

Role of Aspartic Acid 814 in the Function and Expression of *c-kit* Receptor Tyrosine Kinase*

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The *c-kit* receptor tyrosine kinase (KIT) is constitutively activated in three different types of neoplastic mast cell lines by naturally occurring mutations that result in substitutions of Val or Tyr for Asp⁸¹⁴ in the phosphotransferase domain. In an effort to characterize the role of the Asp⁸¹⁴ residue, we have investigated the properties of mutant KITs in which the Asp⁸¹⁴ residue was deleted or mutated to a series of other amino acids. With the exception of rare instances, mutant KITs with substitutions of Asp⁸¹⁴ were found to be constitutively phosphorylated on tyrosine and activated in the absence of the ligand, stem cell factor (SCF), whereas a deletion mutant lacking Asp⁸¹⁴ (KIT^{Del-Asp-814}) did not exhibit tyrosine phosphorylation and activation even after treatment with SCF. In addition to constitutive activation, furthermore, both highly activated substitution mutants (KIT^{Val-814} and KIT^{Tyr-814}) and modestly activated substitution mutants (KIT^{Gly-814} and KIT^{His-814}) were continuously degraded in the absence of SCF, whereas wild-type KIT (KIT^{Wild}) required SCF stimulation to undergo degradation. These results suggested that the Asp⁸¹⁴ residue may play a crucial role in regulating enzymatic activity and expression of KIT and that various types of mutations at the Asp⁸¹⁴ residue may generate oncogenic protein with constitutive activation and degradation.

The *c-kit* proto-oncogene encodes a receptor tyrosine kinase (RTK)¹ that is a member of the same RTK subfamily as the receptors for platelet-derived growth factor and colony-stimulating factor-1 (CSF-1) (1, 2). This RTK subfamily is characterized by the presence of five immunoglobulin-like repeats in the extracellular domain and an insert that splits the cytoplasmic kinase domain into an ATP binding region and the phospho-

transferase domain (1, 2). The enzymatic activity of RTKs is tightly regulated by the binding of their ligands. The binding of ligands promotes receptor dimerization and phosphorylation at specific tyrosine residues, which can serve as docking sites for downstream signal transduction molecules containing Src homology 2 domains (3). The tyrosine-phosphorylated and activated RTKs act as a center for the assemblage of a multiprotein complex that transmits a series of biochemical signals. The activated RTKs are then rapidly internalized and targeted to lysosomes, where both receptors and ligands are degraded.

The *c-kit* RTK (KIT) is encoded by the *W* locus on mouse chromosome 5, whereas its ligand, stem cell factor (SCF), is encoded by the *Sl* locus on mouse chromosome 10. A variety of loss-of-function mutations at either the KIT/*W* or SCF/*Sl* locus have been described, and these mutations have provided insights into KIT function and site of action. The phenotypes of mice bearing the KIT or SCF mutations include melanocyte deficiency, macrocytic anemia, mast cell deficiency, and sterility, emphasizing how essential SCF-regulated enzymatic activity of KIT is for normal hematopoiesis, melanogenesis, and gametogenesis (4). In contrast to loss-of-function mutations, information about gain-of-function mutations of KIT has been very limited. However, we have recently found the presence of constitutively activating mutations of *c-kit* gene in three different types of neoplastic mast cell lines, the human mast cell leukemia cell line (HMC-1) (5), the rat mast cell leukemia cell line (RBL-2H3) (6), and the murine mastocytoma cell line (P-815) (7). The *c-kit* gene of HMC-1 cells was found to carry two constitutively activating mutations, the Val⁵⁶⁰ to Gly mutation in the juxtamembrane domain and the Asp⁸¹⁶ to Val mutation in the phosphotransferase domain (5). In addition, both RBL-2H3 and P-815 cells possessed the constitutively activating mutation of the *c-kit* gene at the corresponding Asp codon in the phosphotransferase domain, resulting in the substitution of Tyr⁸¹⁷ for Asp in RBL-2H3 cells and that of Tyr⁸¹⁴ for Asp in P-815 cells, respectively (6, 7).

The occurrence of the activating mutations at the same Asp codon in the three neoplastic mast cell lines suggested that the Asp codon may be a hot spot for activating mutation of *c-kit*. Furthermore, since the Asp lies near the highly conserved Asp-Gly-Phe sequence and occupies the equivalent position in other members of RTKs such as receptors for platelet-derived growth factor, CSF-1, insulin, and hepatocyte growth factor (8), the Asp region might be important in function and regulation of RTKs, including KIT. To better understand the role of the Asp, we have investigated the properties of murine KITs with the various substitutions and deletion of Asp⁸¹⁴.

MATERIALS AND METHODS

Reagents—Recombinant murine (rm) SCF was a gift of Kirin Brewery Co. Ltd. (Tokyo, Japan). Rat antimouse *c-kit* (ACK2) monoclonal antibody (mAb) (9) and full-length murine *c-kit* cDNA were donated by Dr. S.-I. Nishikawa (Kyoto University, Kyoto, Japan). A rabbit polyclonal antibody against the whole murine KIT was a gift of Dr. P. Besmer (Cornell University Graduate School of Medical Science, New York) (5–7). An anti-KIT polyclonal antibody against synthetic peptide of the C-terminal portion of human KIT was purchased from Oncogene Science, Inc. (Uniondale, NY). Mouse anti-phosphotyrosine mAb generated against phosphotyramine was a gift of Dr. B. Drucker (Oregon Health Sciences University, Portland, OR) (10). The mammalian expression vector pEF-BOS was donated by Dr. S. Nagata (Osaka Bioscience Institute, Osaka, Japan) (11). The human embryonic kidney cell line, 293T, was provided by Dr. D. Baltimore (Rockefeller Univer-

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¹ The abbreviations used are: RTK, receptor tyrosine kinase; CSF-1, colony-stimulating factor-1; KIT, *c-kit* RTK; SCF, stem cell factor; rm, recombinant murine; mAb, monoclonal antibody; DMEM, Dulbecco's modified essential medium; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; IL-3, interleukin-3.

sity, New York) (12) and maintained in Dulbecco's modified essential medium (DMEM, ICN Corp., Costa Mesa, CA) supplemented with 10% fetal calf serum (Nippon Bio-Supply Center, Tokyo, Japan).

Construction of Mutant-type *c-kit* Genes and Transfection—The murine *c-kit*^{Wild}, *c-kit*^{Val-814}, and *c-kit*^{Tyr-814} cDNAs have been cloned into an *EcoRV* site of Bluescript I KS(−) in our laboratory (5, 7). Each blunted *HindIII-EcoRI* fragment of Bluescript I KS(−) containing *c-kit*^{Wild}, *c-kit*^{Val-814}, or *c-kit*^{Tyr-814} cDNA was introduced into the blunted *XbaI* site of pEF-BOS. To generate other types of mutant-type *c-kit* cDNAs, we carried out the site-directed mutagenesis by polymerase chain reaction (PCR) according to the method described by Higuchi *et al.* (13). We synthesized one antisense primer (nucleotides 2669–2698, 5'-CGGGCTGACCATCCGGAAGCCTTCCTTGAT-3') (2) and 18 sense primers (nucleotides 2353–2379, 5'-TTCGGGCTAGCCAGAXXXATCAGGAAT-3'; the mutated portion is shown as three nucleotides XXX) (2). The underlined three nucleotides of sense primers were GAA for *c-kit*^{Glu-814}, AAC for *c-kit*^{Asn-814}, CAG for *c-kit*^{Gln-814}, AGC for *c-kit*^{Ser-814}, ACC for *c-kit*^{Thr-814}, CGC for *c-kit*^{Arg-814}, AAG for *c-kit*^{Lys-814}, CAC for *c-kit*^{His-814}, GGC for *c-kit*^{Gly-814}, GCC for *c-kit*^{Ala-814}, CTC for *c-kit*^{Leu-814}, ATC for *c-kit*^{Ile-814}, TTC for *c-kit*^{Phe-814}, TGG for *c-kit*^{Trp-814}, ATG for *c-kit*^{Met-814}, TGC for *c-kit*^{Cys-814}, and CCC for *c-kit*^{Pro-814} and were deleted for *c-kit*^{Del-Asp-814}. Sense and antisense primers have *NheI* and *MroI* sites, respectively. PCR was performed by conventional technology using *c-kit*^{Wild} cDNA as a template and various combinations of sense and antisense primers. The amplified PCR products were digested with *NheI* and *MroI*. The *NheI-MroI* fragment of *c-kit*^{Wild} cDNA in pEF-BOS was exchanged by the corresponding fragment of various PCR products. The resulting plasmids were sequenced to confirm the mutations.

Each expression vector was transfected into 293T cells by the calcium phosphate method as described previously (14). Two days after transfection, the cells were used for further analysis.

Immunoprecipitation, Immunoblotting, and *In Vitro* Kinase Assay—The procedures of cell lysis, immunoprecipitation, SDS-polyacrylamide gel electrophoresis (PAGE), and immunoblotting were performed as described previously (5–7). Briefly, after depletion of growth factors and serum, the transfected cells were untreated or treated with rmSCF (100 ng/ml) for 15 min at 37 °C. The cells were then washed with cold phosphate-buffered saline and lysed in lysis buffer. After removal of insoluble materials by centrifugation, the lysates were precipitated with an ACK2 mAb and Protein G-Sepharose beads (Pharmacia Biotech, Sweden). The immunoprecipitates were washed five times with lysis buffer and subjected to SDS-PAGE with 5–20% gradient polyacrylamide. Proteins were electrophoretically transferred from the gel onto a polyvinylidene difluoride membrane (Immobilon, Millipore Corp., Bedford, MA), and immunoblotting was performed with either an anti-KIT polyclonal antibody against the C-terminal portion of human KIT or an anti-phosphotyrosine mAb.

For *in vitro* kinase assay, the cell lysates were prepared by lysis buffer and incubated for 45 min at 4 °C with a rabbit polyclonal antibody against the whole murine KIT and Protein G-Sepharose beads to collect antigen-antibody complexes. The immune complexes were washed and incubated in kinase buffer containing γ -[³²P]ATP (DuPont NEN; 20 μ Ci/ml) for 20 min at 25 °C as described previously (15). The immune complexes were then washed and separated by SDS-PAGE with 5–20% gradient polyacrylamide. The gel was dried, and radioactive proteins were detected by autoradiography (5–7).

Degradation Assay—Cells were radiolabeled for 6 h with [³⁵S]methionine (ICN Corp., Costa Mesa, CA; 100 Ci/ml) in 1 ml of methionine-free DMEM (Life Technologies, Inc.) supplemented with dialyzed 10% fetal calf serum. Cells were washed three times with DMEM and incubated in DMEM containing excessive unlabeled methionine for 1 h. After treatment with or without rmSCF (100 ng/ml), the labeled cells were collected at various times and lysed in lysis buffer. [³⁵S]Methionine-labeled KIT were precipitated with an ACK2 mAb and Protein G-Sepharose beads and were subjected to SDS-PAGE with 5–20% gradient polyacrylamide. The gel was dried, and radioactive proteins were detected by autoradiography. The amount of radioactivity in the protein bands corresponding to KIT was determined by a scanning imager (Molecular Dynamics Inc., Sunnyvale, CA) (16).

RESULTS

Effects of Substitutions of Asp⁸¹⁴ on Tyrosine Phosphorylation and Activation of KIT—We have converted Asp⁸¹⁴ to a series of other amino acids to characterize the role of the Asp residue in KIT activation. Because murine KIT can be recognized by murine and rat SCF but not by human SCF, we have

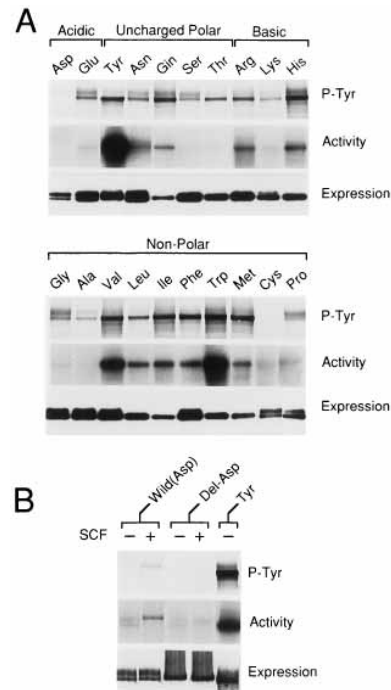


FIG. 1. Tyrosine phosphorylation and activation of mutant-type KIT with substitutions (A) and deletion (B) of Asp⁸¹⁴. A, KITs were immunoprecipitated with an ACK2 mAb from the lysates of transfected cells with wild- and mutant-type *c-kit* cDNAs and subjected to immunoblotting with an anti-phosphotyrosine mAb or an anti-KIT polyclonal antibody. *In vitro* kinase assay for autokinase activity was performed after the immunoprecipitation with a rabbit polyclonal antibody to the whole murine KIT. All samples were not stimulated with rmSCF. B, KIT^{Wild} and KIT^{Del-Asp-814} were also subjected to immunoblotting and *in vitro* kinase assay before and after treatment with rmSCF (100 ng/ml) for 15 min. This experiment was repeated three times producing similar results. P-Tyr, phosphotyrosine.

selected human 293T cells as a recipient for the transfection of murine *c-kit* cDNA. This combination could disregard the effect of human SCF if human SCF were produced from 293T cells after transfection. After transfection into 293T cells, KITs were immunoprecipitated with an ACK2 mAb from cell lysates that were prepared without the addition of rmSCF. The immunoprecipitated KIT was then subjected to immunoblotting with an anti-KIT polyclonal antibody against the C-terminal portion of human KIT or an anti-phosphotyrosine mAb. As shown in Fig. 1A, all transfectants were found to express KIT that was composed of 145-kDa (mature) and 125-kDa (immature) forms, although expression levels varied from transfectant to transfectant. Immunoblotting analysis using an anti-phosphotyrosine mAb showed that KIT^{Wild} was scarcely phosphorylated on tyrosine in the absence of rmSCF. By contrast, all of the mutant-type KITs except KIT^{Cys-814} had an increased amount of tyrosine phosphorylation, particularly in the immature (125 kDa) form, without the addition of rmSCF (Fig. 1A). Furthermore, immune complex kinase assay revealed that most of mutant-type KITs exhibited higher levels of kinase activity than KIT^{Wild}. Among these mutants, markedly elevated kinase activity was observed in KIT^{Tyr-814}, KIT^{Val-814}, KIT^{Leu-814}, KIT^{Ile-814}, and KIT^{Trp-814} (Fig. 1A). Furthermore, in accordance with previous data on KIT^{Val-814} (17), rmSCF had little or no effect on tyrosine phosphorylation and activation of the highly activated KIT^{Val-814} and KIT^{Tyr-814} and the modestly activated KIT^{Gly-814} and KIT^{His-814} (data not shown).

In addition to substitution mutants, we also made a deletion mutant lacking the Asp⁸¹⁴ (KIT^{Del-Asp-814}). In accordance with previous findings, SCF treatment led to a marked increase in tyrosine phosphorylation and kinase activity of KIT^{Wild} (5–7).

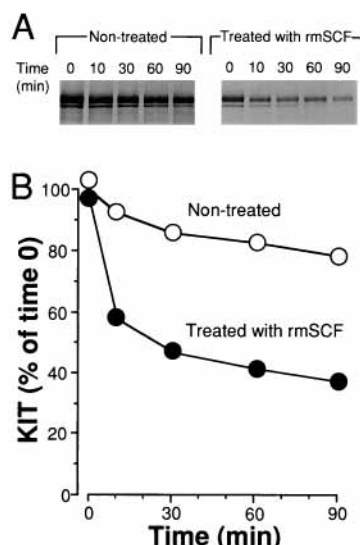


FIG. 2. Time course of SCF-induced degradation of KIT^{Wild} . A, the ^{35}S -labeled KIT in 293T cells are immunoprecipitated with ACK2 mAb after non-treatment or treatment with rmSCF (100 ng/ml) for 15 min and following 0–90 min of incubation at 37 °C. B, the amounts of KIT quantified by densitometric analysis are expressed as a percentage of the value at the starting point. The figure shows the representative results of three experiments.

By contrast, tyrosine phosphorylation and kinase activity of $KIT^{Del-Asp-814}$ were only minimal or absent even after stimulation with rmSCF, suggesting that the deletion of Asp⁸¹⁴ results in impairment of KIT function (Fig. 1B).

Effects of Substitutions of Asp-814 on Degradation of KIT—In addition to constitutive activation of KIT, the substitutions of Asp⁸¹⁴ were suggested to affect expression of KIT, because our previous studies demonstrated that surface expression of mutant-type KIT bearing substitution of Val or Tyr for Asp⁸¹⁴ was constantly lower than that of KIT^{Wild} when introduced into either 293T cells (5–7) or the murine interleukin-3 (IL-3)-dependent cell lines (Ba/F3, FDC-P1, and IC-2) (17, 18). We therefore investigated the effects of substitutions of Asp⁸¹⁴ on KIT degradation. To determine the kinetics of degradation of KIT, 293T cells transfected with wild- and mutant-type *c-kit* cDNAs were labeled with [^{35}S]methionine and incubated with or without rmSCF. The total amount of cellular KIT was analyzed by immunoprecipitation and SDS-PAGE. In the absence of rmSCF, KIT^{Wild} stably expressed on the 293T cells with a half-life of >4 h. The stimulation with rmSCF caused the rapid degradation of KIT^{Wild} at a rate of 4.2% min⁻¹ within the initial 10 min, and the half-time of KIT^{Wild} was 24 min (Fig. 2). By contrast, the highly activated $KIT^{Val-814}$ and $KIT^{Tyr-814}$ were continuously degraded in the absence of rmSCF with a half-life of 47 and 45 min, respectively (Fig. 3). Furthermore, the modestly activated $KIT^{Gly-814}$ and $KIT^{His-814}$ also showed the ligand-independent degradation with a half-life of 57 and 61 min, respectively (Fig. 3). In the presence of rmSCF, the degradation rates of the $KIT^{Val-814}$ and $KIT^{Tyr-814}$ were slightly accelerated (3–4% min⁻¹) within the initial 10 min but were similar to those observed in the absence of rmSCF 10 min after the addition of rmSCF (data not shown).

DISCUSSION

We have previously found that KIT is constitutively activated by naturally occurring mutations of an Asp⁸¹⁴ residue in the phosphotransferase domain of the *c-kit* proto-oncogene, resulting in substitutions of the nonpolar amino acid Val or the uncharged polar amino acid Tyr for the acidic amino acid Asp (5–7). When the *c-kit*^{Val-814} mutant was introduced into cells of

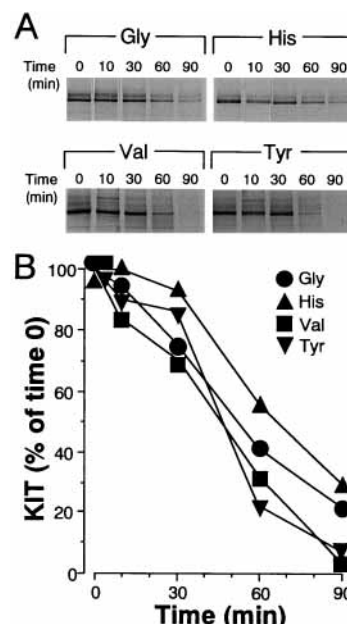


FIG. 3. Time course of SCF-independent degradation of $KIT^{Gly-814}$, $KIT^{His-814}$, $KIT^{Val-814}$ and $KIT^{Tyr-814}$. A, ^{35}S -labeled KIT in 293T cells are immunoprecipitated with ACK2 mAb after 0–90 min of incubation at 37 °C in the absence of rmSCF. B, the amounts of mutant KITs quantified by densitometric analysis are expressed as a percentage of the value at the starting point. This experiment was repeated three times producing similar results.

murine IL-3-dependent cell lines, Ba/F3 (pro-B type), FDC-P1 (myeloid type), and IC-2 (mast cell type), the cells expressing the activated $KIT^{Val-814}$ were found to show a factor-independent growth *in vitro* and to produce large tumors at the injection sites in nude mice (17, 18). Furthermore, IC-2 cells expressing $KIT^{Tyr-814}$ also produced large tumors at the injection sites in nude mice.² These results suggest that the constitutively activating mutations of *c-kit* such as $KIT^{Val-814}$ and $KIT^{Tyr-814}$ could induce a factor-independent and tumorigenic phenotype. In this study, we found that constitutive activation of KIT was also generated by the conversion of Asp⁸¹⁴ to a wide variety of amino acids other than Val and Tyr, including uncharged polar (Asn, Gln), basic (Arg, His), and nonpolar (Leu, Ile, Phe, Trp, Met, Pro) amino acids. In contrast to substitutions, the deletion of Asp⁸¹⁴ was found to abolish tyrosine kinase activity of KIT even after stimulation with rmSCF. These results indicate that the Asp⁸¹⁴ may play a crucial role in regulating enzymatic activity of KIT and suggest that a variety of mutations of the *c-kit* gene at the Asp⁸¹⁴ codon may yield aggressive oncoproteins capable of inducing cell transformations.

Our data also suggest that Asp⁸¹⁴ may be important in regulating expression of KIT. Our previous studies demonstrated that the immature intracellular KIT precursor of ~125 kDa was predominantly observed in cells transfected with *c-kit*^{Val-814} or *c-kit*^{Tyr-814} cDNA, whereas the mature (~145 kDa) form of KIT protein was predominant in cells transfected with *c-kit*^{Wild} cDNA (5–7). Furthermore, surface expression of the activating $KIT^{Val-814}$ was significantly lower than that of KIT^{Wild} after retroviral infection of *c-kit*^{Val-814} and *c-kit*^{Wild} cDNAs into murine IL-3-dependent cell lines, Ba/F3, FDC-P1 and IC-2 (17, 18). These results suggested that activating mutations of Asp⁸¹⁴ affected intracellular transport of mutant-type KIT. In addition to the insufficient transport of mutant-

² Y. Moriyama, T. Tsujimura, K. Hashimoto, M. Morimoto, H. Kitayama, Y. Matsuzawa, Y. Kitamura, and Y. Kanakura, unpublished observations.

type KIT from cytoplasm to cell surface membrane, the present results revealed that the highly activated KIT^{Val-814} and KIT^{Tyr-814} as well as modestly activated KIT^{Gly-814} and KIT^{His-814} were continuously degraded in the absence of rmSCF, whereas KIT^{Wild} required ligand stimulation to undergo rapid degradation. These results are consistent with previous findings that activating mutants of CSF-1 receptor show rapid internalization and degradation in the absence of ligand (19) and suggest that the constitutive low surface expression of mutant-type KIT is attributable, at least in part, to the ligand-independent degradation. Furthermore, these findings raise the possibility that the transforming activity of mutant-type KIT does not result from a failure of the receptor to down-regulate, as described in an internalization-defective mutant of epidermal growth factor receptor (20, 21).

Despite the dramatic effects of the activating mutations at the Asp⁸¹⁴ residue on the function and expression of KIT, the precise mechanisms underlying the constitutive activation and degradation of mutant-type KIT remain to be determined. A number of previous studies have suggested that activating mutations of growth factor receptors tend to involve changes that mimic ligand-stimulated activation, such as receptor dimerization and down-regulation (19, 22, 23). In the case of Val to Gly⁵⁵⁹ mutation in the juxtamembrane of KIT, we found that KIT^{Gly-559} was organized at the plasma membrane in a dimerized form in the absence of rmSCF (17), suggesting that the Gly⁵⁵⁹ mutation may yield receptor dimerization with resulting enzymatic activation. However, a dimeric form of KIT^{Val-814} was barely detectable without the addition of rmSCF, although it was detectable after stimulation with rmSCF (17). It is therefore possible that the *c-kit* mutations at the Asp⁸¹⁴ residue may be unique in causing constitutive activation and degradation independently of receptor dimerization, although it is still possible that the *c-kit* mutations at the Asp⁸¹⁴ residue may induce conformational changes that lead to association of constitutively activating KIT in the cytoplasmic domain.

Interestingly, our preliminary experiments indicate that KIT^{Val-814} is capable of inducing neoplastic transformation of normal hematopoietic stem cells more efficiently than KIT^{Gly-559} ³.

Furthermore, recent surveys on human leukemias suggested the presence of Asp⁸¹⁶ to Val mutation, corresponding to the mouse Val⁸¹⁴ mutation, of *c-kit* in a fraction of human hematological malignancies (24). Molecular identification of the downstream targets of the constitutively activated KIT will provide important insights not only into fundamental mechanisms regulating enzymatic activity and expression of KIT but also into novel signaling events associated with tumorigenesis.

REFERENCES

1. Yarden, Y., Kuang, W. J., Yang-Feng, T., Coussens, L., Munemitsu, S., Dull, T. J., Chen, E., Schlessinger, J., Francke, U., and Ullrich, A. (1987) *EMBO J.* **6**, 3341–3351
2. Qiu, F. H., Ray, P., Brown, K., Barker, P. E., Jhanwar, S., Ruddle, F. H., and Besmer, P. (1988) *EMBO J.* **7**, 1003–1011
3. Carl-Henrik, H. (1995) *Cell* **80**, 213–223
4. Alastair, D. R., and Alan, B. (1991) *Genes and Phenotypes*, Vol. 3, pp. 105–133, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
5. Furitsu, T., Tsujimura, T., Tono, T., Ikeda, H., Kitayama, H., Koshimizu, U., Sugahara, H., Butterfield, J. H., Ashman, L. K., Kanayama, Y., Matsuzawa, Y., Kitamura, Y., and Kanakura, Y. (1993) *J. Clin. Invest.* **92**, 1736–1744
6. Tsujimura, T., Furitsu, T., Morimoto, M., Kanayama, Y., Nomura, S., Matsuzawa, Y., Kitamura, Y., and Kanakura, Y. (1995) *Int. Arch. Allergy Appl. Immunol.* **106**, 377–385
7. Tsujimura, T., Furitsu, T., Morimoto, M., Isozaki, K., Nomura, S., Matsuzawa, Y., Kitamura, Y., and Kanakura, Y. (1994) *Blood* **83**, 2619–2626
8. Steven, K., Anne, M. Q., and Tonny, H. (1988) *Science* **241**, 42–52
9. Ogawa, M., Matsuzaki, Y., Nishikawa, S., Hayashi, S., Kunisada, T., Sudo, T., Kina, T., Nakauchi, H., and Nishikawa, S. (1991) *J. Exp. Med.* **174**, 63–71
10. Kanakura, Y., Druker, B., Cannistra, S. A., Furukawa, Y., Torimoto, Y., and Griffin, J. D. (1990) *Blood* **76**, 706–715
11. Mizushima, S., and Nagata, S. (1990) *Nucleic Acids Res.* **18**, 5322
12. Graham, F. L., Smith, J., Russel, W. C., and Nairn, R. (1977) *J. Gen. Virol.* **36**, 59–74
13. Higuchi, R., Krummel, B., and Saiki, R. K. (1988) *Nucleic Acids Res.* **16**, 7351–7367
14. Southern, P. J., and Berg, P. (1982) *J. Mol. Appl. Genet.* **1**, 327
15. Majumder, S., Brown, K., Qiu, F. H., and Besmer, P. (1988) *Mol. Cell. Biol.* **8**, 4896–4903
16. Miyazawa, K., David, A. W., Gotoh, A., Nishimaki, J., Broxmeyer, H. E., and Toyama, K. (1995) *Blood* **85**, 641–649
17. Kitayama, H., Kanakura, Y., Furitsu, T., Tsujimura, T., Oritani, K., Ikeda, H., Sugahara, H., Mitsui, H., Kanayama, Y., Kitamura, Y., and Matsuzawa, Y. (1995) *Blood* **85**, 790–798
18. Hashimoto, K., Tsujimura, T., Moriyama, Y., Yamatodani, A., Kimura, M., Tohya, K., Morimoto, M., Kitayama, H., Kanakura, Y., and Kitamura, Y. (1996) *Am. J. Pathol.*, in press
19. Li, W., and Stanley, E. R. (1991) *EMBO J.* **10**, 277–288
20. Chen, W. S., Lazar, C. S., Lund, K. A., Welsh, J. B., Chang, C. P., Walton, G. M., Der, C. J., Wiley, H. S., Gill, G. N., and Rosenfeld, M. G. (1989) *Cell* **59**, 33–43
21. Wells, A., Welsh, J. B., Lazar, C. S., Wiley, H. S., Gill, G. N., and Rosenfeld, M. G. (1990) *Science* **247**, 962–964
22. Bargmann, C. I., Hung, M. C., and Weinberg, R. A. (1986) *Cell* **45**, 649–657
23. Bargmann, C. I., and Weinberg, R. A. (1988) *EMBO J.* **7**, 2043–2052
24. Nagata, H., Worobec, A. S., Oh, C. K., Chowdhury, B. A., Tannenbaum, S., Suzuki, Y., and Metcalfe, D. D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 10560–10564

³ H. Kitayama, T. Tsujimura, I. Matsumura, K. Oritani, H. Ikeda, J. Ishikawa, M. Okabe, M. Suzuki, K. Yamamura, Y. Matsuzawa, Y. Kitamura, and Y. Kanakura, unpublished observations.