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

By Hitoshi Kitayama, Yuzuru Kanakura, Takuma Furitsu, Tohru Tsujimura, Kenji Oritani, Hirokazu Ikeda, Hiroyuki Sugahara, Hideki Mitsui, Yoshio Kanayama, Yukihiro Kitamura, and Yuji Matsuzawa

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, Val559 → 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 KITG559 or KITV814 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, KITG559 or KITV814 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 KITG559 underwent dimerization even in the absence of SCF, whereas the phosphorylated KITV814 did not, suggesting the distinct mechanisms underlying constitutive activation of KIT by G559 and V814 mutations. Furthermore, the cells expressing either KITG559 or KITV814 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 KITG559 or KITV814 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 KITG559 or KITV814 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. © 1995 by The American Society of Hematology.

The proto-oncogene c-kit is allelic with the white spotting (W) locus on mouse chromosome 5, 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). 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. To investigate the biochemical consequence and biologic significance of these mutations, retroviral vectors encoding KITG559 or KITV814 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, KITG559 or KITV814 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 KITG559 underwent dimerization even in the absence of SCF, whereas the phosphorylated KITV814 did not, suggesting the distinct mechanisms underlying constitutive activation of KIT by G559 and V814 mutations. Furthermore, the cells expressing either KITG559 or KITV814 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 KITG559 or KITV814 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 KITG559 or KITV814 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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In this study, we introduced wild-type KIT (KIT\textsuperscript{WT}), KIT\textsuperscript{G599S} and KIT\textsuperscript{V814I} into cells of two murine interleukin-3 (IL-3)-dependent cell lines, Ba/F3 (pro-B type)\textsuperscript{35} and FDC-P1 (myeloid type).\textsuperscript{36} Expression of KIT\textsuperscript{G599S} or KIT\textsuperscript{V814I} 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 KIT\textsuperscript{G599S} was organized at the plasma membrane in a dimerized form, whereas KIT\textsuperscript{V814I} 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 onco-
genes.

**MATERIALS AND METHODS**

**Reagents.** Recombinant murine (rm) SCF and IL-3 were generous gifts of Kirin Brewery Co Ltd (Tokyo, Japan). Antiphosphotyrosine antibody,\textsuperscript{37,38} a murine monoclonal antibody (MoAb) generated against phosphotyramine, was generously supplied by Dr B. Drucker (Oregon Health Science University, Portland, OR). Rat-antimouse c-kit MoAb (ACK2) and full length of murine c-kit cDNA were kindly donated from Dr S.-I. Nishikawa (Kyoto University, Kyoto, Japan).\textsuperscript{39} 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 BS\textsubscript{3} from Pierce (Rockford, IL).

**Cells and mice.** Ba/F3,\textsuperscript{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,\textsuperscript{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 rMlL-3 (10 ng/mL). A helper virus-free packaging cell line,\textsuperscript{40} SP2, was maintained in Dulbecco’s modified essential medium (Nakarai tesque) supplemented with 10% FCS. S/SI-3T3, a fibroblast cell line derived from S/SI-mutant mice lacking in SCF expression, was established in our laboratory as described previously.\textsuperscript{41} 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 pM5Gneo,\textsuperscript{42} a derivative of myeloproliferative sarcoma virus (MPSV), was a kind gift from Dr W. Ostertag (University of Oregon Health Science University, Portland, OR). The mutated murine c-kit genes encoding KIT\textsuperscript{G599S} or KIT\textsuperscript{V814I} were constructed by site-directed mutagenesis as described previously.\textsuperscript{32} The full length of wide-type (WT) or mutated c-kit cDNAs were inserted into the EcoRI site of pM5Gneo. Retroviral vector (pM5Gneo) alone or retroviral vectors containing c-kit\textsuperscript{WT}, c-kit\textsuperscript{G599S} or c-kit\textsuperscript{V814I} genes were transfected into packaging cell line (292) by calcium phosphate method, and G418-resistant clones of each packaging cell line were isolated. The virus titer was determined by infecting S/SI-3T3 fibroblasts with serial dilutions of retrovirus-containing supernatant from each clone and by counting the numbers of G418-resistant colonies. The resultant viruses were termed pM5Gneo, pM5Gneo-KIT\textsuperscript{WT}, pM5Gneo-KIT\textsuperscript{G599S}, and pM5Gneo-KIT\textsuperscript{V814I} and had approximately comparable viral titers ranging from 2 to 5 × 10\textsuperscript{5} colony-forming units/mL of supernatant. For gene transfer, Ba/F3 and FDC-P1 cells (2 × 10\textsuperscript{5} cells/each cell line) were cocultured with the virus-producing packaging cells for 48 hours in the presence of rmIL-3 (10 ng/mL) and Polybrene (8 μg/mL). After infection, the cells expressing neomycin-resistant gene were selected by cultivation in a medium containing G418 (0.6 mg/mL) and rmIL-3 (10 ng/mL).

**Flow cytometry.** The cells (5 × 10\textsuperscript{5}) were washed with phosphate-buffered saline (PBS) and resuspended in PBS containing 0.5% bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO) and 0.1% NaN\textsubscript{3}. Cells were incubated with rat antimurine c-kit MoAb (ACK2) or rat monoclonal IgG1 control (Becton Dickinson, Mountain View, CA) at 4°C for 30 minutes, and then washed three times with the buffer. Cells were subsequently incubated with fluorescein-conjugated goat-antirat IgG antibody at 4°C for 30 minutes, and washed two times before analysis using a FACScan flow cytometer (Becton Dickinson).

**Stimulation with factors and cell lysis.** Exponentially growing cells were washed free of serum and growth factors and incubated in serum-free medium (Cosmedium 003; CosmoBio, Tokyo, Japan) for 12 hours at 37°C to factor-deprive the cells. The cells (10\textsuperscript{6} cells suspended in 1 mL of Cosmedium 003) were exposed to SCF (100 ng/mL) at 37°C for 15 minutes. After stimulation with factors, cells were washed with cold PBS and lysed in lysis buffer (20 mmol/L Tris-HCl, pH 8.0, 137 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40) containing protease and phosphatase inhibitors as described previously.\textsuperscript{30,32,37,38} Insoluble material was removed by centrifugation at 10,000 g for 15 minutes at 4°C.

**Immunoprecipitation and immunoblotting.** The procedures of immunoprecipitation, gel electrophoresis, and immunoblotting were performed according to the methods described previously.\textsuperscript{30,32,37,38} Briefly, the lysates were precleared with protein-G Sepharose beads (Pharmacia AB, Uppsala, Sweden) for 2 hours at 4°C. The precleared lysates were incubated with ACK2 and protein-G Sepharose beads to collect antigen-antibody complexes. The immunoprecipitates were washed five times with lysis buffer containing protease and phosphatase inhibitors, and subjected to sodium dodecyl sulphate-polyacryla-
midy gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred from the gel onto a polyvinylidene difluoride membrane (Immobilon, Millipore Corp, Bedford, MA). After blocking residual binding sites on the filter by incubation in TBS (10 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl) containing 1% gelatin (Bio-Rad Laboratories, Richmond, CA), immunoblotting was performed with an antiphosphotyrosine MoAb or anti-c-kit polyclonal antibody.

**Chemical cross-linking experiments.** Dimerization of KIT was analyzed by chemical cross-linking.\textsuperscript{43} Ba/F3 cells expressing KIT\textsuperscript{WT}, KIT\textsuperscript{G599S} or KIT\textsuperscript{V814I} were washed twice and suspended in PBS containing 1 mg/mL BSA. The cells were stimulated with or without rmSCF (300 ng/mL) for 90 minutes at 4°C, and were washed five times in ice-cold PBS. The cells were then incubated in PBS containing 1 mmol/L BS\textsubscript{3} for 30 minutes at 22°C, and the reaction was terminated by washing in ice-cold PBS, followed by the incubation in 150 mmol/L glycine-HCl (pH 7.5) for 5 minutes at 4°C. In these buffers used after stimulation with or without rmSCF, sodium orthovanadate (Na\textsubscript{2}VO\textsubscript{4}, 100 μmol/L) was included to prevent dephosphorylation of KIT. The cells were lysed in lysis buffer, and immunoprecipitations were performed with anti-c-kit polyclonal antibody as described above. For the detection of KIT that was phosphorylated on tyrosine, KIT-containing immunoprecipitates were subjected to SDS-PAGE (4% acrylamide) and immunoblotting with an antiphosphotyrosine MoAb and developed with horseradish-peroxidase–conjugated antimouse IgG (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 bro-
midne, Sigma] rapid colorimetric assay as previously described.\textsuperscript{30,37} Briefly, quadruplicate aliquots of cells (1 × 10\textsuperscript{4} Ba/F3 cells or 2 × 10\textsuperscript{4} 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 'H-thymidine incorporation or cell enumeration as described previously.32,33,34

Tumorigenicity assay. Ba/F3 cells expressing KIT<sup>WT</sup>, KIT<sup>G559</sup>, or KIT<sup>V814</sup> 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 × 10<sup>6</sup> injected site; two sites per mouse) were subcutaneously injected into the posterior flanks 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-kit<sup>WT</sup>, c-kit<sup>G559</sup> or c-kit<sup>V814</sup> genes, which were termed pM5Gneo-KIT<sup>WT</sup>, pM5Gneo-KIT<sup>G559</sup> or pM5Gneo-KIT<sup>V814</sup>, 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 antimouse 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-KIT<sup>WT</sup> (BaF3-KIT<sup>WT</sup>) or pM5Gneo-KIT<sup>G559</sup> (BaF3-KIT<sup>G559</sup>) showed abundant surface expression of KIT<sup>WT</sup> or KIT<sup>G559</sup> on their surface, respectively (Fig 1B). In the cells infected with pM5Gneo-KIT<sup>V814</sup> (BaF3-KIT<sup>V814</sup>), surface expression of KIT<sup>V814</sup> 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-KIT<sup>WT</sup>, pM5Gneo-KIT<sup>G559</sup>, or pM5Gneo-KIT<sup>V814</sup> (Fig 1B): FDC-P1 cells infected with pM5Gneo-KIT<sup>WT</sup> (FDCP1-KIT<sup>WT</sup>) or pM5Gneo-KIT<sup>G559</sup> (FDCP1-KIT<sup>G559</sup>) exhibited high levels of KIT<sup>WT</sup> or KIT<sup>G559</sup> expression; the cells infected with pM5Gneo-KIT<sup>V814</sup> (FDCP1-KIT<sup>V814</sup>) did moderate KIT<sup>V814</sup> expression; and little expression of KIT was observed on the cells infected with pM5Gneo (FDCP1-Vector).

 Constitutive tyrosine phosphorylation of mutant 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 KIT<sup>WT</sup>, KIT<sup>G559</sup>, and KIT<sup>V814</sup> 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 KIT<sup>WT</sup>, particularly in the 145-kD form of KIT<sup>WT</sup>, after treatment with rmSCF (Fig 2, upper panel). By contrast, both 145-kD and 125-kD forms of KIT<sup>G559</sup> or KIT<sup>V814</sup> were strikingly phosphorylated on tyrosine regardless of rmSCF stimulation (Fig 2, upper panel), suggesting constitutive activation of KIT<sup>G559</sup> and KIT<sup>V814</sup> in BaF3-KIT<sup>G559</sup> and BaF3-KIT<sup>V814</sup> cells, respectively. Also in FDC-P1 cells, KIT<sup>WT</sup> was found to be phosphorylated on
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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 phosphorylation 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<sub>3</sub> 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 an ~290-kD homodimeric form even in the absence of rmSCF, although tyrosine phosphorylation of dimerized KIT<sup>G559S/rmSCF</sup> (~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/rmSCF</sup> 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, <sup>3</sup> 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-
ing either KITG559 or KITV814 proliferated in the absence of exogenous rmIL-3 or rmSCF; and rmIL-3 or rmSCF had a minimal effect on proliferation of the cells. To exclude the possibility that factor-independence could be caused by autocrine stimulation by IL-3 or SCF synthesized by the KITGss9, KITV814 expressing cells themselves, we assayed conditioned media from these cells for growth supporting activity. The conditioned media could not induce proliferation or survival of parental IL-3-dependent cell lines or SCF-responsive KITWT-positive lines (data not shown), suggesting 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-

**A. Ba/F3**

![Graph](image1)

**B. FDC-P1**

![Graph](image2)

Fig 4. Proliferation of Ba/F3 (A) and FDC-P1 (B) cells in response to various concentrations of rmIL-3 (left panel) and rmSCF (right panel). Quadruplicate aliquots of the cells expressing vector alone (□), KITWT (■), KITV814 (○), and KITG559 (△) were cultured with each factor and cell proliferation was measured using an MTT colorimetric assay. The results are shown as mean ± SEM for one of three similar experiments.

**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.42 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.32 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-

| Table 1. Tumorigenicity of Ba/F3 Cells Expressing Wild-Type and Mutant KIT |
|--------------------------------------|-----------------|-----------------|
| Type of Cells | No. of Tumors/No. of Injection Sites |
| BaF3-Vector | 0/10 | 0/8§ |
| BaF3-KITWT | 0/10 | 3/8§ |
| BaF3-KITG559 | 10/10 | ND† |
| BaF3-KITV814 | 10/10 | ND† |

Each type of cells (3 x 10⁶ 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-KITWT 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 KIT<sup>V814</sup> exhibited a significantly higher level of tyrosine phosphorylation and activation than KIT<sup>G559</sup> in a transient expression system using 293T cells. However, the results of this study using a stable expression system suggest that both KIT<sup>G559</sup> and KIT<sup>V814</sup> have potential to function as oncogenic proteins.

We also provide the data suggest that high or stable expression of KIT<sup>WT</sup> may play a role in excessive proliferation or transformation of hematopoietic cells. Although proliferation of BaF3-KIT<sup>WT</sup> cells was not supported in vitro in the absence of IL-3 or SCF, the subcutaneous inoculation of BaF3-KIT<sup>WT</sup> cells into nude mice was found to result in development of skin tumors, albeit to a much lesser degree than that of BaF3-KIT<sup>G559</sup> or BaF3-KIT<sup>V814</sup> 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-KIT<sup>WT</sup> 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 KIT<sup>WT</sup> by itself is not sufficient to cause cell transformation, and the continuous stimulation of KIT<sup>WT</sup> 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. In this
study, we have found that a substantial fraction of KitG559<br>exits as a dimerized form even in the absence of rmSCF.<br>This result suggests that G559 mutation may yield receptor<br>merization resulting in enzymatic activation that leads to<br>cell transformation. This finding is comparable with the pre<br>vious results showing that a point mutation of c-erbB/neru,<br>in which glutamic acid substitute for valine at codon 664 in<br>the transmembrane domain, leads to constitutive merization<br>and activation of the tyrosine kinase receptor.27 In con<br>trast to KitG559, cross-linking analysis using BS' showed<br>that a dimeric form of KitV814 was scarcely detectable in<br>the absence of rmSCF, whereas a 330-kD cross-linked form<br>of the KitV814/rmSCF was observed after stimulation with<br>rmSCF. Therefore, it is possible that V814 mutation may be<br>a unique activating mutation that induces factor-independent<br>growth and tumorigenesis independently of receptor merization.<br>Also, despite little evidence for dimerization of RTK<br>in the cytoplasmic domain, it is possible that association of<br>KitV814 receptor may be mediated by the cytoplasmic do<br>main, thereby leading to receptor activation and cell transforma<br>tion. The experiments to test these possibilities are cur<br>rently underway.<br>In addition to Kit, constitutively activating point muta<br>ions have been found in hematopoietic growth factor recep<br>tors such as CSF-1 receptor (CSF-1R; the product of c-fis<br>proto-oncogene)26,27 and erythropoietin receptor (EpoR).53,54<br>Activating mutations in CSF-1R were shown to induce mor<br>phologic transformation, anchorage-independent growth, and<br>tumorigenicity in mouse NIH3T3 cells52,27; activating muta<br>ions have been detected in a fraction of myelodysplas<br>tic syndrome and acute myelocytic leukemia.25 Furthermore,<br>despite no kinase sequences in the cytoplasmic domain, acti<br>vating mutations in EpoR were reported to initiate the de<brvelopment of erythroleukemia in murine experimental models.54<br>However, CSF-1 and Epo, respectively, are thought to act<br>primarily on cells of monocyte/macrophage and erythroid<br>lineages, and neither of them are very potent growth factors<br>for hematopoietic stem cells.55,57 By contrast, Kit is known to<br>express on various types of hematopoietic stem/progenitor<br>cells as well as mast cells2,23,29,28,29 and SCF acts as a potent<br>mitogen to stimulate directly the growth of the cells particu<br>larly in combination with other growth factors like Epo,<br>granulocyte-macrophage CSF, granulocyte CSF, IL-3, and<br>IL-7.5,9,13,14,60-62 Therefore, it is possible that the activatin<br>g mutations in Kit may contribute to aberrant cell growth and<br>leukemogenesis of mast cells and various types of hematopoi<br-etotic stem/progenitor cells. This possibility may be sup<br>ported, at least in part, by our recent findings that constitu<br-tively activating mutations of c-kit gene are detectable in the<br>same Asp codon within the phosphotransferase domain in<br>both a murine mastocytoma cell line (P-815) and a rat mast<br>cell leukemia cell line (RBL-2H3).53,54 Also, the possibility may be additionally supported by our preliminary study<br>showing that retroviral transfer of the c-kit mutant genes<br>into normal hematopoietic stem cells results in formation of<br>various types of colonies in the absence of hematopoietic<br>growth factors.<br>In summary, the results presented here show that introduc<br>tion of the c-kit mutant genes into IL-3-dependent cell lines<br>results in production of a factor-independent and tumorigenic<br>phenotype and suggest that the constitutively activating muta<br>tions of c-kit gene may be involved in some aspect of neoplastic transformation of hematopoietic cells. Identifica<br-tion of molecular mechanisms responsible for the receptor<br>activation and signaling events of the Kit mutants will be<br>important in understanding the role of Kit in normal and<br>abnormal growth of hematopoietic cells.<br><br>REFERENCES<br>1. Chabot B, Stephenson DA, Chapman VM, Besmer P, Berstein<br>A: The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. Nature 335:88, 1988<br>2. Geissler EN, Ryan MA, Housman DE: The dominant-white<br>spotting (W) locus of the mouse encodes the c-kit proto-oncogene.<br>Cell 55:185, 1988<br>3. Yarden Y, Ullrich A: Growth factor receptor tyrosine kinases.<br>Annu Rev Biochem 57:443, 1988<br>4. Ullrich A, Schlessinger J: Signal transduction by receptors with<br>tyrosine kinase activity. Cell 61:203, 1990<br>5. 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