSF cDNA, generously donated by Dr. Donna Hogge, Terry Fox Laboratory, Vancouver.
Cell Lines—The M07E cell line was obtained from Dr. Hermann Zillenier, Biomedical Research Centre, Vancouver, and was originally established by Dr. Luigi Pegoraro and colleagues from the peripheral blood of an infant with acute megakaryocytic leukemia (23). These cells, as well as AML-193 cells, which were obtained from ATCC, were cultured in DMEM containing 10% FCS or 10% FCS for 12-18 h at 4 °C, and then washed 4 times with PSB containing 0.1% Nonidet P-40 and phosphorylated proteins (20). After removal of the primary antibody, blots were incubated with cell lysates for 2 h at 4 °C, and then 20 μl of packed protein A-Sepharose beads (Pharmacia) were added for an additional 2 h at 4 °C. Beads were washed 4 times with PSB containing 0.1% Nonidet P-40 and 0.5% BSA for 12-18 h at 4 °C using 10 mM CAPS, pH 10.0 and 10% methanol. Residual binding of insoluble material was removed by centrifugation at 4 °C for 15 min at 10,000 g. After a 48-h incubation at 37 °C in a humidified atmosphere of 5% CO₂ in air, 1 μCi/ml [³H]thymidine (Du Pont-New England Nuclear, 2 Ci/mmol) was added and cells labeled for 4 h prior to harvesting and counting in an LKB 1205 Betaplate liquid scintillation counter.

Gel Electrophoresis and Immunoblotting—Exponentially growing cells, washed free of serum and growth factors, were incubated with either 1% BSA or 0.5% BSA for 12-18 h at 4 °C (identical results were obtained with these two conditions). 2 h prior to cytokine stimulation, sodium orthovanadate (20 mM) was added to cells, as described previously (20). After treatment for 10 min at 37 °C with control buffer or the cytokines IL-3, GM-CSF, or SF (at levels 5-fold higher than that giving maximal [³H]thymidine incorporation), cells were washed with cold phosphate-buffered saline and gently rocked for 30 min at 4 °C in phosphorylation solubilization buffer (PSB) (50 mM Hepes, pH 7.2, 100 mM NaF, 10 mM NaPPi, 2 mM Na₃V₀₄, 2 mM EDTA, 2 mM Na₂MoO₄, 100 Kallikrein inhibitor units/ml aprotinin, 2 μg/ml leupeptin, and 0.5 mM freshly made phenylmethylsulfonyl fluoride) containing 0.5% Nonidet P-40. Insoluble material was removed by centrifugation at 4 °C for 15 min at 10,000 × g and lysates made 2.3% with sodium dodecyl sulfate (SDS), 5% with β-ME, and 10% with glycerol and boiled for 2 min prior to SDS-polyacrylamide (7.5%) gel electrophoresis (SDS-PAGE). Following SDS-PAGE, proteins were electrophoretically transferred onto Immobilon-P PVDF membranes (Millipore, MA) at 100 V for 2 h at 4 °C using 10 mM CAPS, pH 10.0 and 10% methanol. Residual binding sites on the membranes were blocked by incubation in Tris-buffered saline (TBS, 10 mM Tris-Cl, pH 8.0, 150 mM NaCl) containing 5% BSA for 1 h at 23 °C. Blots were then washed in TBST (TBS with 0.05% Tween-20) and incubated for 2 h in TBST with 1.5 mg/ml of a monoclonal antibody specific for the c-kit receptor (data not shown) since incubation of M07E cells with SF did not induce phosphorylation of such a protein. Instead, SF induced the phosphorylation on tyrosine residues of a diffuse 93-kDa protein (Fig. 2A). Phosphorylation of this protein is apparently specific for these cytokines since incubation of M07E cells with SF did not induce phosphorylation of such a protein. Instead, SF induced the phosphorylation on tyrosine residues of a diffuse 145-165-kDa protein and a relatively discrete 95-kDa protein (Fig. 2A). These results support the concept that IL-3 and GM-CSF share a common set of signal transduction intermediates as has been proposed by several investigators (20, 27), whereas SF stimulates a distinct but overlapping subset of signal transducing molecules (18).

To characterize the nature of the proteins specifically involved in the c-kit pathway, we investigated the identity of the 145-165- and 95-kDa proteins phosphorylated after stimulation of M07E cells by SF. Immunoprecipitation analysis showed that the 145-165-kDa protein is recognized by a monoclonal antibody specific for the c-kit receptor (data not shown). Three human hematopoietic cell lines were first examined for their ability to proliferate in response to SF: the megakaryocytic cell line, M07E, which grows in response to interleukin-3 (IL-3), granulocyte-macrophage-colony stimulating factor (GM-CSF), or interleukin-9 (20, 23); the acute myeloblastic leukemic cell line, AML-193, which proliferates in response to IL-3, GM-CSF, and granulocyte-colony stimulating factor (24); and the recently isolated TF-1 cell line, which was established from the bone marrow of a patient with erythroleukemia and responds to erythropoietin, GM-CSF, and IL-3 (25). As shown in Fig. 1A, M07E and TF-1 cells (but not AML-193 cells) could also proliferate under serum free conditions in response to SF. Moreover, as was found with normal human bone marrow cells (2, 6), SF stimulation of M07E cells was synergistic with IL-3 (or GM-CSF), whereas GM-CSF stimulation was only additive with IL-3 (Fig. 1B). This synergism was also observed, to a lesser extent, with TF-1 cells (data not shown).

In an attempt to explain the different proliferative responses of M07E/1 cells to IL-3, GM-CSF, and SF, we examined the overall pattern of tyrosine phosphorylation induced by each of these cytokines. In agreement with previous observations (20, 26) stimulation of quiescent M07E cells with either IL-3 or GM-CSF induced the phosphorylation on tyrosine residues of a diffuse 93-kDa protein (Fig. 2A). Phosphorylation of this protein is apparently specific for these cytokines since incubation of M07E cells with SF did not induce phosphorylation of such a protein. The mechanisms of SF-induced phosphorylation of such a protein could involve a common set of signal transduction intermediates as has been proposed by several investigators (20, 27), whereas SF stimulates a distinct but overlapping subset of signal transducing molecules (18).
Steel Factor Induces the Tyrosine Phosphorylation of p95^vav

Fig. 2. Identification of phosphotyrosine-containing proteins in M07E cells after cytokine treatment. A, M07E cells were incubated for 10 min at 37 °C with control buffer (C) or with IL-3, GM-CSF (GM), or SF, solubilized, and subjected to Western analysis using the anti-phosphotyrosine mAb 4G10. B, M07E cells were treated with control buffer (C), IL-3, GM-CSF (GM), or SF as indicated above, lysed, immunoprecipitated with rabbit anti-p95^vav polyclonal antibodies (21) and phosphotyrosine-containing proteins identified by immunoblotting as in A. C, the immunoblot shown in B was reprobed with a second anti-p95^vav antibody that recognizes the cysteine-rich region of p95^vav (22). D, M07E cells, incubated with SF (lanes 2 and 4–6) or control buffer (lanes 1 and 3) for 10 min at 37 °C, were lysed and either subjected directly to SDS-PAGE (lanes 1 and 2) or immunoprecipitated prior to SDS-PAGE with anti-p95^vav antiserum (lanes 3 and 4), preimmune serum (lane 5), or anti-p95^vav antiserum in the presence of 20 μg/ml immunizing peptide (lane 6). The immunoblot on the left, carried out using an anti-phosphotyrosine mAb, was reprobed with an anti-p95^vav antibody recognizing the cysteine-rich region of p95^vav (right panel). The migration of p95^vav and the IgG heavy chain is indicated by a solid and an open arrow, respectively.

Fig. 3. Time course of p95^vav tyrosine phosphorylation in SF-stimulated M07E cells. Cells were treated with control buffer (C) or SF for the indicated times. Cell lysates were immunoprecipitated using anti-p95^vav antibodies and tyrosine phosphorylated p95^vav detected by Western blotting with anti-phosphotyrosine antibodies as indicated in the legend to Fig. 2. Reblotting of the blot with anti-p95^vav antibodies established that all lanes contained the same amount of p95^vav protein.

The migration of p95^vav and the IgG heavy chain is indicated by a solid and an open arrow, respectively. The migration of p95^vav and the IgG heavy chain is indicated by a solid and an open arrow, respectively.

shown), indicating that this protein is the tyrosine-phosphorylated c-kit receptor (17). A candidate for the 95-kDa protein is the product of the vav proto-oncogene, p95^vav, a protein present in hematopoietic cells of lymphoid, myeloid, and erythroid lineages (21, 28, 29). p95^vav contains an SH2 domain characteristic of many proteins known to be substrates for tyrosine protein kinases (30). Indeed, p95^vav becomes phosphorylated on tyrosine residues upon co-activation of the T-cell receptor with the auxiliary CD4 protein (31). Similar results have been obtained in mature and immature B-cells upon engagement of their surface IgM molecules (22) and in IgE-activated RBL-2H3 basophil cells (32).

Western blot analysis of p95^vav immunoprecipitates using anti-phosphotyrosine antibodies suggested that the 95-kDa protein tyrosine phosphorylated in response to SF was the product of the vav proto-oncogene, p95^vav (Fig. 2B). IL-3 and GM-CSF also appeared to stimulate a small but consistent (i.e. approximately 2-fold) increase in tyrosine phosphorylation of this immunoprecipitated protein. To confirm that this protein was indeed p95^vav, the blot shown in Fig. 2B was reprobed with a second anti-p95^vav antiserum elicited against a different epitope (31). As shown in Fig. 2C, a protein of identical size was detected in all lanes indicating that the phosphotyrosine protein identified in Fig. 2B was p95^vav. Moreover, each of the lanes exhibited the same intensity, demonstrating that the different levels of tyrosine phosphorylation observed in Fig. 2B were due to differential phosphorylation and not to unequal levels of p95^vav protein. The specificity of these observations was further illustrated by incubating total cell lysates from SF treated M07E cells with either preimmune serum, anti-p95^vav antiserum or anti-p95^vav antiserum together with the immunizing peptide (Fig. 2D). As expected, the 95-kDa tyrosine phosphorylated protein was only detected in the lysate incubated with anti-p95^vav antiserum in the absence of competing peptide.

To define the timing of p95^vav tyrosine phosphorylation through the signaling processes mediated by c-kit activation, we analyzed the kinetics of p95^vav tyrosine phosphorylation upon SF-stimulation of M07E cells. As illustrated in Fig. 3, the vav proto-oncogene product became substantially phosphorylated within 1 min of exposure of M07E cells to SF at 37 °C, reached maximal levels within 2 min, and began to decline by 30 min. Reblotting with anti-p95^vav antibodies indicated that the observed induction is due to the specific phosphorylation of p95^vav on tyrosine residues and not to an increase in its level of expression (data not shown).

To determine if SF-induced tyrosine phosphorylation of p95^vav was unique to M07E cells or was a general property of other SF-responsive cells, experiments similar to those described above were carried out using the SF-responsive TF-1 and the non-responsive AML-193 cell lines. As shown in Fig. 4, SF stimulated a marked increase in the tyrosine phosphorylation of p95^vav in M07E and TF-1 cells, but not in AML-193 cells. The levels of tyrosine-phosphorylated p95^vav in all three cell lines were also increased in response to IL-3 and GM-CSF. However, in M07E and TF-1 cells, the observed increase was less than that induced by SF (data not shown). Interestingly, the AML-193 cells, which do not express c-kit receptors on their cell surface,2 had an apparently higher level

2 P. Lansdorp, personal communication.
The rapid phosphorylation of p95<sup>tyr</sup> on tyrosine residues and the presence of an SH2 domain in this protein suggested that p95<sup>tyr</sup> might be physically associated with the c-kit receptor, as has been found previously for the activated epidermal growth factor and platelet-derived growth factor receptors (22, 31, 32). To investigate this possibility, the anti-c-kit monoclonal antibody YB5-B8 (19) was used to immunoprecipitate c-kit receptors from untreated and SF-treated M07E cells. Western analysis carried out using anti-phosphotyrosine antibodies revealed a major SF-induced 145-165-kDa band and a minor band at 95 kDa (Fig. 5A). The latter band, however, was also present in the unstimulated and in the SF-stimulated sample immunoprecipitated with an irrelevant antibody, thus suggesting that this protein was not p95<sup>tyr</sup>. This was confirmed by reblotting with anti-p95<sup>tyr</sup> antibodies (Fig. 5B). Thus, if there is a direct interaction between the c-kit and p95<sup>tyr</sup>, it is apparently too weak to withstand the detergent solubilization conditions used. Alternatively, tyrosine phosphorylation of p95<sup>tyr</sup> might be an indirect step mediated by the activation of some cytoplasmic tyrosine kinase by c-kit. For instance, various members of the c-src family of tyrosine kinases have been shown to be substrates for the platelet-derived growth factor receptor (33). Further studies using cell lines expressing higher levels of c-kit and p95<sup>tyr</sup> proteins should help to clarify the mechanism by which p95<sup>tyr</sup> becomes phosphorylated on tyrosine residues upon SF stimulation of c-kit receptors.

The identification of the gene encoding the c-kit receptor as the Dominant White Spotting (W) locus has provided conclusive evidence that this receptor plays an important role in a variety of developmental processes, specifically those related to the development of the gonadal, pigment, and hematopoietic cell lineages (13). However, little is known about the signaling process initiated by the activated receptor. Recent studies have indicated that SF-activated c-kit binds to the regulatory subunit of the phosphatidylinositol 3'-kinase and to phospholipase C-γ1 (17). Our results have identified p95<sup>tyr</sup> as a downstream component of this signaling process, although it is not clear yet whether p95<sup>tyr</sup> is a direct substrate of c-kit. Moreover, the specific function of p95<sup>tyr</sup> in the c-kit pathway remains to be determined. p95<sup>tyr</sup> contains a complex array of structural motifs, which include a helix-loop-helix/leucine zipper-like domain with limited homology of the Myc protein family, a cysteine-rich region with homology to those present in protein kinase C, c-Raf, and n-chimaerin, and one SH2 and two SH3 regions (21, 28-30). Additionally, p95<sup>tyr</sup> contains a region of homology with the products of the abl oncogene, the bcr gene, and the yeast CDC24 protein (34). Since both CDC24 and abl appear to have GDP/GTP exchange activities for the ras-like protein CDC42 (35), it is possible that p95<sup>tyr</sup> acts as a linker between cell surface tyrosine phosphorylation and the downstream signaling pathways regulated by the ras-like family of GTP-binding proteins.

p95<sup>tyr</sup> is phosphorylated on tyrosine residues in a great variety of cell types upon activation of different cell surface receptors. Recent studies have implicated p95<sup>tyr</sup> in the signaling processes initiated by the TCR/CD4 complex in T cells (31), the IgM antigen receptor in B-cells (22), and the IgE allergen receptor in mast cells (32). The present results include c-kit as an additional cell surface receptor whose signaling may be mediated by p95<sup>tyr</sup>. These observations, taken together, suggest the vac gene may play a central role in the signal transduction pathways responsible for the activation of hemopoietic cells.

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Steel Factor Induces the Tyrosine Phosphorylation of p95


