Expression of Isoforms of the Human Receptor Tyrosine Kinase c-kit in Leukemic Cell Lines and Acute Myeloid Leukemia

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Because mutations in receptor tyrosine kinases may contribute to cellular transformation, studies were undertaken to examine c-kit in human leukemia. Isoforms of c-kit have been characterized in the human megakaryoblastic leukemia cell line M-07. Deletion of the four amino acids Gly-Asn-Asn-Lys in the extracellular domain represents an alternatively spliced isoform that has been shown by others, in mice, to be associated with constitutive receptor autophosphorylation (Reith et al., EMBO J. 10:2451, 1991). Additional isoforms differ in the inclusion or exclusion of a serine residue in the interkinase domain, a region that contains the binding site for phosphatidylinositol 3-kinase. By RNase protection analysis, we have shown coexpression of the Gly-Asn-Asn-Lys+ and Gly-Asn-Asn-Lys- isoforms, with dominance of the Gly-Asn-Asn-Lys− isoform, in normal human bone marrow, normal melanocytes, a range of tumor cell lines, and the blasts of 23 patients with acute myeloid leukemia. Analysis of transcripts for the Ser+ and Ser- isoforms also showed coexpression in all normal and leukemic cells examined. The ratios of isoform expression for both the Gly-Asn-Asn-Lys and Ser variants were relatively constant, providing no evidence in the tumors examined that upregulation of one isoform contributes to the neoplastic process.

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The proto-oncogene c-kit encodes a transmembrane tyrosine kinase that is structurally similar to the receptors for platelet-derived growth factor and macrophage colony-stimulating factor. These receptors share a number of common features including a glycosylated extracellular domain containing 5 Ig-like repeats, a single hydrophobic transmembrane domain, and a cytoplasmic region containing a split tyrosine kinase catalytic domain. It has been shown that c-kit and the murine dominant white spotting allele, W, are allelic. The ligand of the c-kit receptor has been identified and is encoded by the murine Steel (Sl) locus.

Mutations at the W locus in mice lead to intrinsic defects in stem cells of the hematopoietic, germ cell and melanocyte lineages, and c-kit expression has been shown in tissues affected by these mutations. Human c-kit mRNA has been detected in placenta, bone marrow, and cell lines derived from a glioblastoma and an erythroleukemia. Expression of human c-kit has also been shown in primary myeloid leukemic blast cells, but not in cells from patients with acute lymphoblastic leukemia. Human activated T lymphocytes and leukemic cell lines of granulocytic, monocytic, and lymphoid origin do not express detectable levels of c-kit.

A number of abnormalities within the W locus have been molecularly characterized, and the different W alleles vary in the severity of the phenotype they confer. The mildly dominant, homozygous viable alleles W44 and W57 express reduced levels of an apparently normal c-kit protein. In contrast, c-kit defects conferred by the moderately dominant homozygous alleles W41 W59 and W60 or the homozygous lethal alleles W59 and W62 have been shown to cause sense mutations within the kinase domain of the receptor. The homozygous lethal W mutation is the result of a 78 amino acid deletion that includes the transmembrane domain of c-kit.

Recently, abnormalities in human c-kit have been shown in individuals with piebaldism. In one study, a Gly→Arg substitution at codon 664, within the kinase domain, was shown to be linked to the piebald phenotype in the proband’s family. A heterozygous deletion that encompassed the entire coding region for c-kit was found in another subject with piebald trait.

Activating mutations in receptor tyrosine kinases that allow constitutive tyrosine kinase activity in the absence of ligand have been described and reviewed. It is thought that these mutations may result in enhancement of ligand-independent dimerization and cross-phosphorylation. In this study, we initially sought to determine whether mutations within human c-kit might be present in a human leukemic cell line. In this report we show that the human megakaryoblastic leukemia cell line M-07 expresses isoforms of c-kit and that these isoforms are also expressed in normal tissues, other tumor cell lines, and the blasts from patients with acute myeloid leukemia (AML). One of these isoforms is similar to that described in mouse where a 12-bp sequence at the 3'-end of the exon upstream of the exon encoding the transmembrane domain of c-kit, has been shown to be alternatively spliced. The second isoform has not been previously described and results from alternative 3' splice acceptor site usage involving exon 15 that has recently been shown to encode part of the interkinase domain of c-kit.

Materials and Methods

Leukemic cells, bone marrow, and melanocytes. Leukemic blast cells were obtained from diagnostic samples of peripheral blood or bone marrow, and normal bone marrow was obtained from healthy volunteers. Mononuclear cells were separated on Ficoll-Hypaque (Sigma Chemical Co, St Louis, MO). Human melanocytes and the Cancer Society of New Zealand, the Leukaemia and Blood Foundation of New Zealand, and the Cancer Society of New Zealand, Inc.

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cytes were grown from neonatal foreskin specimens according to an established protocol. All samples were provided with informed consent, obtained according to protocols approved by the Auckland Area Health Board Ethics Committee (Auckland, New Zealand).

**Human cell lines.** M-07 is a factor-dependent cell line that is maintained in human interleukin 3 (IL-3) and was established from the peripheral blood of an infant with acute megakaryoblastic leukemia. TF-1 is a factor-dependent cell line established from a patient with AML (French-American-British [FAB] subtype, M2) that had evolved from a myelodysplastic syndrome. These 3 cell lines were maintained at a cell density of 3 × 10^5 mL in the presence of 100 U/mL of human IL-3 in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mmol/L L-glutamine, and 10% heat-inactivated fetal calf serum (Life Technologies Ltd, Auckland, New Zealand). Cultures were incubated at 37°C in the presence of 10% CO2 in air.

**Human cell lines.** Isolation of cDNA clones encoding c-kit from an M-07 library. A cDNA library, constructed from M-07-derived RNA, was screened on nitrocellulose filters with a 50-mer oligonucleotide probe, complementary to part of the extracellular domain of human placental c-kit. Hybridization was performed at 65°C in a solution that contained 4X SSC (1X SSC; 150 mmol/L sodium chloride/15 mmol/L sodium citrate, pH 7.4), 5X Denhardt's reagent, 0.5% sodium dodecyl sulphate (SDS), and 100 μg/mL salmon sperm DNA. Filters were washed at 65°C in 2X SSC that contained 0.1% SDS. Twenty-five clones that hybridized to the c-kit probe were selected and analyzed further. One 5-kb clone was completely sequenced using the di-deoxy chain termination method, and 4 additional clones were sequenced through the region flanked by the 12- and 3-bp deletions.

**RNase protection analysis.** Total RNA was prepared from cells by acid guanidinium isothiocyanate extraction. Poly(A)+ RNA was selected from total RNA by oligo(dT) cellulose chromatography. RNase protection analysis was performed by hybridization of 2.5 μg of poly(A)+ RNA or 10 μg of total RNA to RNA probes that encoded sequences of human placental c-kit and /3 actin, overnight at 52°C. RNase digestion was performed with RNase T1 (1.75 μg/mL) and RNase A (35 μg/mL) at 37°C for 1 hour. The reaction was stopped with proteinase K (333 μg/mL) and SDS (0.3%). The protected products were run on a 6% urea/acrylamide gel, and the autoradiograph exposed at -70°C.

![Diagram](attachment:image.png)

**Fig 1.** Identification of isoforms of c-kit expressed in the M-07 cell line. The positions of the 4 amino acid and single amino acid deletions, when compared with the sequence for human placental c-kit, are shown for 1 clone (25B) isolated from an M-07 cDNA library. The domains of human c-kit are shown; the solid area represents the transmembrane domain and the shaded areas in the cytoplasmic region define the split kinase domain, bisected by the interkinase region. The Gly-Asn-Asn-Lys* isoform: A 12-bp segment in the extracellular domain, encoding Gly-Asn-Asn-Lys (amino acids 510 to 513 of the published human placental c-kit sequence), was absent in all 5 M-07 cDNA clones analyzed. The consensus 5 splice junction sequences AAG are shown as shaded areas flanking the 12-bp segment. The Ser- isoform: In the interkinase region of the cytoplasmic domain, a 3-bp sequence that encoded Ser at amino acid position 715 was absent in 2 of 5 M-07 cDNA clones analyzed.
C-KIT ISOFORM EXPRESSION

The probe for analysis of the Gly-Asn-Asn-Lys isoform was derived from nucleotides 1297 to 1759 of the human placental sequence. It included nucleotides that encode amino acids 510 to 513 (Gly-Asn-Asn-Lys) of the c-kit protein. The probe template was obtained by amplification of cDNA that had been derived from RNA of the human embryonal carcinoma cell line NT2/D1, using the polymerase chain reaction and was subcloned into pSP72 (Promega, Madison, WI). In an RNase protection assay, the free probe yielded a 520-nucleotide (nt) band, and c-kit transcripts that contained the 12-bp sequence protected a fragment of 463 nt. Partially protected fragments, corresponding to the presence of transcripts that lacked the 12-bp sequence, yielded bands of 252 nt and 199 nt.

A probe for analysis of the 3-bp deletion encompassing nt 2164 to 2166 of human placental c-kit was derived by subcloning an Apa I-Bgl II fragment (nt 2100 to 2238) from an M-07 cDNA clone that contained the 3 nt that encode a serine residue at position 715 of the c-kit protein, into pSP72. In an RNase protection assay, the free probe yielded a 293-nt band, and c-kit transcripts that contained the 3-bp sequence protected a fragment of 234 nt. Partially protected fragments, corresponding to the presence of transcripts that lacked the 3-bp sequence, yielded bands of 152 nt and 79 nt. A riboprobe was also constructed from a Sal I-Sma I fragment of human β actin. The length of the free probe was 132 nt, and β-actin transcripts protected a 54-nt fragment.

RESULTS

Identification of isoforms of c-kit expressed in the M-07 cell line. Work to characterize c-kit in a human leukemic cell population initially established that the M-07 cell line expressed abundant RNA transcripts for c-kit as assessed by Northern analysis, expressed c-kit protein as determined by staining with the monoclonal antibody YB5.B8, and proliferated in response to SF factor, establishing that c-kit in this cell line was functional (data not shown). To further characterize c-kit in M-07 cells, 200,000 clones from an M-07 cDNA expression library were screened with a 50-mer oligonucleotide that encoded part of the extracellular domain of human placental c-kit. A total of 25 positive clones were selected for further analysis. Of these, 1 clone termed 25B was completely sequenced through the coding and noncoding regions. Two alterations from the published sequence for human placental c-kit were observed in this clone, and are shown in Fig 1.

In the region that encodes the extracellular domain, a sequence of 12 bp that encompasses the amino acids Gly-Asn-Asn-Lys was absent. An additional 4 clones from the M-07 cDNA library were partially sequenced through the region encompassing the 12-bp and 3-bp (see below) variations. All 4 independent clones were Gly-Asn-Asn-Lys. In the sequence of Yarden et al, the consensus 5′ splice junction sequence AAG flanks this 12-bp segment. It has been shown that these 12-bp reside at the 3′ end of the exon immediately upstream of the exon that encodes the transmembrane domain and that this region is alternatively spliced to yield products that are either Gly-Asn-Asn-Lys or Gly-Asn-Asn-Lys21.

Figure 1 also shows details of a second alteration in the sequence of c-kit within the M-07 cDNA clone 25B. A deletion in the cytoplasmic domain encompassed nt 2164 through 2166 of the sequence of the human placental c-kit, a region in the mid-segment of the split kinase domain that encodes a Ser residue at position 715. Analysis of the genomic sequence of human c-kit22,23 reveals that the 3′ splice junction involving exon 15 contains the repeated sequence AGC. As deduced from the cDNA sequence of human placental c-kit, the more 5′ AGC defines the intron-exon
boundary, whereas the second AGC encodes a Ser residue. Therefore, it is likely that the Ser\(^-\) transcript of c-kit results from alternative 3' splice acceptor site use. Two of 5 clones analyzed from the M-07 cDNA library showed this 3-bp deletion and were also Gly-Asn-Asn-Lys\(^+\).

Expression of the Gly-Asn-Asn-Lys isoforms of human c-kit in normal tissues, tumor cell lines, and AML blasts. To determine the pattern of expression of the Gly-Asn-Asn-Lys isoforms of human c-kit, RNase protection analysis was undertaken using a probe that differentiated between transcripts representative of the Gly-Asn-Asn-Lys\(^+\) (protected fragment of 463 nt) and Gly-Asn-Asn-Lys\(^-\) (protected fragments of 252 nt and 199 nt) isoforms, respectively. RNase protection analysis was performed with RNA from human bone marrow mononuclear cells, melanocytes (Fig 2), and the tumor cell lines M-07, HL60, K562, M-07, A172, TF-1, and GI-6 cell lines (A) and with total RNA from the cell lines HEL and M-07 (B). Details of the markers, probes, and controls are as described for Fig 2.

Table 1. Expression of c-kit in Human AML

<table>
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<tr>
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Abbreviations: MDS, myelodysplastic syndrome; MPD, myeloproliferative disorder.

\(^*\) FAB subtypes for AML follow standard definitions.

\(^\dagger\) The presence (+) or absence (−) of mRNA transcripts for c-kit as determined in an RNase protection assay.

Expression of the Ser isoform of human c-kit in bone marrow, leukemic cell lines, and AML blasts. Analysis of the pattern of expression of the Ser isoforms of human c-kit was also performed using RNase protection. RNA from human bone marrow mononuclear cells, from the leukemic cell lines M-07, HEL, and TF-1, and from the blasts of 10 patients with AML was examined (Fig 5). The probe was designed so that a 234-nt fragment was protected by the Ser\(^+\) transcript, and fragments of 152 nt and 79 nt were protected by Ser\(^-\) transcripts. Coexpression of the Ser\(^+\) and Ser\(^-\) isoforms, with dominance of the Ser\(^+\) isoform, was shown in all cells that expressed c-kit.
Fig 4. Expression of the Gly-Asn-Asn-Lys isoforms of c-kit in human AML blasts. RNase protection analysis was performed with total RNA isolated from the blast cells of patients with a range of FAB subtypes of AML. Results of analysis of a representative series of samples are shown. Details of the markers, probes, and controls are as described for Fig. 2.

DISCUSSION

In this study we describe the identification and the patterns of expression of isoforms of the human receptor tyrosine kinase c-kit. These involve the presence or absence of the sequence Gly-Asn-Asn-Lys in a region of the extracellular domain adjacent to the transmembrane domain and the presence or absence of a Ser residue in the interkinase domain.

Isoforms that vary with respect to the Gly-Asn-Asn-Lys sequence have been reported previously in studies of murine c-kit and have been shown to result from alternative splicing of the c-kit gene.20,21 This Asn-rich segment involved has been termed NRI (Asn-rich insert).20 By analyzing the intron-exon structure of the murine c-kit gene, it was shown that the 12-bp sequence that encodes Gly-Asn-Asn-Lys lies at the 3' end of an exon that is immediately upstream of the exon that encodes the transmembrane region.21 This 12-bp segment is flanked by the splice donor sequence AAG. It is of interest that the 5' splice junction involved in the formation of the isoforms is the same as that proposed to be used in the aberrant RNA splicing associated with the original W mutation.15 Analysis of genomic sequences derived from cells of the W mouse has shown that there is a single base substitution (GT → AT) at the 5' splice donor site of the exon that encodes the transmembrane domain, which results in the skipping of either one or two exons.21

Murine c-kit Gly-Asn-Asn-Lys isoforms have been identified in placenta, brain, and in IL-3–dependent mast cells derived from adult and fetal mouse tissues.20,21 The predominant isoform in these tissues was shown to be the Gly-Asn-Asn-Lys splice variant. It was also noted that both isoforms were expressed in human glioblastoma and erythroleukemia cell lines.20 When the Gly-Asn-Asn-Lys isoforms were transiently expressed in COS-1 cells, both were able to be activated by soluble SF factor and were associated with the same set of substrates including phosphatidylinositol 3-kinase (PI 3-kinase) and phospholipase C,1 (PLC,1).20 Neither isoform was associated with guanosine triphosphatase activating protein (GAP) or the GAP-associated proteins p62 and p190. However, in the absence of ligand, the 2 isoforms had distinct signal transduction activities. The Gly-Asn-Asn-Lys– variant but not the Gly-Asn-Asn-Lys+ showed constitutive tyrosine phosphorylation that was accompanied by a low constitutive level of association with PI 3-kinase and PLC,1. This suggested diversity in normal c-kit signaling pathways.

This study shows that the Gly-Asn-Asn-Lys isoforms also exist in normal human tissues (bone marrow and melanocytes). The pattern of expression is similar to that observed in mouse20,21 in that the normal tissues that expressed c-kit showed dominance of the Gly-Asn-Asn-Lys+ isoform. Because all 5 cDNA clones derived from M-07 cells were Gly-Asn-Asn-Lys+ and because this isoform had been shown to be associated with increased constitutive phosphorylation, we proceeded to examine expression of the 2 isoforms in a range of human tumor cell lines and in AML blasts. Initially, it was found that the M-07 cell line expressed functional c-kit receptors, as has been shown by others.30,31 As in normal tissues, all tumor cells that expressed c-kit were found to contain both isoforms, with dominance of the Gly-Asn-Asn-Lys– variant. The ratio of the 2 isoforms remained relatively constant in all tumor cell lines and AML blasts examined and showed no evidence of upregulation of the Gly-Asn-Asn-Lys+ isoform.
Fig 5. Expression of the Ser isoform of c-kit in bone marrow, leukemic cell lines, and AML blasts. RNase protection analysis was performed with total RNA from normal human bone-marrow mononuclear cells, cell lines HEL, TF-1, and M-07, and from the blasts of patients with a range of AML subtypes. Transfer RNA (10 μg) was analyzed as a control, and bands shared by this and the sample lanes were regarded as nonspecific. The markers were pBR322 digested with Msp I. The size of the free c-kit probe, which contained sequences of the polylinker of pSP72, was 293 nt. A fully protected fragment representing the Ser+ isoform was 234 nt in length, and partially protected fragments representing the Ser- isoform were 152 nt and 79 nt. The free β-actin probe was 132 nt in length and the 54-nt β-actin probe fragment is shown for each lane as a control for the amount of RNA analyzed.

In this analysis of c-kit in de novo AML and transformed myelodysplastic or myeloproliferative disorders, expression of the receptor was shown in all but one case, a patient with M2 AML. This high frequency of c-kit expression observed in AML blasts and the lack of correlation between the level of expression and FAB subtype are in agreement with published studies.11-13 We describe additional isoforms of c-kit involving the presence or absence of a 3-bp sequence that encodes a Ser residue at position 715 in the mid-segment of the split kinase domain.

As described above, the Ser- isoforms are generated by alternative 3’ splice acceptor site usage. It is interesting that in the published sequence of murine c-kit,7 the nucleotides that encode this Ser residue in human cells, are absent. The preceding codon (TGT) in the mouse cDNA predicts that the more 5’ splice junction used in human does not exist in mouse. In v-kit,33 an Asn residue is encoded at the position equivalent to position 715 in human, and the sequence (AAC) suggests that the 3’ splice acceptor site for v-kit is equivalent to the upstream human c-kit acceptor site. The substitution of Asn for Ser is conservative. This study showed that the Ser+ and Ser- isoforms were coexpressed in
all normal and tumor tissues examined that expressed c-kit with dominance of the Ser+ isoform.

The functional significance of these Ser isoforms remains to be determined; however, it is noteworthy that single amino acid changes in the cytoplasmic domain of c-kit, with profound effects on receptor function, have been described in the W mouse developmental mutants W41, W55, W6, W7, and W42.13-15 It is possible that the single amino acid deletion in the split kinase domain may alter the signaling activity of this receptor variant.

One of the characteristics of the tyrosine kinase family of receptors is the presence of a split kinase domain that is bisected by a variable length hydrophilic amino acid insert. It has recently been shown that this 76 amino acid interkinase domain of human c-kit contains the binding site for PI 3-kinase and that phosphorylation of tyrosine residues within the kinase insert is essential for association with the effector protein.34 The site of variation at amino acid position 715 is close to putative tyrosine autophosphorylation sites located at amino acid positions 721 and 730. Furthermore, there is evidence that sequences flanking the phosphorylation site participate in recognition, in that the association of c-kit with PI 3-kinase could not be inhibited by antibodies to phosphotyrosine alone.34 It is possible that inclusion or exclusion of a serine residue at position 715 may alter PI 3-kinase binding, and work will be required to explore this further.

The biological roles of the Gly-Asn-Asn-Lys+/- and Ser+/- isoforms of c-kit remain unclear. The finding that the Gly-Asn-Asn-Lys isoforms are conserved between mouse and human and have been shown to possess different signal transduction properties in the absence of ligand suggests a biologic role for these receptor variants. Further analysis of the phosphorylation differences and abilities of the isoforms to interact with cellular substrates should provide additional understanding of the role of c-kit variants during normal development and possibly in the process of oncogenesis.

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