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 (KITDel-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 (KITVal-814 and KITTyr-814) and modestly activated substitution mutants (KITGly-814 and KITHis-814) were continuously degraded in the absence of SCF, whereas wild-type KIT (KITWild) 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) 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 phosphotransferase 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 assembly of a multicomponent complex that transmits a series of biochemical signals. The activated RTKs 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 S1 locus on mouse chromosome 10. A variety of loss-of-function mutations at either the KIT/SCF or S1 loci 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 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—Racombinant murine (rm) SCF was a gift of Kirin Brewery Co. Ltd. (Tokyo, Japan). Rat antinouse 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 phosphotyrosine 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 University).
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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 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 KITWild was scarcely phosphorylated on tyrosine in the absence of rmSCF. By contrast, all of the mutant-type KITs except KITCys814 had a 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 the mutant-type KITs exhibited higher levels of kinase activity than KITWild. Among these mutants, markedly elevated kinase activity was observed in KITTyr-814, KITVal-814, KITILE-814, KITHis-814, and KITTrp-814 (Fig. 1A). Furthermore, in accordance with previous data on KITVal-814 and KITTyr-814 (17), rmSCF had little or no effect on tyrosine phosphorylation and activation of the highly activated KITVal-814 and KITTyr-814 and the modestly activated KITHis-814 and KITHIs-814 (data not shown).

In addition to substitution mutants, we also made a deletion mutant lacking the Asp814 (KITDel-Asp814). In accordance with previous findings, SCF treatment led to a marked increase in tyrosine phosphorylation and kinase activity of KITWild (5–7).
Results of three experiments. 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<sup>Del-Asp-814</sup> 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 Asp<sup>814</sup> 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 Asp<sup>814</sup> was constantly lower than that of KIT<sup>Wild</sup> 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 Asp<sup>814</sup> 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 <sup>35</sup>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<sup>Wild</sup> stably expressed on the 293T cells with a half-life of >4 h. The stimulation with rmSCF caused the rapid degradation of KIT<sup>Wild</sup> at a rate of 4.2% min<sup>−1</sup> within the initial 10 min, and the half-time of KIT<sup>Wild</sup> was 24 min (Fig. 2). By contrast, the highly activated KIT<sup>Val-814</sup> and KIT<sup>Tyr-814</sup> 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<sup>Gly-814</sup> and KIT<sup>His-814</sup> 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 KIT<sup>Val-814</sup> and KIT<sup>Tyr-814</sup> were slightly accelerated (3–4% min<sup>−1</sup>) 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 Asp<sup>814</sup> 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<sup>Val-814</sup> mutant was introduced into cells of 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<sup>Val-814</sup> 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<sup>Tyr-814</sup> also produced large tumors at the injection sites in nude mice. These results suggest that the constitutively activating mutations of c-kit such as KIT<sup>Val-814</sup> and KIT<sup>Tyr-814</sup> 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 Asp<sup>814</sup> 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 Asp<sup>814</sup> was found to abolish tyrosine kinase activity of KIT even after stimulation with rmSCF. These results indicate that the Asp<sup>814</sup> 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 Asp<sup>814</sup> codon may yield aggressive oncoproteins capable of inducing cell transformations.

Our data also suggest that Asp<sup>814</sup> 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<sup>Val-814</sup> or c-kit<sup>Tyr-814</sup> cDNA, whereas the mature (∼145 kDa) form of KIT protein was predominant in cells transfected with c-kit<sup>Wild</sup> cDNA (5–7). Furthermore, surface expression of the activating KIT<sup>Val-814</sup> was significantly lower than that of KIT<sup>Wild</sup> after retroviral infection of c-kit<sup>Val-814</sup> and c-kit<sup>Wild</sup> cDNAs into murine IL-3-dependent cell lines, Ba/F3, FDC-P1 and IC-2 (17, 18). These results suggest that activating mutations of Asp<sup>814</sup> affected intracellular transport of mutant-type KIT. In addition to the insufficient transport of mutant-
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Furthermore, recent surveys on human leukemias suggested the presence of Asp<sup>B14</sup> to Val mutation, corresponding to the mouse Val<sup>B14</sup> 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 Phenoypes, 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., Matsuza, Y., Kitamura, Y., and Kanakura, Y. (1993) J. Clin. Invest. 92, 1736–1744
6. Tsujimura, T., Furitsu, T., Morimoto, M., Kanayama, Y., Nomura, S., Matsuza, Y., Kitamura, Y., and Kanakura, Y. (1995) Int. Arch. Allergy Appl. Immunol. 106, 377–385
7. Tsujimura, T., Furitsu, T., Morimoto, M., Itozaki, K., Nomura, S., Matsuzawa, Y., Kitamura, Y., and Kanakura, Y. (1994) Blood 83, 2619–2626
8. Steven, K., Anne, M. O., and Tony, H. (1988) Science 241, 42–52
9. Ogawa, M., Matsuzaka, Y., Nishikawa, S., Hayashi, S., Kinuia, T., Sudo, T., Kina, T., Nakachi, K., and Nishikawa, S. (1991) J. Exp. Med. 174, 63–71
10. Kanakura, Y., Druker, B., Cannon, 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 Sai, K. R. (1988) Nucl. 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, 2496–2503
16. Miyazawa, K., David, A. W., Gotot, A., Nishikawa, J., Broxmeyer, H. E., and Toyama, K. (1995) Blood 85, 641–649
17. Kijima, H., Kanda, Y., Furitsu, T., Tsujimura, T., Oritani, K., Ikeda, H., Sugahara, H., Mitsu, H., Kanayama, Y., Kitamura, Y., and Matsuza, Y. (1995) Blood 85, 790–798
18. Hashimoto, K., Tsujimura, T., Moriyama, Y., Yamashita, A., Kimura, M., Tohya, K., Morimoto, M., Kitamura, Y., Kanakura, Y., and Kitamura, Y. (1998) 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., Wobiec, 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. Kitayama, T., Tsujimura, T., Matsuzawa, K., Oritani, H., Ikeda, J., Ishikawa, M., Okabe, M., Suzuki, K., Yamamura, Y., Matsuzawa, Y., Kitamura, and Y. Kanakura, unpublished observations.
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