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Tec Kinase Associates with c-kit and Is Tyrosine Phosphorylated and Activated following Stem Cell Factor Binding

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Stem cell factor (SCF) plays a crucial role in hematopoiesis through its interaction with the receptor tyrosine kinase c-kit. However, the signaling events that are activated by this interaction and involved in the control of growth or differentiation are not completely understood. We demonstrate here that Tec, a cytoplasmic, src-related kinase, physically associates with c-kit through a region that contains a proline-rich motif, amino terminal of the SH3 domain. Following SCF binding, Tec is tyrosine phosphorylated and its in vitro kinase activity is increased. Tyrosine phosphorylation of Tec is not detected in the response to other cytokines controlling hematopoiesis, including colony-stimulating factor-1 (CSF-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-3 (IL-3). Conversely, the cytoplasmic kinase JAK2 is activated by IL-3 but not by SCF stimulation. The activation of distinct cytoplasmic kinases may account for the synergy seen in the actions of SCF and IL-3 on hematopoietic stem cells.

The proliferation and differentiation of hematopoietic cells are regulated through the interaction of hematopoietic growth factors with their receptors (4, 17). The receptors for colony-stimulating factor 1 (CSF-1), c-fms, and stem cell factor (SCF), c-kit, are members of the receptor protein tyrosine kinases which couple ligand binding to activation of their intrinsic protein tyrosine kinase activity. In contrast, a number of hematopoietic growth factors, including interleukin-3 (IL-3), utilize receptors of the cytokine receptor superfamily (1). These receptors do not contain intrinsic protein tyrosine kinase catalytic activity but nevertheless couple ligand binding to induction of protein tyrosine phosphorylation (11, 12). Recent studies have shown that members of a novel family of cytoplasmic protein tyrosine kinases termed the Janus kinases (JAKs) associate with the membrane-proximal region of the cytoplasmic domains of these receptors (13). Following ligand binding and receptor dimerization, the JAKs become tyrosine phosphorylated and their in vitro kinase activity is activated.

One of the consistent properties of SCF and IL-3 is their ability to synergize in the proliferation and/or differentiation of normal hematopoietic cells or growth factor-dependent cell lines (2, 8, 10, 16, 25). The basis for this synergy is not known, although in cell lines, IL-3 and SCF induce the rapid tyrosine phosphorylation of a number of substrates which are both unique and shared (9). The basis for these differences could be related to the substrate preferences of c-kit and JAK2 as well as other downstream kinases activated in the individual responses.

Tec kinase was identified as a src-related cytoplasmic protein tyrosine kinase expressed in liver (14); however, Tec is highly expressed in hematopoietic lineages (15). The *Tec* gene resides on murine chromosome 5 and is tightly linked with c-*kit* (15). Tec kinase belongs to a structurally related subfamily of src kinases which includes itk (21) and Bpk/atk/btk (26, 27), which are predominantly expressed in T and B cells, respectively. The importance of this subfamily is indicated by the association of mutations in Bpk/atk/btk with X-linked agammaglobulinemia in humans and X-linked immunodeficiency in mice (19, 24), thus implicating Bpk/atk/btk in B-cell signaling.

In order to begin to address the possible role for Tec kinase in hematopoiesis, we have looked for conditions under which Tec is inducibly tyrosine phosphorylated and its kinase activity is increased. The results demonstrate that in Mo7e cells, Tec is specifically tyrosine phosphorylated and its in vitro kinase activity increases in response to SCF but not to other hematopoietic growth factors, including IL-3. In addition, Tec was found to physically associate with c-kit through an amino-terminal domain containing a proline-rich region. These studies support the hypothesis that Tec uniquely participates in the response of hematopoietic cells to SCF and may contribute to the synergy of the response of hematopoietic cells to IL-3 and SCF.

MATERIALS AND METHODS

Cells, culture conditions, and lysis conditions. Mo7e cells were cultured in growth factor-free Dulbecco's modified Eagle's medium (DMEM) containing 1% fetal bovine serum for 12 h. The cells were washed once in phosphate-buffered saline and resuspended in DMEM with 1% fetal bovine serum at 10^7 cells per ml. Cells were stimulated with recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (200 ng/ml), IL-3 (200 ng/ml), or SCF (100 ng/ml) for 5 min at 37°C. Following stimulation, the cells were collected by centrifugation and suspended in cold lysis buffer (20 mM Tris-HCl [pH 7.4]–150 mM NaCl–10% glycerol–1% Triton X-100–0.2 mM Na_3VO_4–50 mM NaF containing 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, and 10 μg of aprotinin per ml) and incubated on ice for 15 min. Insoluble

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material was removed by centrifugation at 10,000 × *g* at 4°C for 15 min.

Immunoprecipitations. For immunoprecipitations, the lysates were mixed with a polyclonal antibody against Tec (1:500 dilution), a polyclonal antibody against JAK2 (1:500 dilution), or a monoclonal antibody to phosphotyrosine (4G10, 2 μg/ml; UBI) and incubated for 1 h on ice. The antibody for Btk was kindly provided by Mary Ellen Conley, Department of Immunology, St. Jude Children's Research Hospital, and has been previously described (26). Protein A-Sepharose (BL-4; Pharmacia) was added, and the samples were rotated at 4°C for 30 min. Immunoprecipitates were extensively washed with lysis buffer before suspension in Laemmli's sample buffer. For Western blotting (immunoblotting), the proteins in the total cell lysate or the immunoprecipitates were separated in a sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS–7.5% PAGE) gel and electrotransferred to nitrocellulose membranes. The membranes were washed twice in TBS buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) and incubated for 2 h in TBS containing 5% nonfat milk. For detection of phosphotyrosine-containing proteins, membranes were incubated in 1 μg of a monoclonal antibody against phosphotyrosine (4G10) per ml in 5% milk–TBS for 1 h and subsequently probed with a 1:1,500 dilution of peroxidase-conjugated anti-mouse antibody (Amersham). For Western blotting of Tec or JAK, membranes were incubated with a 1:5,000 dilution of polyclonal antipeptide antibody against Tec or JAK2 and then immunoblotted with a sheep anti-rabbit peroxidase-conjugated antibody (Amersham). After incubation, the membranes were washed four times with TBS and subjected to electrochemiluminescence detection (Amersham) according to the manufacturer's recommendations. Rabbit polyclonal antiserum was raised against Tec by using a synthetic peptide corresponding to amino acid residues 165 to 182 (KRRPPPPIPPEEENTEEI). This sequence is immediately upstream of the SH3 domain and is quite distinct for Tec relative to the Tec subfamily members Btk and itk.

In vitro kinase assays. For in vitro kinase assays, immunoprecipitates were washed once in kinase buffer (20 mM Tris-HCl, 150 mM NaCl, 10 mM $MnCl_2$, and 10 mM $MgCl_2$) and resuspended in 30 μl of kinase buffer in the presence of 10 μCi of [γ-^{32}P]ATP. The reaction mixtures were incubated at 30°C for 20 min, and reactions were stopped by the addition of 1 ml of wash buffer (0.3% SDS, 100 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% Triton X-100).

GST-Tec fusion proteins. The GST-Tec fusion proteins were produced in bacteria from the pGEX/2T expression vector (Pharmacia). The expression constructs contained fragments of Tec as indicated (see Fig. 6 and 7). For expression of c-kit, 5×10^5 COS7 cells were seeded into 100-mm-diameter dishes. After 12 h, the cells were transfected with either the pLXSN or the pLXSN/c-kit retroviral expression construct by using the lipofectAMINE reagent (Gibco, BRL) according to the manufacturer's protocol. After 60 h, the cells were lysed in 1 ml of lysis buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid] [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, and 10 μg of leupeptin per ml). Cell extracts were clarified by centrifugation and incubated with GST or the GST-Tec fusion protein immobilized to glutathionine-agarose at 4°C for 4 h with gentle rotation. The beads were washed four times with washing buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100), and the samples were suspended in SDS-PAGE loading buffer, denatured by boiling, and loaded onto SDS–7.5% PAGE gels.

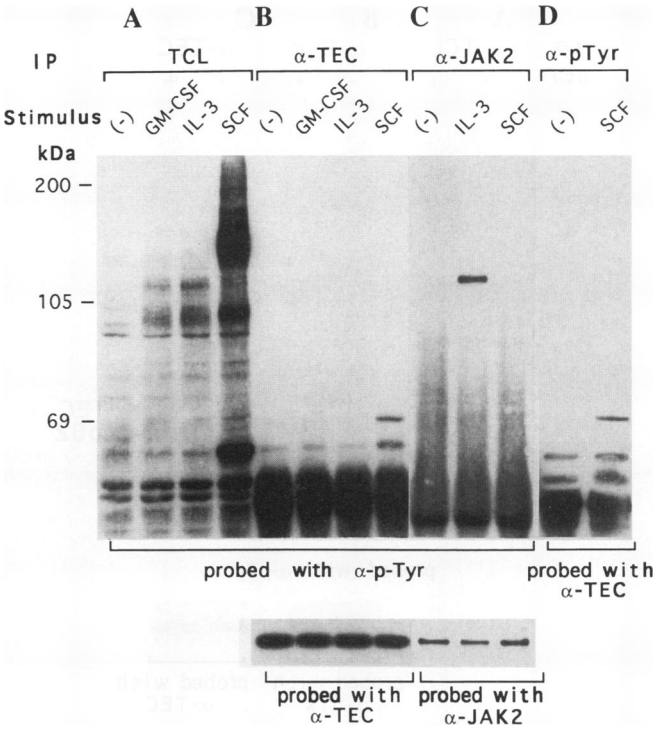

FIG. 1. SCF stimulates Tec tyrosine phosphorylation. The effects of SCF, GM-CSF, and IL-3 stimulation of Mo7e cells on tyrosine phosphorylations were examined. The effects of GM-CSF, IL-3, and SCF on total cellular tyrosine phosphorylation are shown in panel A; the effects of GM-CSF, IL-3, and SCF on Tec tyrosine phosphorylation are shown in panel B; while the effects of IL-3 and SCF on JAK2 tyrosine phosphorylation are shown in panel C. The effects on immunoprecipitable Tec and JAK2 are shown below panels B and C, respectively. In panel D, cell extracts were immunoprecipitated with a monoclonal antibody against phosphotyrosine (4G10) (α-pTyr), and these were probed for the presence of Tec by Western blotting. IP, immunoprecipitate; TCL, total cell lysates. The positions of the migrations of molecular mass standards are indicated.

RESULTS

Tec is specifically tyrosine phosphorylated in the response to SCF. To initially explore the involvement of Tec in hematopoietic growth factor signaling, we examined the effects of GM-CSF, IL-3, and SCF on Tec tyrosine phosphorylation in the growth factor-dependent human megakaryocytic cell line Mo7e (Fig. 1). Each growth factor induced tyrosine phosphorylation, and the patterns seen with GM-CSF and IL-3 were similar to but different from the SCF response (Fig. 1A). When extracts were immunoprecipitated with an antipeptide antiserum against Tec and immunoblotted with an antiphosphotyrosine antibody, Tec phosphorylation (70 kDa) was readily detected following stimulation with SCF but not with IL-3 or GM-CSF (Fig. 1B). Reprobing with an antiserum against Tec confirmed that the changes were not due to alterations of protein levels. The inducible tyrosine phosphorylation of Tec was also examined by affinity isolation of phosphotyrosine-containing proteins with the monoclonal antibody 4G10 (Fig. 1D). Tec protein was not detected in the 4G10-bound fraction with extracts from unstimulated cells, but it was detected in extracts from SCF-stimulated cells. Lastly, SCF stimulation of Mo7e cells did not induce tyrosine phosphorylation of the Tec-related kinase, Btk, indicating the specificity of the response (Fig. 2B).

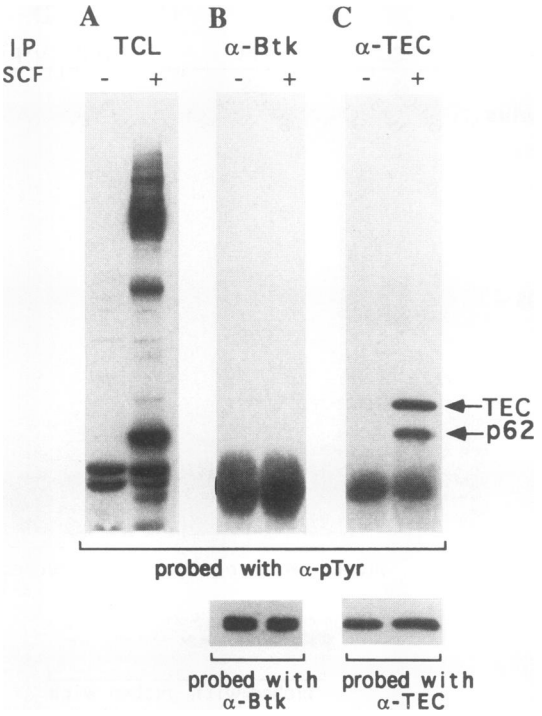

FIG. 2. SCF induces Tec tyrosine phosphorylation but not that of Btk. Effects of SCF on total cellular tyrosine phosphorylation (A), Btk tyrosine phosphorylation (B), and Tec tyrosine phosphorylation (C) are shown. The bottom panels show the effects on immunoprecipitable Btk and Tec. The positions of migration of Tec and p62 are indicated. IP, immunoprecipitate; TCL, total cell lysates; α-pTyr, antiphosphotyrosine antibody.

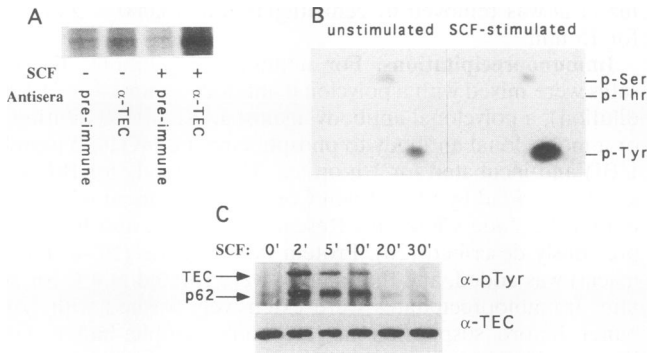

FIG. 3. Tec is rapidly phosphorylated on tyrosine following SCF stimulation. (A) Mo7e cells were deprived of growth factors overnight, incubated in phosphate-free media containing $^{32}P_i$ (1 mCi/ml) for 3 h at 37°C, and stimulated (+) with SCF for 5 min or left unstimulated (−). Extracts were immunoprecipitated with control, preimmune serum, or with antiserum against Tec, and the proteins were resolved by SDS-PAGE. (B) The labeled Tec protein was isolated from the gels and hydrolyzed, and the phosphoamino acids were resolved by two-dimensional electrophoresis as previously described (3). The positions of migration of the control phosphoamino acids (phosphoserine [p-Ser], p-Thr, and p-Tyr) are indicated. (C) The kinetics of Tec phosphorylation were examined by obtaining lysates from Mo7e cells stimulated for the indicated times (minutes) with SCF. Tec was immunoprecipitated, the proteins were resolved by SDS-PAGE and blotted to nitrocellulose, and the membranes were probed with an antiphosphotyrosine monoclonal antibody (4G10) (α-pTyr), as described for Fig. 1. As a control, the membranes were also probed with an antiserum against Tec (lower panel).

In addition to Tec, a 62-kDa protein is consistently detected in Tec immunoprecipitates probed with antiphosphotyrosine antibodies, as indicated in Fig. 1B and 2C. This protein is not detected with antisera to different regions of Tec and thus is not a Tec isoform and is not observed in Tec immunoprecipitates used in competition experiments with the peptide used for immunization (data not shown). The 62-kDa protein was not detected with antisera against c-src, lyn, or SHC (data not shown), nor was it detected with antisera against the GAP-associated p62 protein (30). The data suggest that a novel 62-kDa phosphoprotein associates with Tec and becomes tyrosine phosphorylated following SCF stimulation with kinetics that are comparable to those of Tec (see below).

The 130-kDa cytoplasmic kinase, JAK2, is tyrosine phosphorylated and activated following IL-3 or Epo stimulation (22, 28). We therefore examined SCF-stimulated cells for the induction of tyrosine phosphorylation of JAK2. IL-3 stimulation of Mo7e cells induced tyrosine phosphorylation of JAK2 (Fig. 1C). However, JAK2 was not detectably tyrosine phosphorylated in the response to SCF. In addition, neither JAK1 nor TYK2 was inducibly tyrosine phosphorylated in the response to SCF or IL-3 (data not shown).

c-kit is related to c-fms, the CSF-1 receptor, which is primarily expressed in macrophages (20). Studies have shown that src, fyn, and yes associate with c-fms following ligand binding and that their in vitro kinase activity increases two- to threefold (5). We utilized the CSF-1-responsive macrophage cell line, Bac1 (18), to determine whether CSF-1 induced Tec tyrosine phosphorylation. No detectable tyrosine phosphorylation of Tec occurred following CSF-1 stimulation (data not shown). Therefore, among several cytokines, the induction of tyrosine phosphorylation of Tec was specific for the response to SCF.

To confirm tyrosine phosphorylation of Tec, cells were labeled with $^{32}P_i$ and stimulated with SCF, after which Tec was immunoprecipitated and the immunoprecipitates were resolved by SDS-PAGE. Consistent with the above data, there was an increase in phosphorylation of Tec following SCF stimulation (Fig. 3A). The changes in specific phosphoamino acids were determined by isolating Tec, hydrolyzing the samples, and analyzing the phosphoamino acids by two-dimensional analysis (Fig. 3B). In unstimulated cells, both phosphoserine and phosphotyrosine were detected. Following SCF stimulation, there was an approximately sevenfold increase in phosphotyrosine and no detectable increase in phosphoserine. The kinetics of the phosphorylation are shown in Fig. 3C. Tec was phosphorylated by 2 min following stimulation and persisted until approximately 20 min. The kinetics of the associated 62-kDa phosphoprotein were comparable and were similar to the general pattern of phosphorylation, including that of c-kit (data not shown).

SCF stimulation induces an increase in Tec in vitro kinase activity. We next examined the in vitro kinase activity of Tec (Fig. 4A). In vitro kinase assays of immunoprecipitates from stimulated cells showed a significant increase in the phosphorylation of a protein migrating at the position of Tec. The specificity for the reactions is indicated by the lack of detectable phosphorylation in immunoprecipitates with control serum or immune serum in the presence of the peptide against which the antiserum was made (Fig. 4B). By phosphoamino acid analysis, labeling was exclusively on tyrosine (data not shown). For comparison, we also examined the activity in lyn immunoprecipitates (Fig. 4A). The major phosphoproteins

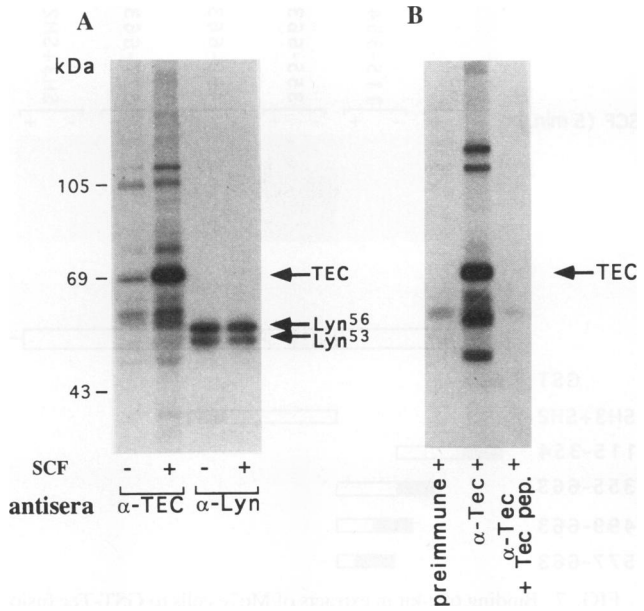

FIG. 4. SCF induces Tec kinase activity in Mo7e cells. Cells were deprived of growth factors overnight and stimulated with SCF for 5 min (+) or left unstimulated (−). (A) The cells were lysed, and Tec or lyn was immunoprecipitated. (B) Cell extracts were incubated with preimmune serum, antiserum against Tec, and antiserum against Tec in the presence of a peptide that was used to raise the Tec antiserum (left, middle, and right lanes, respectively). The immunoprecipitates were subjected to an in vitro kinase reaction as described previously (28). The phosphorylated proteins were then resolved by SDS-PAGE and visualized by autoradiography. α-TEC, antiserum against Tec; Tec pep, Tec peptide.

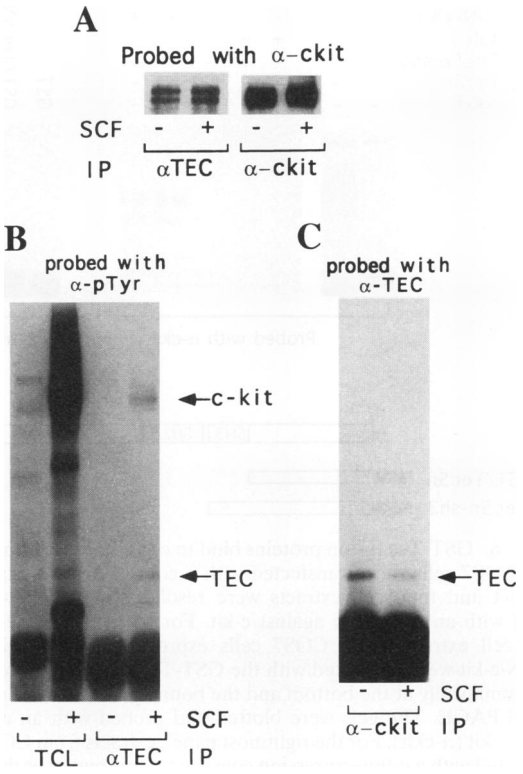

FIG. 5. Tec is physically associated with c-kit. Mo7e cells were cultured overnight in the absence of growth factors and were stimulated with SCF for 5 min (+) or left unstimulated (−). The cells were lysed in the lysis buffer described in Materials and Methods, with the exception that 0.1% Triton X-100 was used. (A) Extracts were immunoprecipitated with antisera against Tec or c-kit and the blots were probed with the antiserum against c-kit. (B). Total cell lysates or immunoprecipitates of Tec were probed with an antibody against phosphotyrosine. The positions of migration of c-kit and Tec are indicated. (C) Extracts were immunoprecipitated with an antiserum against c-kit and the blots were probed with an antiserum against Tec. The position of migration of Tec is indicated. IP, immunoprecipitate; TCL, total cell lysates.

seen in these reactions migrated with the two forms of lyn (56 and 53 kDa) expressed in myeloid cells (31). Stimulation of the cells with SCF had little, if any, effect on lyn in vitro kinase activity.

Tec physically associates with c-kit through a region containing a proline-rich motif. To determine whether Tec physically associates with c-kit, c-kit or Tec was immunoprecipitated and the immunoprecipitates were examined for c-kit or Tec. Experiments which utilized the lysis conditions described above failed to detect any coimmunoprecipitation. However, by lowering the Triton concentration to 0.1%, association was readily detectable. As shown in Fig. 5A, immunoprecipitates of Tec from unstimulated or stimulated cells contained immunoreactive c-kit protein. The specificity of coimmunoprecipitation was indicated by the lack of precipitation of Tec or c-kit under identical conditions with an irrelevant antibody (data not shown). The results obtained when immunoprecipitates of Tec were probed with an antiphosphotyrosine antibody are shown in Fig. 5B. As indicated, a phosphoprotein migrating at the position of c-kit was detected in stimulated but not in unstimulated cells. Conversely, immunoprecipitates of c-kit contained a 70-kDa protein that was detected with antiserum against Tec in both unstimulated and stimulated cells (Fig. 5C). As described above, the specificity was indicated by the lack of precipitation of Tec under identical conditions with an irrelevant antiserum (data not shown). Although a decrease in c-kit-associated Tec was evident in this experiment following SCF stimulation, this has not been a consistent observation.

The association of c-kit with Tec was further examined in COS cells transiently transfected with a c-kit expression construct. Expression of c-kit in COS cells resulted in the production of protein that was detectable by immunoprecipitation and Western blotting with an antiserum against c-kit (Fig. 6). Under these conditions, c-kit was not detectably tyrosine phosphorylated, as determined by lack of reactivity in Western blotting with a monoclonal antibody against phosphotyrosine (data not shown). As shown in Fig. 6, c-kit could be specifically isolated with a GST fusion protein containing the region amino terminal of the SH3 domain of Tec from cells transfected with the c-kit expression construct but not with GST alone. A fusion protein containing the amino-terminal region of Tec, including the SH3 domain, also bound c-kit. Lastly, the fusion protein did not bind to c-fms in cells transiently transfected with a c-fms expression construct. These data substantiate the specific association of Tec with the non-tyrosine-phosphorylated form of c-kit and indicated that the association occurs through sequences in the amino terminus of Tec.

To further define the binding region, a series of GST fusion proteins were examined for their ability to bind to c-kit in extracts of Mo7e cells (Fig. 7). Binding was readily detected with a fusion protein that contained amino acids (aa) 81 to 183 (bp 355 to 663), a region that is immediately amino terminal of

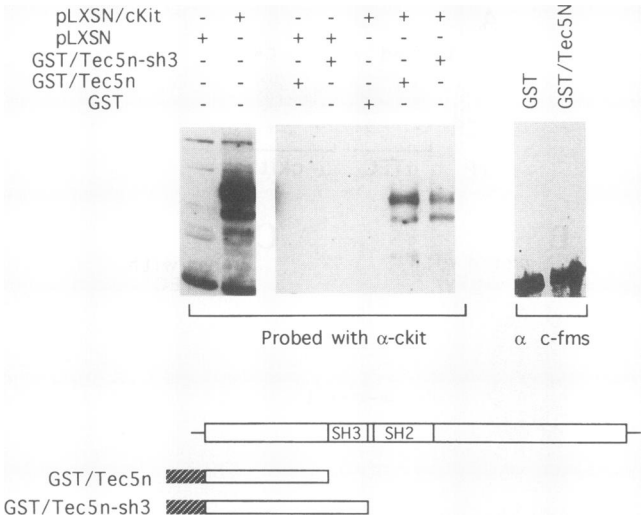

FIG. 6. GST-Tec fusion proteins bind to c-kit. For the leftmost two lanes, COS7 cells were transfected with a control or c-kit expression construct and total cell extracts were resolved by SDS-PAGE and blotted with an antiserum against c-kit. For the center panel of five lanes, cell extracts from COS7 cells expressing either pLXSN or PLXSN/c-kit were incubated with the GST-Tec fusion proteins shown diagrammatically at the bottom and the bound proteins were resolved by SDS-PAGE. The gels were blotted and probed with an antibody against c-kit (α-ckit). For the rightmost panel, extracts from COS7 cells transfected with a c-fms expression construct were treated as described above with a GST fusion protein containing the amino-terminal region of Tec.

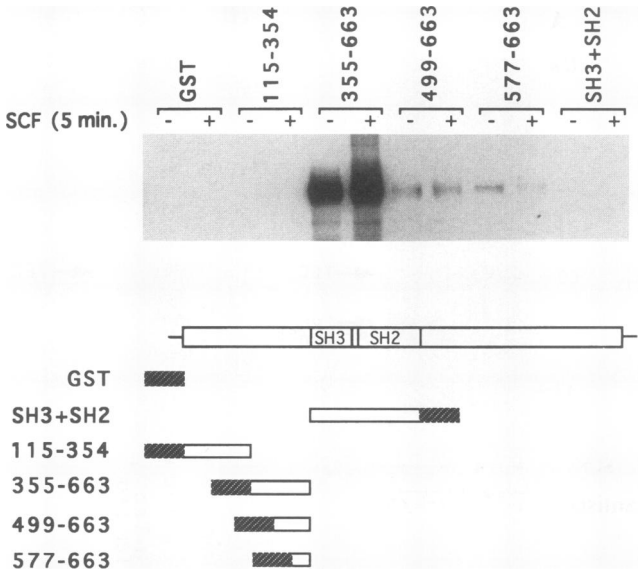

FIG. 7. Binding of c-kit in extracts of Mo7e cells to GST-Tec fusion proteins. Cell extracts of SCF-treated (+) or untreated (−) Mo7e cells were prepared as described in Materials and Methods and were incubated with the GST fusion proteins shown diagrammatically at the bottom. The bound proteins were eluted and resolved by SDS-PAGE. The proteins were transferred to filters, and the filters were probed with an antiserum against c-kit. The fusions are indicated by the Tec nucleotide positions. The corresponding amino acid positions are as follows: bp 115 to 354, aa 1 to 81; bp 355 to 663, aa 81 to 183; bp 499 to 663, aa 129 to 183; bp 577 to 663, aa 155 to 183. The SH3-SH2 fusion protein contains amino acids 184 to 344.

the SH3 domain. This binding was not dependent upon the prior stimulation of the cells with SCF. In contrast, no binding was seen with fusion proteins containing the entire SH3 and SH2 domains or aa 1 to 81 (bp 115 to 354) from the amino terminus. Fusion proteins containing the regions from aa 129 to 663 (bp 499 to 663) or aa 155 to 183 (bp 577 to 663) also had detectable binding activity but were clearly less efficient than the bp 355-to-663 fusion protein. The localization of binding to the region containing amino acids 155 to 183 was further indicated by the inability of a GST fusion protein containing amino acids 81 to 128 to bind c-kit (data not shown).

DISCUSSION

The results implicate Tec kinase in the signaling pathways initiated by SCF through its interaction with c-kit. The possible role of Tec in hematopoietic signaling was of interest for two reasons. First, Tec is predominantly expressed in hematopoietic cells, suggesting a lineage-specific function. Curiously, the other family members, Btk and Itk, show a similarly restricted pattern of expression (6, 21, 26). Secondly, Btk has been implicated in B-cell signaling through the observation that mutations in Btk are associated with X-linked agammaglobulinemia in humans and X-linked immunodeficiency in mice (19, 24, 26).

The results demonstrate that SCF induces the tyrosine phosphorylation of Tec and that this is associated with an apparent increase in in vitro Tec kinase activity. It should be noted, however, that additional bands are detected in the immunoprecipitate kinase reactions (Fig. 4); therefore, it is possible that the increases seen are due to a coimmunoprecipitating kinase that also phosphorylates Tec. To attempt to address this possibility, we have tried a number of conditions under which to look for in-gel kinase activity following renaturation. However, under none of the conditions was an induced kinase activity detectable.

The activation of Tec was quite specific for SCF relative to CSF-1, IL-3, and GM-CSF, as well as hepatocyte growth factor (HGF), G-CSF, Epo, IL-2, IL-4, and immunoglobulin E (data not shown). In contrast, IL-3, GM-CSF, Epo, and G-CSF activate JAK2 (13), while IL-2 and IL-4 activate JAK3 (29). Conversely, SCF activation was specific for Tec relative to the closely related family member, Btk. Thus, it can be hypothesized that Tec is involved in cellular responses that are specific for SCF, which might include responses uniquely associated with differentiation.

Throughout the studies, a 62-kDa protein which is inducibly tyrosine phosphorylated with kinetics that are comparable to those of Tec has been consistently detected in Tec immunoprecipitates. This protein is not detected by antisera against c-src or lyn. This protein is also not detected by antisera against SHC. Lastly, it was not detected with antiserum against the GAP-associated p62 protein (30). The last observation was of particular interest since recent studies have demonstrated that the GAP-associated p62 protein is a cell cycle-dependent target for c-src (7, 23). Regardless, efforts to clone and identify the Tec-associated phosphoprotein are continuing.

The ability of SCF to uniquely activate Tec may contribute to the synergy that has been seen between SCF and a number of hematopoietic growth factors, including IL-3. The most striking synergies are observed in the proliferation and differentiation of normal progenitors (2, 8, 10, 16, 25). However, it has been observed that SCF and IL-3 synergize in supporting the proliferation of Mo7e cells (10).

Tec constitutively associates with c-kit, in contrast to the association of src kinases with other receptor protein tyrosine kinases. It should be noted, however, that the possibility that

the c-kit–Tec association is mediated through an adaptor protein has not been formally excluded. The region required for association is immediately amino terminal of the SH3 domain and contains a highly charged, proline-rich region (RKALPPAPETKKRRPPAPIP). Whether the proline-rich region is specifically involved in binding is not known, partly because of the loss of binding activity with the smaller constructs. This could reflect the requirement for a relatively large binding domain, which includes the proline-rich motif, or the larger constructs may be required to provide the conformation necessary to present the appropriate binding site, such as the proline-rich region. It should also be noted that the possibility exists that the region of association defined by the GST fusion protein studies may not be the region involved in association in vivo. Experiments are currently in progress to examine the effects of various mutants of Tec in cells, including the ability of the mutants to associate with c-kit. The significance of this region in the function of Tec is not known. The Arg→Cys mutation that occurs in Btk in immunodeficient XID mice occurs at the equivalent Arg at amino acid position 29 in Tec (19), a region that does not associate with c-kit in a fusion construct. Experiments are in progress to define the region of c-kit with which Tec associates.

ACKNOWLEDGMENTS

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REFERENCES

1. **Bazan, J. F.** 1990. Structural design and molecular evolution of a cytokine receptor superfamily. Proc. Natl. Acad. Sci. USA **87:** 6934–6938.
2. **Bernstein, I. D., R. G. Andrews, and K. M. Zsebo.** 1991. Recombinant human stem cell factor enhances the formation of CD34+ and CD34+lin− cells and the generation of colony-forming cell progeny from CD34+lin− cells cultured with interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor. Blood **77:**2316–2321.
3. **Boyle, W. J., P. van der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. **201:** 110–149.
4. **Clark, S. C., and R. Kamen.** 1987. The human hematopoietic colony-stimulating factors. Science **236:**1229–1237.
5. **Courtneidge, S. A., R. Dhand, D. Pilat, G. M. Twamley, M. D. Waterfield, and M. F. Roussel.** 1993. Activation of Src family kinases by colony stimulating factor-1, and their association with its receptor. EMBO J. **12:**943–950.
6. **Dymecki, S. M., J. E. Niederhuber, and S. V. Desiderio.** 1990. Specific expression of a tyrosine kinase gene, blk, in B lymphoid cells. Science **247:**332–336.
7. **Fumagalli, S., N. F. Totty, J. J. Hsuan, and S. A. Courtneidge.** 1994. A target for Src in mitosis. Nature (London) **368:**871–874.
8. **Galli, S. J., K. M. Zsebo, and E. N. Geissler.** 1994. The kit ligand, stem cell factor. Adv. Immunol. **55:**1–96.
9. **Hallek, M., B. Druker, E. M. Lepisto, K. W. Wood, T. J. Ernst, and J. D. Griffin.** 1992. Granulocyte-macrophage colony-stimulating factor and steel factor induce phosphorylation of both unique and overlapping signal transduction intermediates in a human factor-dependent hematopoietic cell line. J. Cell. Physiol. **153:**176–186.
10. **Hendrie, P. C., K. Miyazawa, Y. Yang, C. D. Langefeld, and H. E. Broxmeyer.** 1991. Mast cell growth factor (c-kit ligand) enhances cytokine stimulation of proliferation of the human factor-dependent cell line, MO7e. Exp. Hematol. **19:**1031–1037.
11. **Ihle, J. N., F. W. Quelle, and O. Miura.** 1993. Signal transduction through the receptor for erythropoietin. Semin. Immunol. **5:**375–389.
12. **Ihle, J. N., B. Witthuhn, B. Tang, T. Yi, and F. W. Quelle.** 1994. Cytokine receptors and signal transduction. Bailliere's Clin. Haematol. **7:**17–48.
13. **Ihle, J. N., B. A. Witthuhn, F. W. Quelle, K. Yamamoto, W. E. Thierfelder, B. Kreider, and O. Silvennoinen.** 1994. Signaling by the cytokine receptor superfamily: JAKs and STATs. Trends Biochem. Sci. **19:**222–227.
14. **Mano, H., F. Ishikawa, J. Nishida, H. Hirai, and F. Takaku.** 1990. A novel protein-tyrosine kinase, tec, is preferentially expressed in liver. Oncogene **5:**1781–1786.
15. **Mano, H., K. Mano, B. Tang, M. Koehler, T. Yi, D. J. Gilbert, N. A. Jenkins, N. G. Copeland, and J. N. Ihle.** 1993. Expression of a novel form of tec kinase in hematopoietic cells and mapping of the gene to chromosome 5 near kit. Oncogene **8:**417–424.
16. **McNiece, I. K., K. E. Langley, and K. M. Zsebo.** 1991. Recombinant human stem cell factor synergizes with GM-CSF, G-CSF, IL-3 and Epo to stimulate human progenitor cells of the myeloid and erythroid lineages. Exp. Hematol. **19:**226–231.
17. **Metcalf, D.** 1989. The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. Nature (London) **339:**27–30.
18. **Morgan, C., J. W. Pollard, and E. R. Stanley.** 1987. Isolation and characterization of a cloned growth factor dependent macrophage cell line, BAC1.2F5. J. Cell. Physiol. **130:**420–427.
19. **Rawlings, D. J., D. C. Saffran, S. Tsukada, D. A. Largaespada, J. C. Grimaldi, L. Cohen, R. N. Mohr, J. F. Bazan, M. Howard, N. G. Copeland, N. A. Jenkins, and O. N. Witte.** 1993. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. Science **261:**358–361.
20. **Sherr, C. J.** 1990. Colony-stimulating factor-1 receptor. Blood **75:**1–12.
21. **Silicano, J. D., T. A. Morrow, and S. V. Desiderio.** 1992. itk, a T-cell-specific tyrosine kinase gene inducible by interleukin 2. Proc. Natl. Acad. Sci. USA **89:**11194–11198.
22. **Silvennoinen, O., B. Witthuhn, F. W. Quelle, J. L. Cleveland, T. Yi, and J. N. Ihle.** 1993. Structure of the JAK2 protein tyrosine kinase and its role in IL-3 signal transduction. Proc. Natl. Acad. Sci. USA **90:**8429–8433.
23. **Taylor, S. J., and D. Shalloway.** 1994. An RNA-binding protein associated with Src through its SH2 and SH3 domains in mitosis. Nature (London) **368:**867–871.
24. **Thomas, J. D., P. Sideras, C. I. Smith, I. Vorechovsky, V. Chapman, and W. E. Paul.** 1993. Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. Science **261:** 355–358.
25. **Tsuji, K., K. M. Zsebo, and M. Ogawa.** 1991. Enhancement of murine blast cell colony formation in culture by recombinant rat stem cell factor, ligand for *c-kit*. Blood **78:**1223–1229.
26. **Tsukada, S., D. C. Saffran, D. J. Rawlings, O. Parolini, R. C. Allen, I. Klisak, H. Kubagawa, T. Mohandas, S. Quan, J. W. Belmont, M. D. Cooper, M. E. Conley, and O. N. Witte.** 1993. Deficient expression of a B-cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. Cell **72:**279–290.
27. **Vetrie, D., I. Vorechovsky, P. Sideras, J. Holland, A. Davies, F. Flinter, L. Hammarstrom, C. Kinnon, R. Levinsky, M. Bobrow, C. I. E. Smith, and D. R. Bentley.** 1993. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. Nature (London) **361:**226–233.
28. **Witthuhn, B., F. W. Quelle, O. Silvennoinen, T. Yi, B. Tang, O. Miura, and J. N. Ihle.** 1993. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following EPO stimulation. Cell **74:**227–236.
29. **Witthuhn, B. A., O. Silvennoinen, O. Miura, K. S. Lai, C. Cwik, E. T. Liu, and J. N. Ihle.** 1994. Involvement of the JAK3 Janus kinase in IL-2 and IL-4 signalling in lymphoid and myeloid cells. Nature (London) **370:**153–157.
30. **Wong, G., O. Muller, R. Clark, L. Conroy, M. F. Moran, P. Polakis, and F. McCormick.** 1992. Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. Cell **69:**551–558.
31. **Yi, T., J. B. Bolen, and J. N. Ihle.** 1991. Hematopoietic cells express two forms of lyn kinase differing by 21 amino acids in the amino terminus. Mol. Cell. Biol. **11:**2391–2398.