Constitutively Activating Mutations of c-kit Receptor Tyrosine Kinase
Confer Factor-Independent Growth and Tumorigenicity of Factor-Dependent Hematopoietic Cell Lines

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The c-kit receptor tyrosine kinase (KIT) is activated upon ligand binding, thereby leading to a variety of signaling events that play a fundamental role in hematopoiesis. In addition to ligand-dependent activation, we have previously shown that KIT is constitutively activated in a ligand-independent manner by two point mutations, Val560→Gly (G559) mutation in the juxtamembrane domain and Asp814→Val (V814) mutation in the phosphotransferase domain. To investigate the biochemical consequence and biologic significance of these mutations, retroviral vectors encoding KIT(G559) or KIT(V814) were introduced into murine pro-B-type Ba/F3 cells and myeloid FDC-P1 cells, both of which require interleukin-3 (IL-3) for their growth and survival. In the cells, KIT(G559) or KIT(V814) were found to be constitutively phosphorylated on tyrosine in the absence of stem cell factor (SCF) that is a ligand for KIT. Chemical cross-linking analysis showed that a substantial fraction of the phosphorylated KIT(G559) underwent dimerization even in the absence of SCF, whereas the phosphorylated KIT(V814) did not, suggesting the distinct mechanisms underlying constitutive activation of KIT by G559 and V814 mutations. Furthermore, the cells expressing either KIT(G559) or KIT(V814) were found to show a factor-independent growth, whereas the cells expressing wild-type KIT (KITWT) proliferated in response to SCF as well as IL-3. Moreover, subcutaneous injection of Ba/F3 cells expressing KIT(G559) or KIT(V814) into nude mice resulted in production of large tumors at all sites of the injection within 2 weeks, and all nude mice quickly succumbed to leukemia and died. These results suggest that, although the mechanisms underlying constitutive activation of KIT(G559) or KIT(V814) may be different, both of the activating mutations have a function to induce a factor-independent and tumorigenic phenotype. Also, the data of this study raise the possibility that the constitutively activating mutations of c-kit may play a causal role in development of hematologic malignancies.

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T HE PROTO-ONCOGENE c-kit is allelic with the white spotting (W) locus on mouse chromosome 5,1,2 and it encodes a receptor tyrosine kinase (RTK) that is a member of the same RTK subfamily (type III RTK) as the receptors for platelet-derived growth factor and colony-stimulating factor 1 (CSF-1).3,4 This RTK subfamily is characterized by the presence of five Ig-like repeats in the extracellular domain and an insert that splits the cytoplasmic kinase domain into an adenine triphosphate (ATP)–binding region and the phosphotransferase domain.5-7 The ligand for c-kit product has been genetically mapped at the steel (Sl) locus on mouse chromosome 10, and is variously designated as stem cell factor (SCF), mast cell growth factor, kit ligand, or steel factor.8-10

Binding of SCF to c-kit receptor tyrosine kinase (KIT) triggers a series of rapid events, including KIT dimerization and activation, association of KIT with certain cytoplasmic enzymes, and phosphorylation of other cellular proteins, thereby initiating a signaling cascade that leads to various cellular responses.4,11-14 This KIT-mediated signal transduction is known to play a crucial role in proliferation, differentiation, migration, and survival of hematopoietic stem cells, mast cells, neural crest–derived melanocytes and germ cells.11-14 For example, dominant-negative or loss-of-function mutations at KIT/W locus impair the receptor function and thereby give rise to hypoplastic anemia and a deletion of mast cells in hematopoietic system.11-14 In addition to a fundamental role of KIT in hematopoiesis, KIT may potentially function as an oncogenic protein, because c-kit was originally defined as a cellular homologue of feline sarcoma virus (v-kit).15,16 Furthermore, activating mutations of other RTKs such as c-fms and c-erbB2/neu17-19 have been shown to have transforming activity.

To test this possibility, we have previously examined the expression and state of tyrosine phosphorylation of KIT in a series of leukemia cells,20-22 and have found that KIT is constitutively activated in a ligand-independent manner in a human mast-cell leukemia cell line, HMC-1.22 Sequencing analysis of c-kit gene in HMC-1 cells showed the previously unidentified two point mutations, the Val560→Gly (G559) mutation in the juxtamembrane domain and the Asp816→Val (V816) mutation in the phosphotransferase domain.22 Transfection of murine c-kit(559→G559) or c-kit(V814→V814) mutants, corresponding to human c-kit(G559) or c-kit(V814) genes, into a human embryonic kidney cell line (293T) resulted in a ligand-independent activation of KIT(G559) or KIT(V814), suggesting the presence of two constitutively activating mutations in c-kit gene.22 In addition, we have recently found that both a murine mastocytoma cell line (P-815) and a rat mast cell leukemia cell line (RBL-2H3) carry constitutively activating mutations of c-kit gene in the same Asp codon in the phosphotransferase domain.32 These results suggested that the activating mutations of c-kit gene could be involved in some aspect of neoplastic transformation of KIT-positive hematopoietic cells, including mast cells. However, the biologic significance and biochemical consequence of these activating mutations have yet to be determined.

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Submitted June 20, 1994; accepted October 17, 1994.

Supported in part by grants from the Ministry of Education, Science and Culture, the Inamori Foundation, Senri Life Science Foundation, and Mochida Memorial Foundation.

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0006-4971/95/8503-0024$3.00/0

Blood, Vol 85, No 3 (February 1), 1995: pp 790-798

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In this study, we introduced wild-type KIT (KITWT), KITG99 and KITV814 into cells of two murine interleukin-3 (IL-3)-dependent cell lines, Ba/F3 (pro-B type) and FDC-P1 (myeloid type). Expression of KITG99 or KITV814 with constitutive tyrosine kinase activity was found not only to abrogate IL-3 requirement of the cells, but also to cause them to become tumorigenic in nude mice. Furthermore, chemical cross-linking analysis showed that KITG99 was organized at the plasma membrane in a dimerized form, whereas KITV814 was not. Thus, the results of these studies provide new insight into potential role of the activating mutations of c-kit proto-oncogene in receptor activation and oncogenesis.

MATERIALS AND METHODS

Reagents. Recombinant murine (rm) SCF and IL-3 were generous gifts of Kirin Brewery Co Ltd (Tokyo, Japan). Antiphosphotyrosine antibody,72,73 a murine monoclonal antibody (MoAb) generated against phosphotyramine, was generously supplied by Dr B. Drucker (Oregon Health Science University, Portland, OR). Rat-animouse c-kit MoAb (ACK2) and full length of murine c-kit cDNA were kindly donated from Dr S.-I. Nishikawa (Kyoto University, Kyoto, Japan). Anti-c-kit polyclonal antibody against synthetic peptide of C-terminal of human KIT was purchased from Oncogene Science, Inc (New York, NY); this antibody was found to react with murine KIT as well as human KIT. G418 (geneticin) and Polybrene were purchased from Sigma Chemical CO (St Louis, MO), and chemical cross-linker BS3 from Pierce (Rockford, IL).

Cells and mice. Ba/F3,35 a murine IL-3-dependent pro-B lymphoid cell line, was cultured in RPMI 1640 medium (Nakarai tesque, Kyoto, Japan) supplemented with 10% fetal calf serum (FCS; Flow Lab, North Ryde, Australia) and rmIL-3 at the concentration of 10 ng/mL. FDC-P1,36 a murine IL-3-dependent myeloid cell line, was maintained in the Fischer's medium (GIBCO, Grand Island, NY) supplemented with 20% horse serum (GIBCO) in the presence of rmIL-3 (10 ng/mL). A helper virus-free packaging cell line,42 ψ2, was maintained in Dulbecco's modified essential medium (Nakarai tesque) supplemented with 10% FCS. SUSl-3T3, a fibroblast cell line derived from SUSl-mutant mice lacking in SCF expression, was established in our laboratory as described previously.42 BALB/c nu/nu (nude) mice were obtained from Nippon SL C (Shizuoka, Japan); all mice were female and were 8 weeks of age at the time of cell injection.

Construction and retroviral transfer of mutated c-kit gene. A retroviral vector pm5Gneo42, a derivative of myeloproliferative sarcoma virus (MPSV), was a kind gift from Dr W. Ostertag (Universität Hamburg, Hamburg, Germany). The mutated murine c-kit genes encoding KITG99 or KITV814 were constructed by site-directed mutagenesis as described previously.32 The full-length of wild-type (WT) or mutated c-kit cDNAs were inserted into the EcoRI site of pm5Gneo. Retroviral vector (pm5Gneo) alone or retroviral vectors containing c-kitWT, c-kitG99 or c-kitV814 were transfected into packaging cell line (ψ2) by calcium phosphate method, and G418-resistant clones of each packaging cell line were isolated. The resultant retrovirus-containing supernatant from each clone and packaging cell line ($2) by calcium phosphate method, and G418-resistant clones of each packaging cell line were isolated. The virus encoding c-kit MoAb (ACK2) and full length of murine KIT as well as human KIT. G418 (geneticin) and Polybrene were kindly donated from Dr K. Sato (Kirin Brewery CO Ltd, Tokyo, Japan). Antiphosphotyramine, was generously supplied by Dr. B. Drucker (Promega, Madison, WI) and chemiluminescence reagent (Renaissance, DuPont, Boston, MA).

Cell proliferation assay. To quantitate cell proliferation, we used an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma] rapid colorimetric assay as previously described.30 Briefly, quadruplicate aliquots of cells (1 × 105) Ba/F3 cells or 2 × 105 FDC-P1 cells suspended in 100 μL of the medium supplemented...
with 10% FCS) were cultured in 96-well microtiter plates for 72 hours at 37°C in the presence or absence of various concentrations of rmSCF or rmIL-3. MTT (10 μL of 5 mg/mL solution of MTT in PBS) was added to all wells for the final 4 hours of culture. Acid isopropanol (100 μL of 0.04 N HCl in isopropanol) was added to all wells, and mixed thoroughly to dissolve the dark blue crystals. The optical density (OD) was then measured on a Microelisa plate reader (Corona Electric Co, Ibaragi, Japan) with a test wavelength of 540 nm and a reference wavelength of 620 nm. This assay was found to give equivalent results obtained by 3H-thymidine incorporation or cell enumeration as described previously.30,31,34

Tumorigenicity assay. Ba/F3 cells expressing KITWT, KITG559, or KITV814 were washed and resuspended in serum-free RPMI-1640 media. In addition, Ba/F3 cells transfected with pM5Gneo were used as a negative control. Cells (3 × 106 injected site; two sites per mouse) were subcutaneously injected into the posterior flank of nude mice that had received 2.5 Gy (250 rads) of x-ray irradiation 1 day before the injection. Mice were carefully monitored for their signs of palpable or visible tumors at the sites of injection. The tumor tissues were fixed by 10% formamide overnight and embedded in paraffin. The sections (4-μm thick) were histopathologically analyzed after staining with hematoxylin and eosin.

RESULTS

Retroviral transfer of wild-type and mutant KIT into murine IL-3-dependent cell lines. To investigate the role of constitutively activating mutations of c-kit proto-oncogene in factor-independent growth and tumorigenicity, we infected two murine IL-3-dependent cell lines, Ba/F3 (pro-B type) and FDC-P1 (myeloid type), with replication-defective retroviruses containing c-kitW, c-kitG559 or c-kitV814 genes, which were termed pM5Gneo-KITWT, pM5Gneo-KITG559 or pM5Gneo-KITV814, respectively (Fig 1A). As a control, viruses carrying pM5Gneo retroviral vector alone (pM5Gneo) were infected into the cells. After selection in a G418-containing medium for 2 weeks, surface expression of KIT on the infected cells was examined with a rat antimmunoglobulin c-kit MoAb ACK2, which recognizes the extracellular domain of murine KIT. Flow cytometric analysis showed that, although infection of Ba/F3 cells with pM5Gneo vector alone (BaF3-Vector) resulted in no expression of KIT, Ba/F3 cells infected with pM5Gneo-KITWT (BaF3-KITWT) or pM5Gneo-KITG559 (BaF3-KITG559) showed abundant surface expression of KITWT or KITG559 on their surface, respectively (Fig 1B). In the cells infected with pM5Gneo-KITV814 (BaF3-KITV814), surface expression of KITV814 was also detectable, albeit to a lesser degree (Fig 1B). Similar results were obtained from flow cytometric analyses of FDC-P1 cells infected with pM5Gneo, pM5Gneo-KITWT, pM5Gneo-KITG559, or pM5Gneo-KITV814 (Fig 1B): FDC-P1 cells infected with pM5Gneo-KITWT (FDCP1-KITWT) or pM5Gneo-KITG559 (FDCP1-KITG559) exhibited high levels of KITWT or KITG559 expression; the cells infected with pM5Gneo-KITV814 (FDCP1-KITV814) did moderate KITV814 expression; and little expression of KIT was observed on the cells infected with pM5Gneo (FDCP1-Vector).

Constitutive tyrosine phosphorylation of mutated KIT in factor-dependent cell lines. To examine the state of KIT-tyrosyl phosphorylation in the infected cells, the cells were deprived of serum and growth factors for 12 hours and stimulated with or without rmSCF (100 ng/mL) for 15 minutes. KIT was then immunoprecipitated and assayed by immunoblotting with either antiphosphotyrosine MoAb or anti-c-kit polyclonal antibody. As shown in Fig 2 (lower panel), all of KITWT, KITG559, and KITV814 were found to be composed of 145-kD (mature) and 125-kD (immature) forms of KIT protein in the infected Ba/F3 cells. Immunoblotting with an antiphosphotyrosine MoAb showed that increased phosphotyrosine was observed in KITWT, particularly in the 145-kD form of KITWT, after treatment with rmSCF (Fig 2, upper panel). By contrast, both 145-kD and 125-kD forms of KITG559 or KITV814 were strikingly phosphorylated on tyrosine regardless of rmSCF stimulation (Fig 2, upper panel), suggesting constitutive activation of KITG559 and KITV814 in BaF3-KITG559 and BaF3-KITV814 cells, respectively. Also in FDC-P1 cells, KITWT was found to be phosphorylated on...
Fig 2. Tyrosine phosphorylation of KIT in Ba/F3 cells stably expressing KIT<sup>WT</sup>, KIT<sup>G559S</sup>, and KIT<sup>V814F</sup>. KIT was immunoprecipitated with anti-c-kit MoAb (ACK2) from lysates of the indicated cells before and after stimulation with rmSCF (100 ng/mL). The immunoprecipitates were divided into two aliquots, separated by SDS-PAGE, and subjected to immunoblotting with antiphosphotyrosine (anti-P-Tyr) MoAb (upper panel) or with anti-c-kit polyclonal antibody (lower panel). The cells infected with pMSGneo vector alone (Vector) were used as a negative control. The mobilities of the mature (145 kD) and immature (125 kD) forms of KIT are indicated at right.

tyrosine in a ligand-dependent manner, whereas KIT<sup>G559S</sup> and KIT<sup>V814F</sup> showed ligand-independent, constitutive tyrosine phosphorylation (data not shown).

Dimerization of KIT. All of RTKs have been shown to undergo ligand-dependent dimerization that plays an important role in activation of the intrinsic protein kinase activity. To determine if the activating mutations of c-kit led to receptor dimerization regardless of stimulation with SCF, we performed cross-linking analysis of wild-type and mutant KIT (Fig 3). Ba/F3 cells expressing KIT<sup>WT</sup>, KIT<sup>G559S</sup>, or KIT<sup>V814F</sup> were stimulated with or without rmSCF (300 ng/mL) and treated with the water-soluble cross-linker BS<sup>3</sup> that hardly enters the cytosol. Lysates for the cells were then immunoprecipitated with anti-c-kit polyclonal antibody and were subjected to immunoblotting with antiphosphotyrosine MoAb. As expected, rmSCF induced tyrosine phosphorylation of KIT<sup>WT</sup> in Ba/F3-KIT<sup>WT</sup> cells, and the phosphorylated form of KIT<sup>WT</sup> was detected in proteins at an approximate molecular mass of 330 kD that represented a cross-linked dimer of KIT<sup>WT</sup> and rmSCF (KIT<sup>WT</sup>/rmSCF) (Fig 3, left panel). By contrast, a substantial fraction of tyrosine-phosphorylated KIT<sup>G559S</sup> was detectable in a ~290-kD homodimeric form even in the absence of rmSCF, although tyrosine phosphorylation of dimerized KIT<sup>G559S</sup>/rmSCF (~330 kD) was slightly intensified by the treatment with rmSCF (Fig 3, middle panel). Notably, KIT<sup>V814F</sup> was barely detectable in a ~290-kD dimeric form without the addition of rmSCF, whereas an ~330-kD cross-linked form of KIT<sup>V814F</sup>/rmSCF was observed after stimulation with rmSCF (Fig 3, right panel). These results suggest that G559 mutation may result in dimerization of KIT<sup>G559S</sup>, whereas V814 mutation may not induce receptor association at least in the extracellular domain.

Role of constitutively activating mutations in factor-independent growth. To determine if KIT<sup>G559S</sup> and KIT<sup>V814F</sup> could induce factor-independent growth, Ba/F3 and FDC-P1 cells expressing KIT<sup>WT</sup>, KIT<sup>G559S</sup>, or KIT<sup>V814F</sup> were cultured in the presence of 0 to 10 ng/mL rmIL-3 or 0 to 500 ng/mL rmSCF for 72 hours, followed by measurement of cell proliferation using an MTT colorimetric assay (Fig 4). As was the case for each parental cell line, rmIL-3 induced a dose-dependent proliferation of pMSGneo-infected Ba/F3 and FDC-P1 cells; and rmSCF did not have any effects on proliferation of the cells. In addition to the expected proliferative response to rmIL-3, Ba/F3 or FDC-P1 cells expressing KIT<sup>WT</sup> showed a dose-dependent proliferation in response to rmSCF over the range of 0.1 to 100 ng/mL, indicating functional expression of KIT<sup>WT</sup>. By contrast, Ba/F3 or FDC-P1 cells express-
infiltration of BaF3-KITG559 and BaF3-KITV814 cells into bone marrow, spleen, and liver (Fig 5). The tumor cells isolated from bone marrow and spleen were similar to the injected cells in the expression patterns of KIT and B-220 antigen, suggesting that the tumors were originated from the injected cells (data not shown). In the mice injected with BaF3-KITWT cells, no detectable tumors were noted within 2 weeks, but small nodules developed at 3 of 8 injection sites at day 21 (Table 1). Tumors developed at only two thirds of the BaF3-KITWT-injected sites after an extended latency (3 to 6 weeks), and the mice were all alive within the 6-week observation period.

**DISCUSSION**

It is well established that a large number of structural alterations of KIT identified so far in mice, rats, and humans cause either the diminished levels of KIT expression or the qualitative defects in KIT tyrosine kinase activity, thereby leading to a decrease in tyrosine kinase activity of KIT. In addition to the loss-of-function mutations, we have recently provided evidence that KIT can be activated in a ligand-independent manner by two activating mutations resulting in intracellular amino acid substitutions of Gly-559 for Val and Val-814 for Asp. However, the importance of these activating mutations in cell transformation has not been clarified.

In this study, we have investigated the effects of these mutations on the state of KIT expression and also on the cell growth and tumorigenesis. The results show that G559 and V814 mutations of the c-kit proto-oncogene result not only in constitutive tyrosine phosphorylation of KIT proteins without the addition of exogenous ligand, but also in ligand-independent growth of IL-3–dependent murine hematopoietic cell lines, Ba/F3 and FDC-P1, at an almost similar level. In addition, both of these mutations were found to render BaF3 cells tumorigenic in nude mice. This almost equal ability of KITG559 and KITV814 to induce a factor-independent and tumorigenic phenotype was partially unexpected, be-

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<th>Type of Cells</th>
<th>No. of Tumors/No. of Injection Sites</th>
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<tr>
<td>BaF3-Vector</td>
<td>0/10</td>
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<tr>
<td>BaF3-KIT&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>0/10</td>
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<tr>
<td>BaF3-KITG559</td>
<td>10/10</td>
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<td>BaF3-KITV814</td>
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Each type of cells (3 x 10<sup>6</sup> cells/injection site) was inoculated subcutaneously into the posterior flank of five nude mice (two sites/mouse).

Abbreviation: ND, not determined.

* At days 14 and 21, mouse was killed and pathologically analyzed. Pathological analysis performed at day 21 showed that BaF3-KIT<sup>WT</sup> cells were observed in tumors at the site of injection, but not in bone marrow or spleen, whereas BaF3-Vector cells were not detected at any sections.

† All mice died within 2 to 3 weeks.
cause our previous study showed that KITV814 exhibited a significantly higher level of tyrosine phosphorylation and activation than KITG559 in a transient expression system using 293T cells. However, the results of this study using a stable expression system suggest that both KITG559 and KITV814 have potential to function as oncogenic proteins. We also provide the data suggest that high or stable expression of KITG559 may play a role in excessive proliferation or transformation of hematopoietic cells. Although proliferation of BaF3-KITG559 cells was not supported in vitro in the absence of IL-3 or SCF, the subcutaneous inoculation of BaF3-KITG559 cells into nude mice was found to result in development of skin tumors, albeit to a much lesser degree than that of BaF3-KITV814 or BaF3-KITV814 cells. Nude mice are known to lack T cells that are a major source of IL-3, but to exhibit normal number of mast cells in the connective tissues, indicating the absence of IL-3, but the presence of SCF in nude mice. Therefore, it is suggested that the slow but detectable growth of BaF3-KITG559 cells may be generated by their interaction with SCF that is presumably produced by certain mesenchymal cells as a membrane-bound or soluble form. Also, it is suggested that the enhanced expression of KITV814 by itself is not sufficient to cause cell transformation, and the continuous stimulation of KITV814 by ligand is necessary for exhibiting the transformed phenotype.

Despite the dramatic effects of G559 and V814 mutations on the factor-independent and tumorigenic phenotypes, the precise mechanisms by which the c-kit mutations activate KIT tyrosine kinase and transmit oncogenic signals into cells are not completely understood. Although some evidence has been presented that epidermal growth factor receptor can be activated through an intramolecular mechanism and does not necessarily require receptor dimerization, a large number of studies have suggested that receptor dimerization plays an essential role in the activation of intrinsic protein kinase activity and also in signal transduction. Furthermore, there is increasing evidence that the transphosphorylation between the dimerized receptor kinase domains can occur after ligand binding and the receptor cross-phosphorylation may underlie the natural mechanism of receptor activation.
study, we have found that a substantial fraction of KIT559 exists as a dimerized form even in the absence of rmSCF.

This result suggests that G559 mutation may yield receptor dimerization resulting in enzymatic activation that leads to cell transformation. This finding is comparable with the previous results showing that a point mutation of c-erbB/neru, in which glutamic acid substitute for valine at codon 664 in the transmembrane domain, leads to constitutive dimerization and activation of the tyrosine kinase receptor.26 In contrast to KIT559, cross-linking analysis using BS’ showed that a dimeric form of KITV814 was scarcely detectable in the absence of rmSCF, whereas a 330-kD cross-linked form of the KITV814/rmSCF was observed after stimulation with rmSCF. Therefore, it is possible that V814 mutation may be a unique activating mutation that induces factor-independent growth and tumorigenesis independently of receptor dimerization. Also, despite little evidence for dimerization of RTK in the cytoplasmic domain, it is possible that association of KIT444 receptor may be mediated by the cytoplasmic domain, thereby leading to receptor activation and cell transformation. The experiments to test these possibilities are currently underway.

In addition to KIT, constitutively activating point mutations have been found in hematopoietic growth factor receptors such as CSF-1 receptor (CSF-1R; the product of c-fis proto-oncogene) and erythropoietin receptor (EpoR).27,28 Activating mutations in CSF-1R were shown to induce morphologic transformation, anchorage-independent growth, and tumorigenicity in mouse NIH3T3 cells26,27; activating mutations have been detected in a fraction of myelodysplastic syndrome and acute myelocytic leukemia.29 Furthermore, despite no kinase sequences in the cytoplasmic domain, activating mutations in EpoR were reported to initiate the development of erythroleukemia in murine experimental models.30 However, CSF-1 and Epo, respectively, are thought to act primarily on cells of monocyte/macrophage and erythroid lineages, and neither of them are very potent growth factors for hematopoietic stem cells.31,32 By contrast, KIT is known to be important in understanding the role of KIT in normal and abnormal growth of hematopoietic cells.

results in production of a factor-independent and tumorigenic phenotype and suggest that the constitutively activating mutations of c-kit gene may be involved in some aspect of neoplastic transformation of hematopoietic cells. Identification of molecular mechanisms responsible for the receptor activation and signaling events of the KIT mutants will be important in understanding the role of KIT in normal and abnormal growth of hematopoietic cells.

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Constitutively activating mutations of c-kit receptor tyrosine kinase confer factor-independent growth and tumorigenicity of factor-dependent hematopoietic cell lines

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