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(54) **LIGAND FOR THE C-KIT RECEPTOR AND METHODS OF USE THEREOF**

(76) Inventors: **PETER BESMER**, NEW YORK, NY (US); **KARL NOCKA**, LEONIA, NJ (US); **JOCHEN BUCK**, NEW YORK, NY (US)

Correspondence Address:
JOHN P. WHITE
COOPER AND DUNHAM, LLP
1185 AVENUES OF AMERICAS
NEW YORK, NY 10036 (US)

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(52) **U.S. Cl.** **530/350**; 514/2

(57) **ABSTRACT**

This invention provides an isolated nucleic acid molecule which encodes an amino acid sequence corresponding to a c-kit ligand (KL) and a purified c-kit ligand (KL) polypeptide, or a soluble fragment thereof. A pharmaceutical composition which comprises the c-kit ligand (KL) purified by applicants or produced by applicants' recombinant methods and a pharmaceutically acceptable carrier is further provided as well as methods of treating patients which comprise administering to the patient the pharmaceutical composition of this invention.

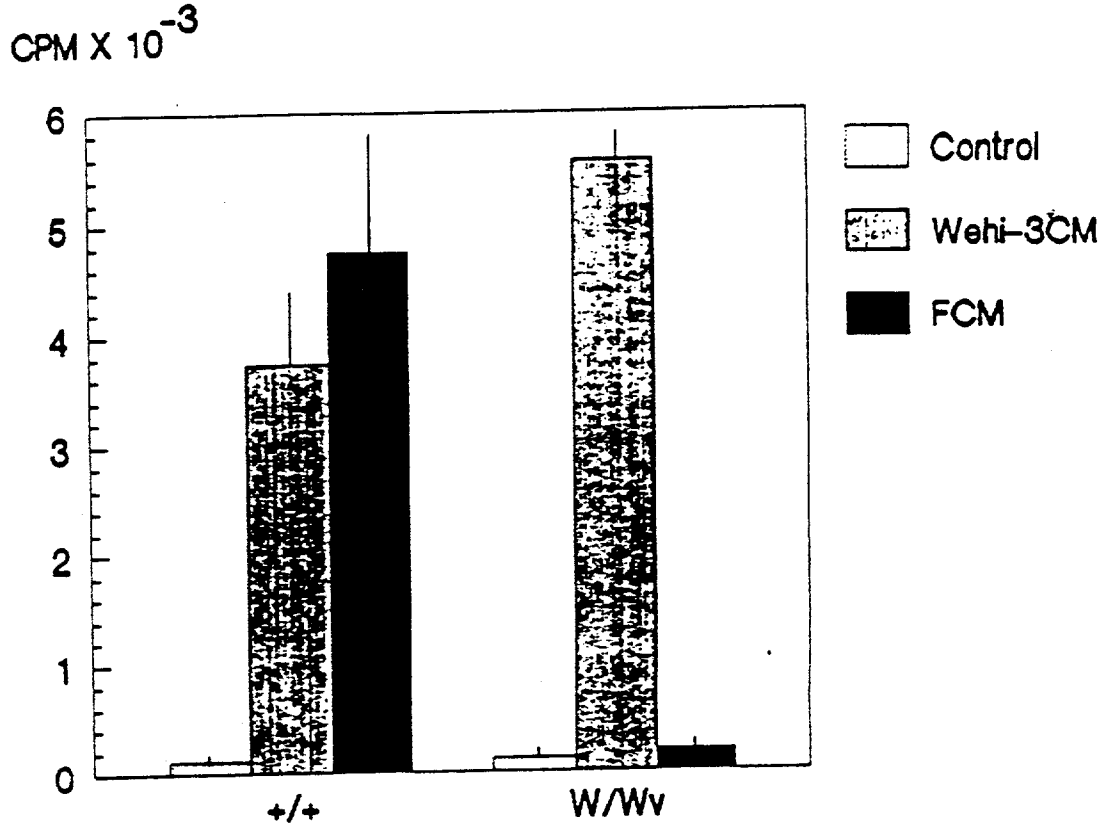

FIGURE 1

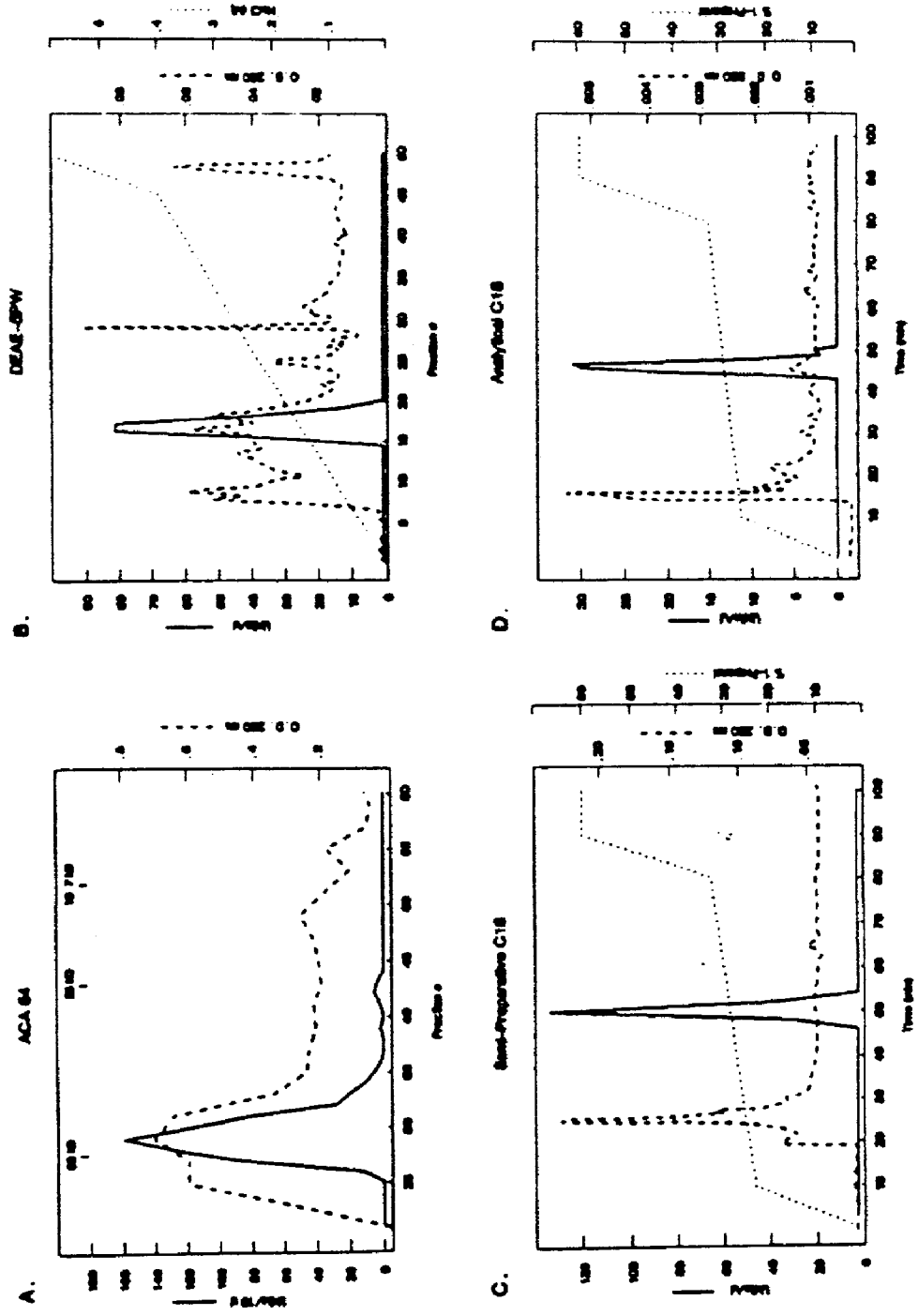

FIGURE 2

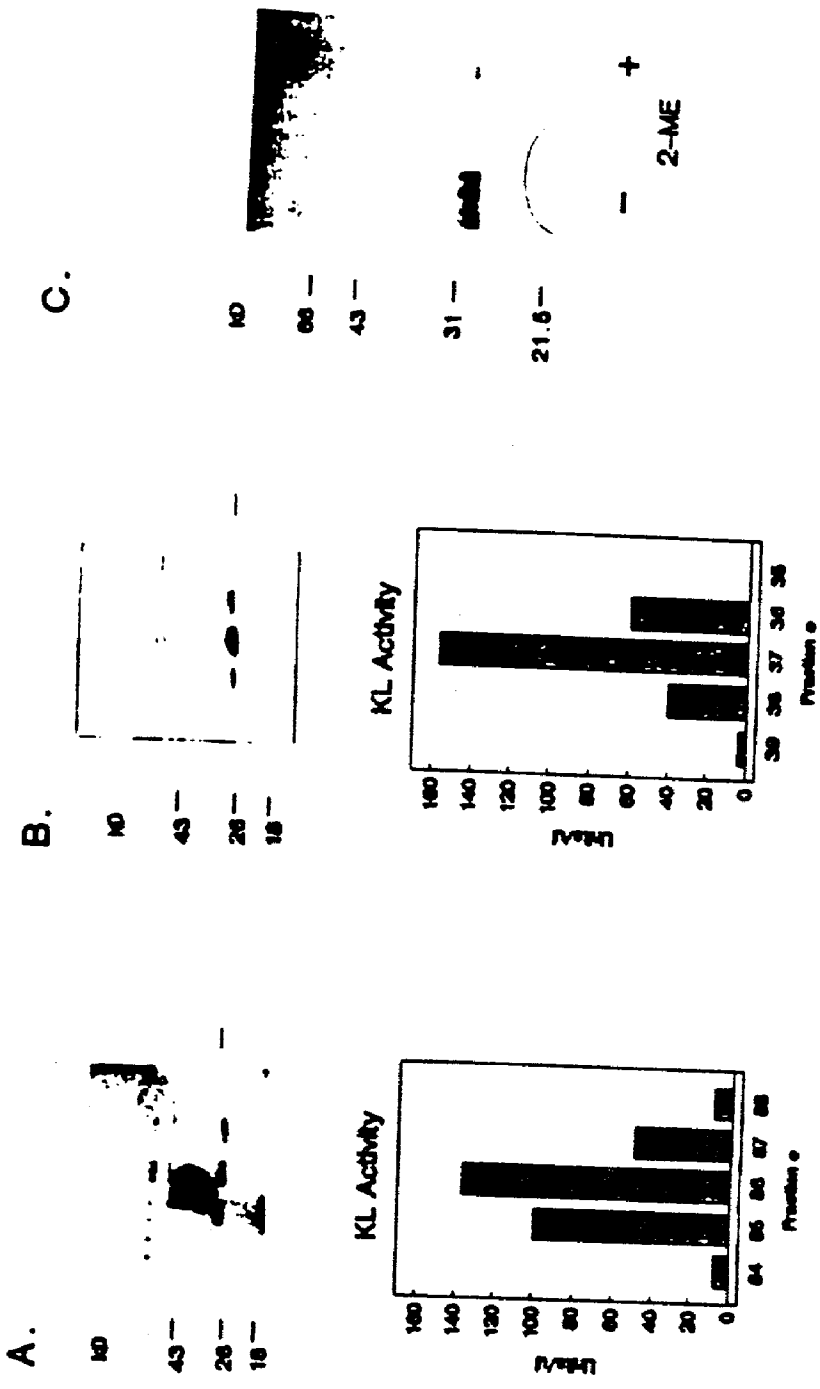
C.
2-ME
+
-
92
66
43
31
21.5
B.
KL Activity
92
43
26
18
A.
KL Activity
92
43
26
18

FIGURE 3

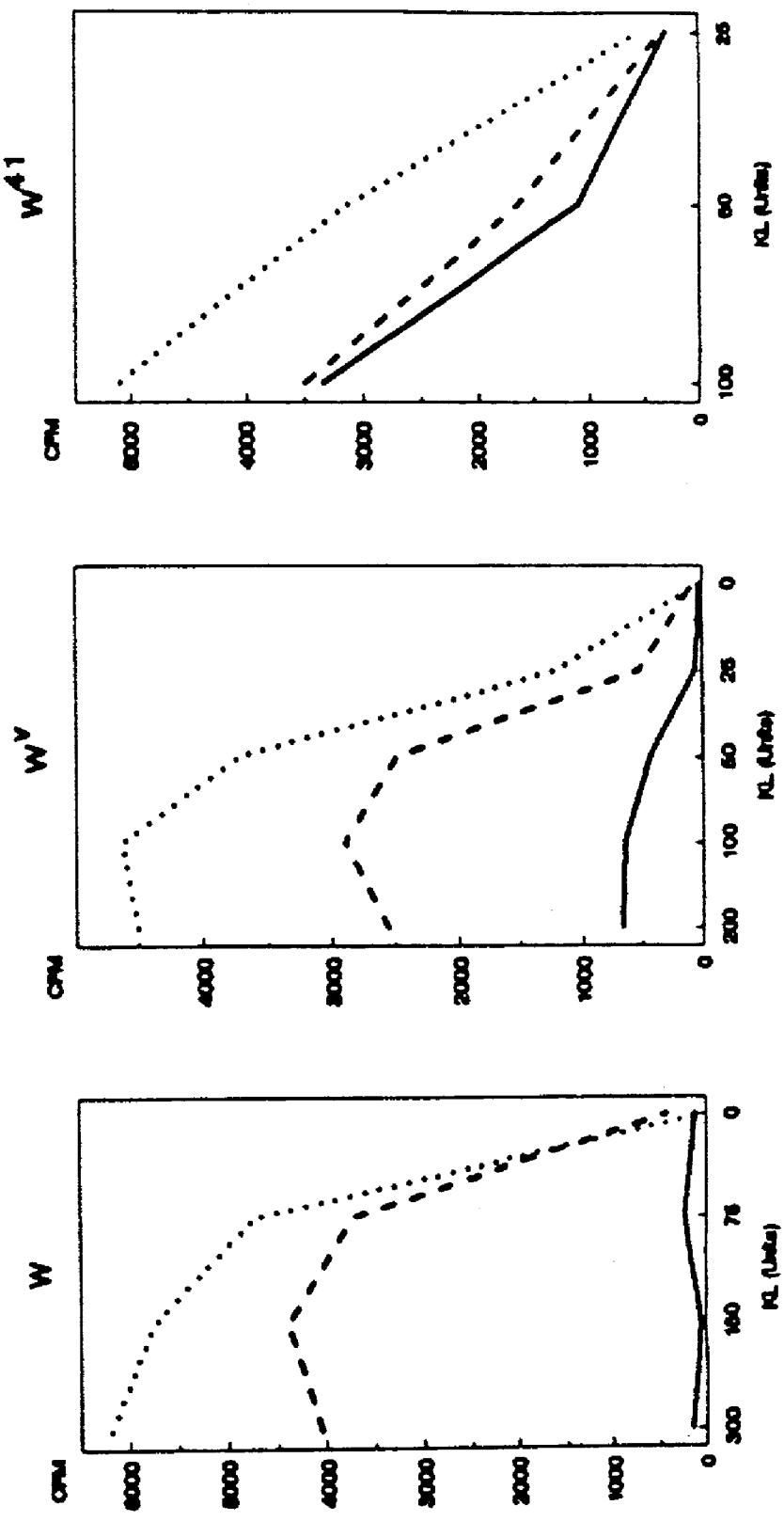
W^4.1
CPM
8000
6000
4000
3000
2000
1000
0
100
50
25
KL (Joules)
W^V
CPM
4000
3000
2000
1000
0
200
100
50
25
0
KL (Joules)
W
CPM
8000
6000
4000
3000
2000
1000
0
300
150
75
0
KL (Joules)

FIGURE 4

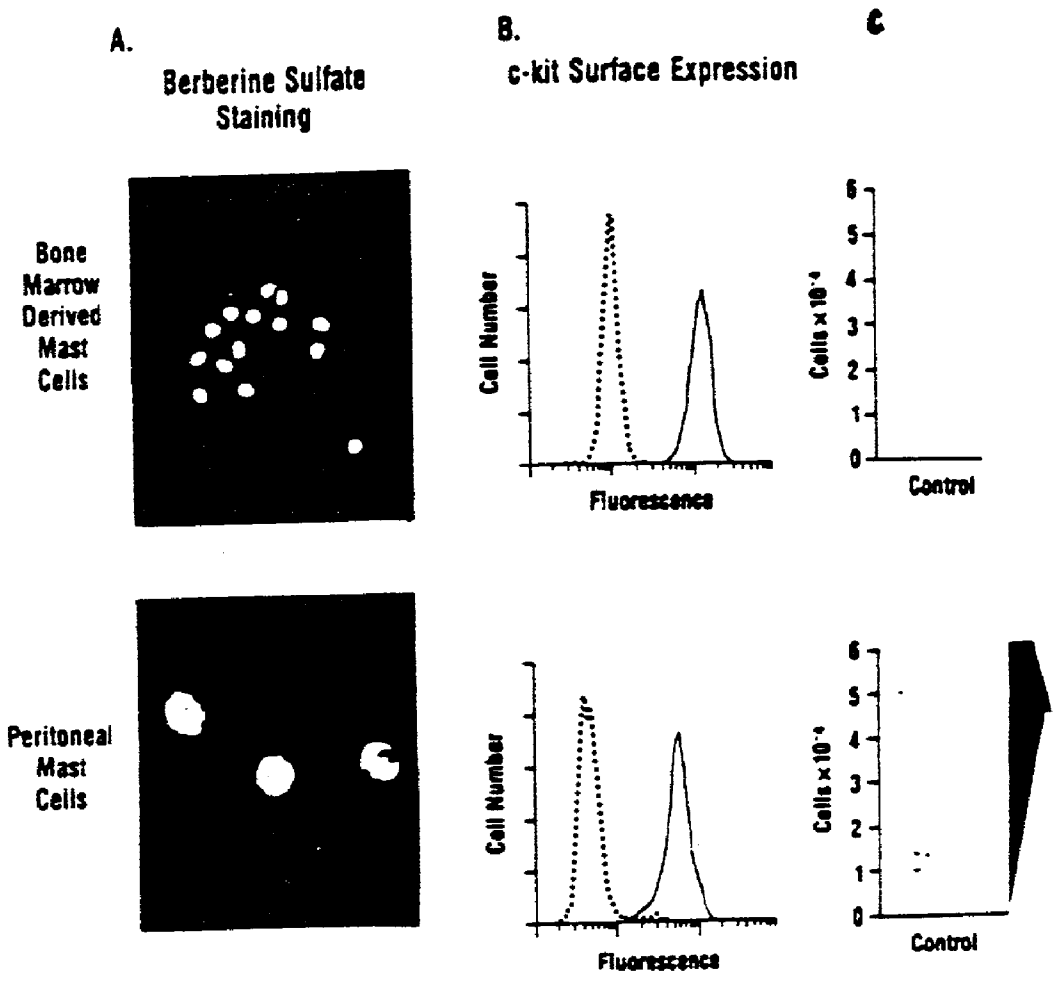
A.
Berberine Sulfate Staining
B.
c-kit Surface Expression
C
Bone Marrow Derived Mast Cells
Peritoneal Mast Cells
Cell Number
Fluorescence
Cells x 10⁻⁴
Control
0
1
2
3
4
5
6

FIGURE 5

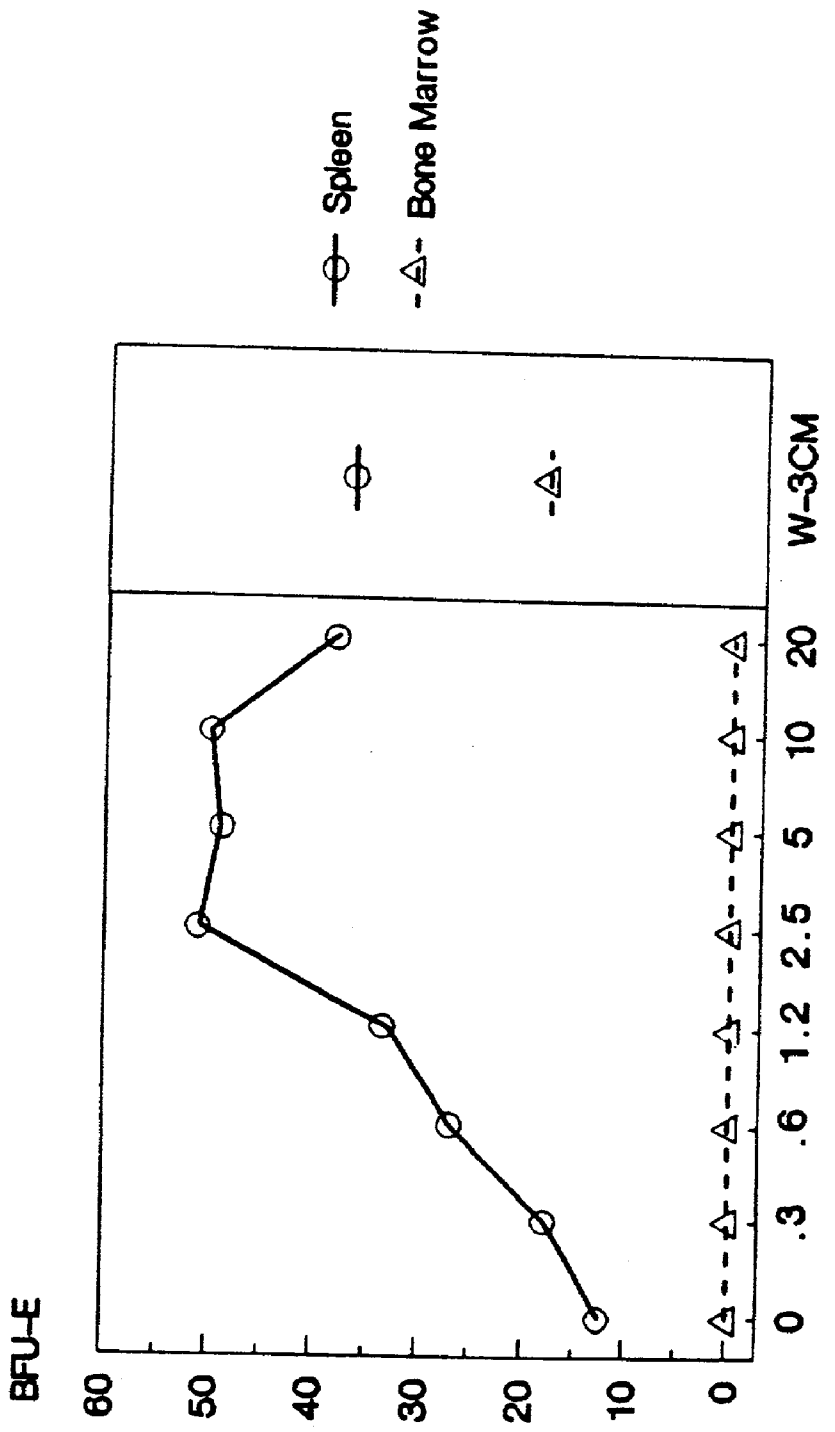
Spleen
Bone Marrow
BFU-E
80
50
40
30
20
10
0
0 .3 .6 1.2 2.5 5 10 20
KL (ng/ml)
W-3CM

FIGURE 6

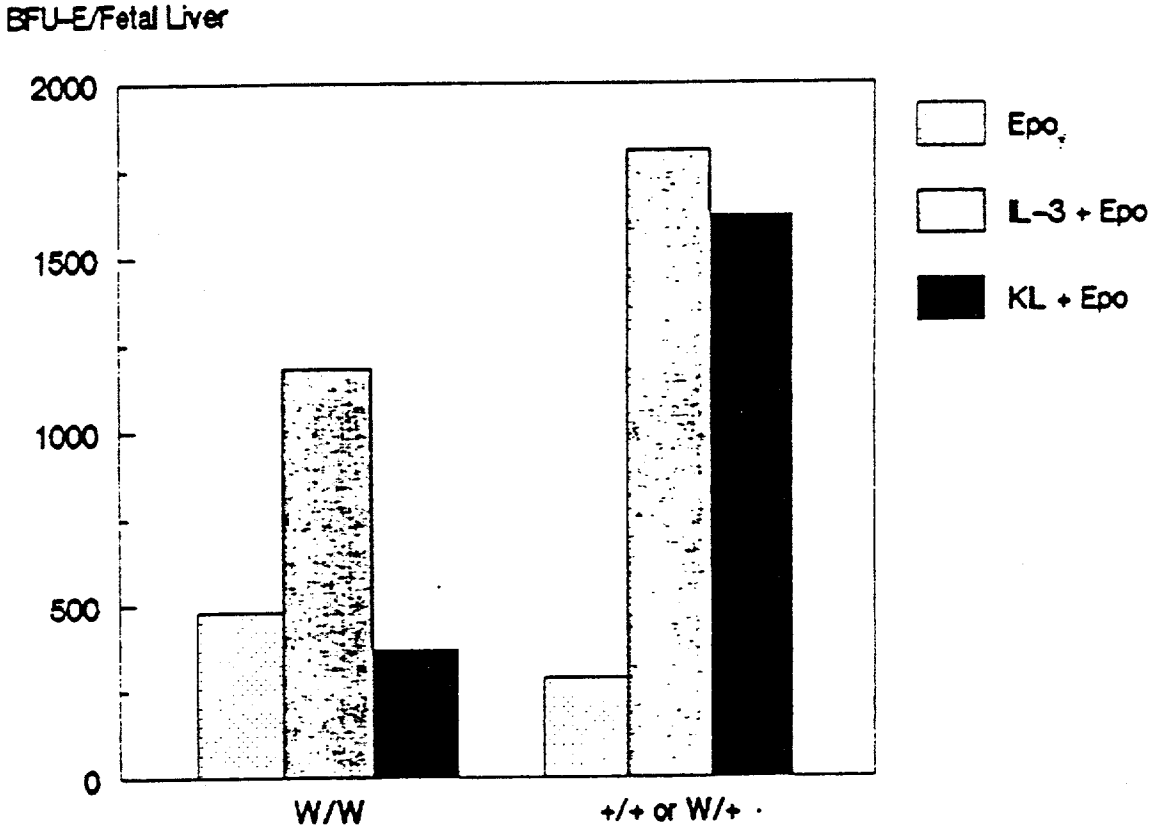

FIGURE 7

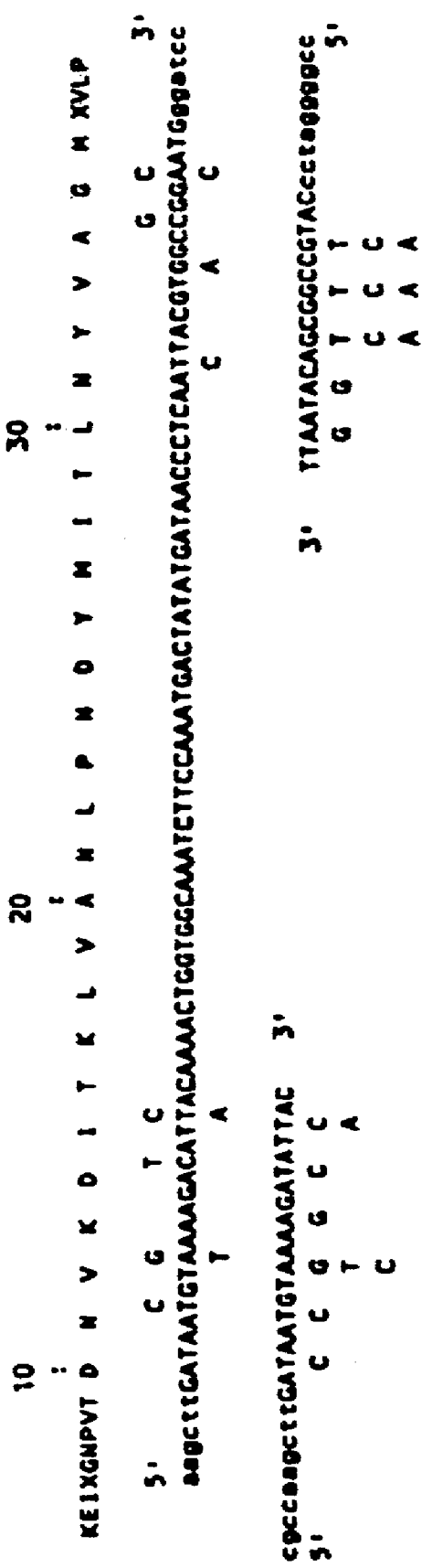
10
20
30
KE1XGNPVT D N V K D I T K L V A N L P N D Y M I T L N Y V A G M XVLP
5'
3'
3'
5'

FIGURE 8

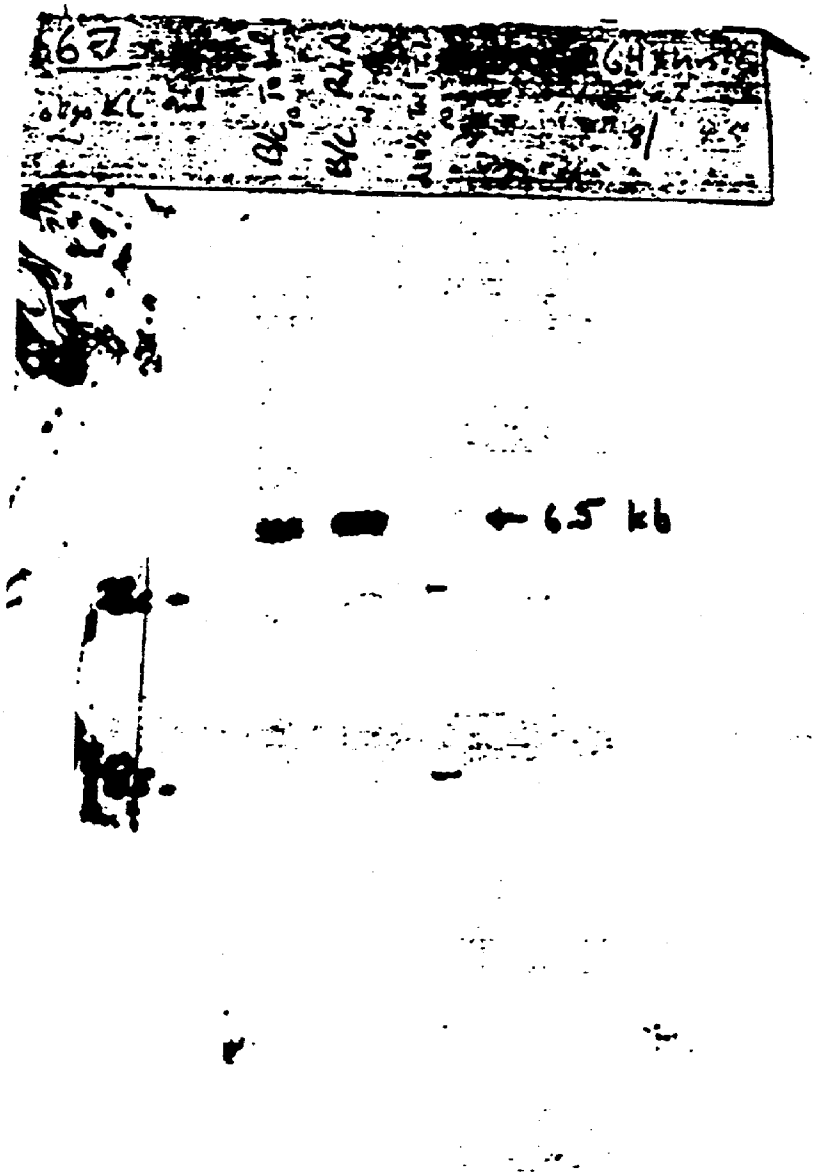

FIGURE 9

FIGURE 10

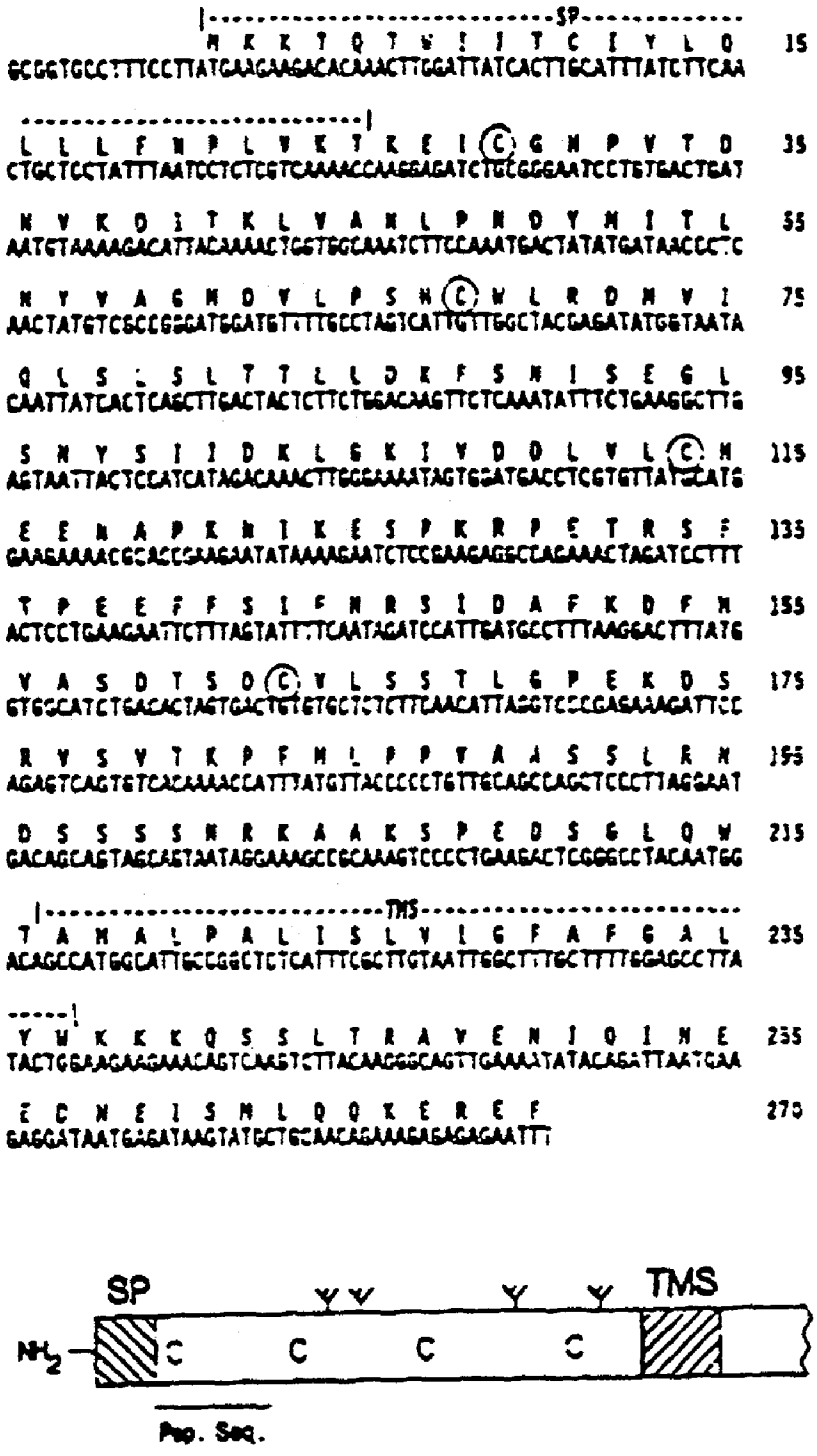

FIGURE 11

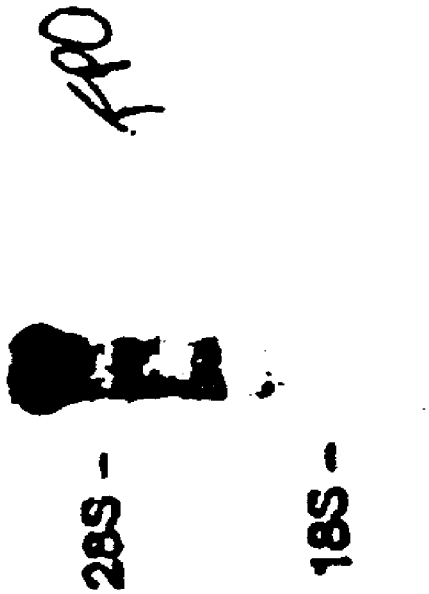

FIGURE 12

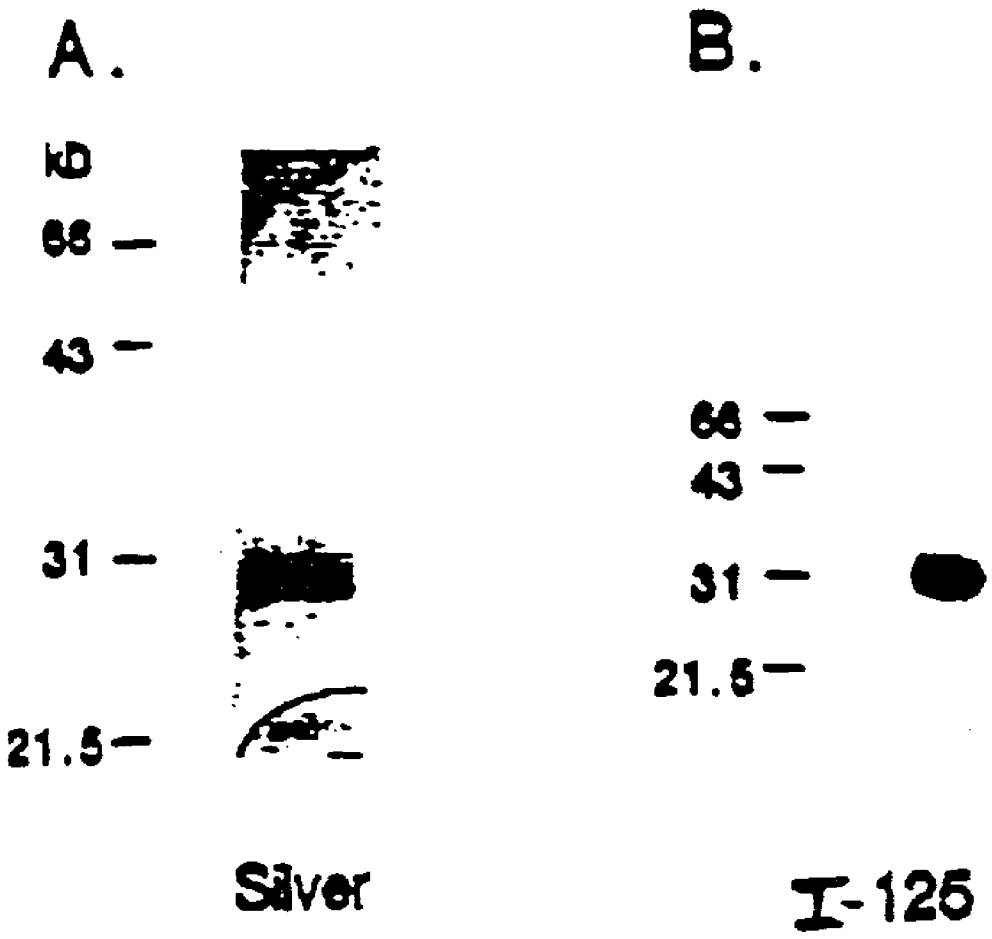

FIGURE 13

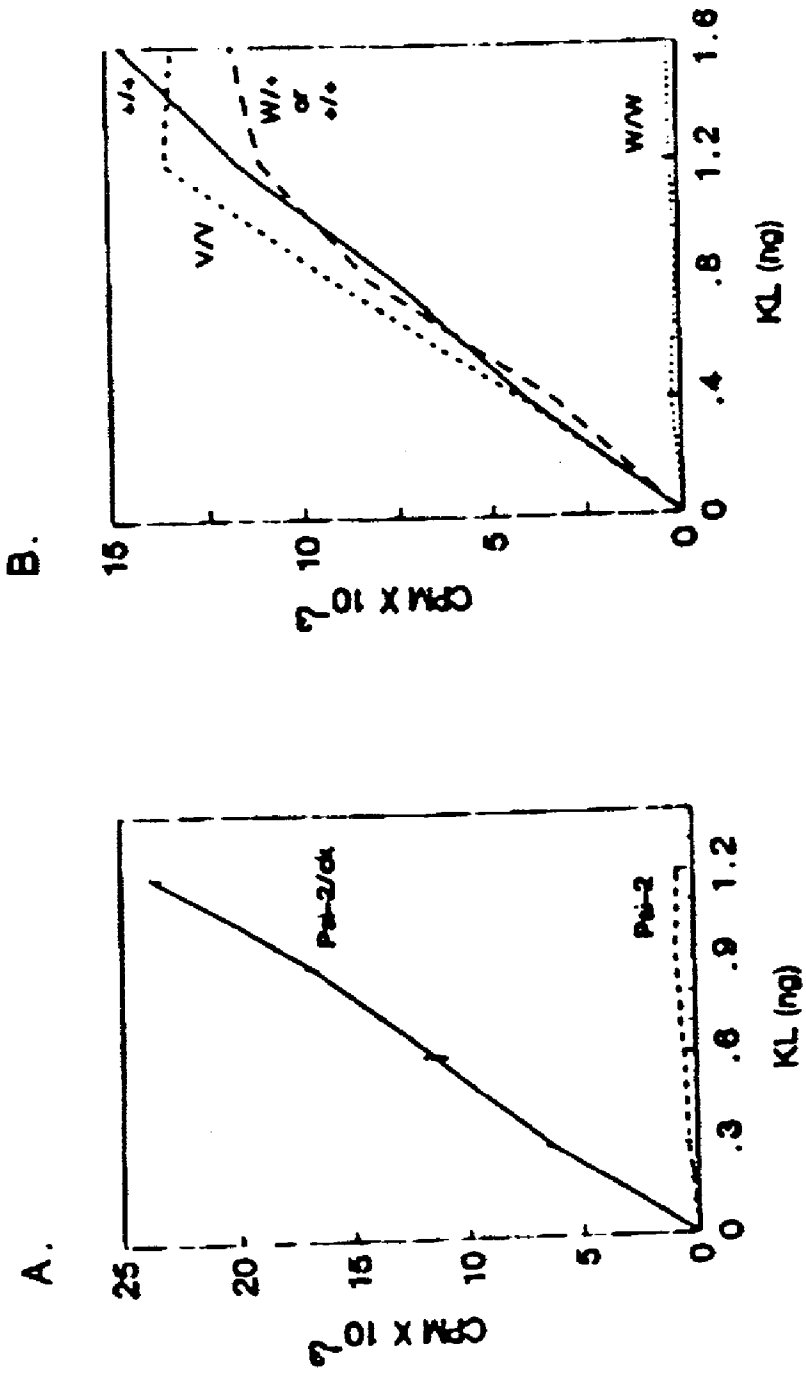
B.
15
10
5
0
0
.4
.8
1.2
1.8
KL (ng)
+/+
W/+ or +/+
V/V
W/W
A.
25
20
15
10
5
0
0
.3
.6
.9
1.2
KL (ng)
Pai-2/cl.
Pai-2

FIGURE 14

FIGURE 15

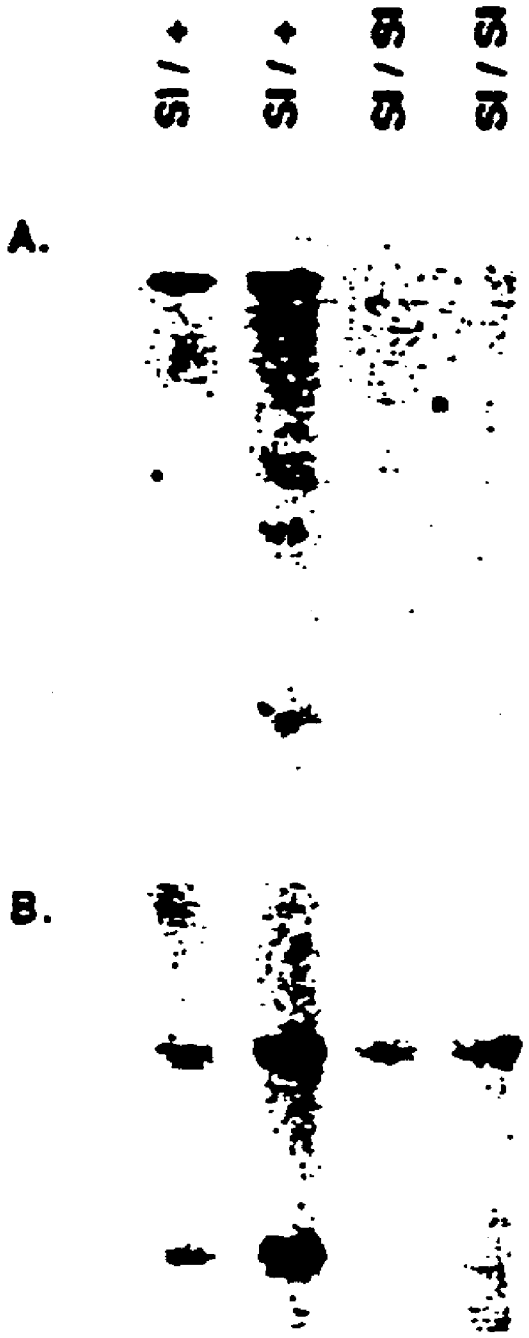

FIGURE 16

LIGAND FOR THE C-KIT RECEPTOR AND METHODS OF USE THEREOF

[0001] This application is a continuation-in-part application of U.S. Ser. No. 573,483, filed Aug. 27, 1990,1 the contents of which are hereby incorporated by reference into the present application.

[0002] The invention described herein was made in the course of work under Grant No. RO1-CA 32926 and ACS MV246D from the National Institute of Health and American Cancer Society, respectively. The United States Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0003] Throughout this application various publications are referred to within parenthesis. Full bibliographic citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures for these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

[0004] The c-kit proto-oncogene encodes a transmembrane tyrosine kinase receptor for an unidentified ligand and is a member of the colony stimulating factor-1 (CSF-1)—platelet-derived growth factor (PDGF)—kit receptor subfamily (Besmer et al., 1986, Qiu et al., 1988; Yarden et al., 1987; Majumder et al., 1988). c-kit was recently shown to be allelic with the white-spotting (W) locus of the mouse (Chabot et al., 1988; Geissler et al., 1988; Nocka et al., 1989). Mutations at the W locus affect proliferation and/or migration and differentiation of germ cells, pigment cells and distinct cell populations of the hematopoietic system during development and in adult life (Russell, 1979; Silvers, 1979). The effects on hematopoiesis are on the erythroid and mast cell lineages as well as on stem cells, resulting in a macrocytic anemia which is lethal for homozygotes of the most severe W alleles (Russell, 1970), and a complete absence of connective tissue and mucosal mast cells (Kitamura and Go, 1978). W mutations exert their effects in a cell autonomous manner (Mayer and Green, 1968; Russell, 1970), and in agreement with this property, c-kit RNA transcripts were shown to be expressed in targets of W mutations (Nocka et al., 1989). High levels of c-kit RNA transcripts were found in primary bone marrow derived mast cells and mast cell lines. Somewhat lower levels were found in melanocytes and erythroid cell lines.

[0005] The identification of the ligand for c-kit is of great significance and interest because of the pleiotropic effects it might have on the different cell types which express c-kit and which are affected by W mutations in vivo. Important insight about cell types which may produce the c-kit ligand can be derived from the knowledge of the function of c-kit/W. The lack of mast cells both in the connective tissue and the gastrointestinal mucosa of W/W^V mice indicated a function for c-kit in mast cell development. Mast cells derived from bone marrow (BMMC) are dependent on interleukin 3 (IL-3) and resemble mast cells found in the gastrointestinal mucosa (MMC) (Yung et al., 1981; Stevens and Austen, 1989). Connective tissue mast cells derived from the peritoneal cavity (CTMC) in vitro require both IL-3 and IL-4 for proliferation (Hamaguchi, et al., 1987; Tsuji, et al., 1990a). The interleukins IL-3 and IL-4 are well characterized hematopoietic growth factors which are produced by activated T-cells and by activated mast cells (Yung et al., 1981; Schrader, 1981; Smith and Rennick, 1986; Brown et al., 1987; Plaut et al., 1989). An additional mast cell growth factor has been predicted which is produced by fibroblasts (Levi-Schaffer et al., 1985). In the absence of IL-3, BMMC and CTMC derived from the peritoneal cavity can be maintained by co-culture with 3T3 fibroblasts (Levi-Schaffer et al., 1986; Dayton et al., 1988). However, BMMC from W/W^V mice as well as mice homozygous for a number of other W alleles are unable to proliferate in the fibroblast co-culture system in the absence of IL-3 (Fujita et al., 1988; Tan et al., 1990; Nocka et al., 1990). This suggested a function for the c-kit receptor in mature mast cells and implied that the ligand of the c-kit receptor is produced by fibroblasts. Huff and coworkers recently reported the stimulation of mast cell colonies from lymph node cells of mice infected with the nematode *Nippostronglyus brasiliensis* by using concentrated conditioned medium from NIH 3T3 fibroblasts (Jarobe et al., 1989). A short term mast cell proliferation assay was developed which means to purify a fibroblast derived activity (designated KL) which, in the absence of IL-3, supports the proliferation of normal BMMC's and peritoneal mast cells, but not W/W^V BMMC's . In addition, KL was shown to facilitate the formation of erythroid bursts (BFU-E). The biological properties of KL are in agreement with those expected of the c-kit ligand with regard to mast cell biology and aspects of erythropoiesis. The defect W mutations exert is cell autonomous; in agreement with this property, there is evidence for c-kit RNA expression in cellular targets of W mutations (Nocka et al., 1989; Orr-Urtreger et al., 1990). The recent characterization of the molecular lesions of several mutant alleles indicated that they are loss-of-function mutations that disrupt the normal activity or expression of the c-kit receptor (Nocka et al., 1989; Tan et al., 1990; Reith et al., 1990; Nocka et al., 1990a).

[0006] Mutations at the steel locus (Sl) on chromosome 10 of the mouse result in phenotypic characteristics that are very similar to those seen in mice carrying W mutations, i.e., they affect hematopoiesis, gametogenesis, and melanogenesis (Bennett, 1956; Russell, 1979; Silvers, 1979). Many alleles are known at the Sl locus; they are semidominant mutations, and the different alleles vary in their effects on the different cell lineages and their degree of severity (Silvers, 1979; Russell, 1979). The original Sl allele is a severe mutation. SIISI homozygotes are deficient in germ cells, are devoid of coat pigment, and die perinatally of macrocytic anemia (Bennett, 1956; Sarvella and Russell, 1956). Mice homozygous for the Sl allele, although viable, have severe macrocytic anemia, lack coat pigment, and are sterile. Both SII^+ and $Sl^d/+$heterozygotes have a diluted coat color and a moderate macrocytic anemia but are fertile, although their gonads are reduced in size. In contrast to W mutations, Sl mutations are not cell autonomous and are thought to be caused by a defect in the micro-environment of the targets of these mutations (Mayer and Green, 1968; McCulloch et al., 1965; Dexter and Moore, 1977). Because of the parallel and complementary characteristics of mice carrying Sl and W mutations, it had been hypothesized that the Sl gene product is the ligand of the c-kit receptor (Russell, 1979; Chabot et al., 1988).

SUMMARY OF THE INVENTION

[0007] This invention provides an isolated nucleic acid molecule which encodes an amino acid sequence corresponding to a c-kit ligand (KL) and a purified c-kit ligand (KL) polypeptide.

[0008] A pharmaceutical compositions which comprise the c-kit ligand (KL) purified by applicants or produced by applicants' recombinant methods and a pharmaceutically acceptable carrier is further provided as well as methods of treating patients which comprise administering to the patient the pharmaceutical compositions of this invention.

BRIEF DESCRIPTION OF THE FIGURES

[0009] **FIG. 1**. Proliferative response of +/+ and W/W^V BMMC to fibroblast conditioned medium and IL-3. Mast cells derived from +/+ or W/W^V bone marrow were cultured in the presence of 1% 3 CM, 10% FCM (20×concentrated), or medium alone. Incorporation of ^{3}H-thymidine was determined from 24-30 hours of culture.

[0010] **FIG. 2**. Chromatographic profiles of the purification of KL.

[0011] A. Gel filtration chromatography on ACA 54 Ultrogel. Absorbance at 280 nm is shown by a broken line and bio-activity by a solid line. The position of the elution of protein size markers is indicated in kD.

[0012] B. Anion exchange FPLC on a DEAE-5PW column.

[0013] The NaCl gradient is indicated by a dotted line.

[0014] C. Separation on semi-preparative C18 column.

[0015] The 1-propanol gradient is indicated by a dotted line.

[0016] D. Separation on analytical C18 column.

[0017] **FIG. 3**. Electrophoretic analysis of KL. Material from individual fractions was separated by SDS/PAGE (12%) and stained with silver. The position of KL (28-30 kD) is indicated by an arrow. KL activity of corresponding fractions is shown below.

[0018] A. Analysis of 0.5 ml fractions from analytical C18 column eluted with ammonium acetate buffer and 1-propanol gradient.

[0019] B. Analysis of 0.5 ml fractions from analytical C4 column eluted with aqueous 0.1% TFA and absence of 2-mercapto-ethanol.

[0020] **FIG. 4**. Proliferation of W* mutant mast cells in response to KL. Mast cells were derived from individual fetal livers from W/+×W/+ mating, or bone marrow of wildtype, W^V and W^{41} heterozygotes and homozygoses. The proliferation characteristics of mutant mast cells was determined by using increasing concentrations of KL in a proliferation assay. Homozygous mutant mast cells are indicated by a solid line, heterozygotes mutant mast cells by a broken line and wildtype mast cells by a dotted line, except for W where normal fetuses may be either +/+ or W/+.

[0021] **FIG. 5**. Comparison of c-kit expression and growth factor responsiveness in BMMC and peritoneal mast cells (CTMC/PMC).

[0022] A. Fluorescent staining of heparin proteoglycans in purified PMC and BMMC by using berberine sulfate.

[0023] B. Determination of c-kit cell surface expression in PMC and BMMC by FACS using c-kit antibodies. Anti-c-kit serum is indicated by a solid line and non-immune control serum by a dotted line.

[0024] C. Determination of the proliferation potential of PMC to KL. 5000 cells were plated in 0.5 ml, in the presence of 1000 U/ml of KL, 10% Wehi-3CM or RPMI-C alone and the number of viable cells was determined two weeks later.

[0025] **FIG. 6**. Determination of burst promoting activity of KL. Bone marrow and spleen cells were plated in the presence of erythropoietin (2U/ml) and pure KL was added at the concentrations shown. The number of BFU-E was determined on day 7 of culture. This data represents the mean of two separate experiments, each with two replicates per concentration of KL.

[0026] **FIG. 7**. Determination of KL dependent BFU-E formation from W/W fetal livers. Fetuses from mating W/+animals were collected at day 16.5 of gestation. One fetus out of four was a W/W homozygote. Liver cells were plated at 10^5 cells/ml in the presence of either control medium, IL-3 (50 U/ml) or KL (2.5 ng/ml). All cultures contained erythropoietin (2U/ml). Data is expressed as the number of BFU-E/liver and is the mean of 2 replicate plates. The data for +/+ or W/+fetuses is the mean from the three normal fetuses in the liver.

[0027] **FIG. 8**. N-terminal amino acid sequence of KL and deduction of the corresponding nucleic acid sequence by PCR. Top line: N-terminal amino acid sequence (residues 10-36) of KL. Middle Line: Nucleotide sequences of three cDNAs obtained by cloning the 101 bp PCR product (see **FIG. 10**) into M13 and subsequent sequence determination. Bottom Line: sequences of the degenerate sense and antisense primers used for first-strand cDNA synthesis and PCR. The amino acid sequence also is identified as SEQ ID:NO: 2.

[0028] **FIG. 9**. Northern blot analysis using the PCR generated oligonucleotide probes corresponding to the isolated c-kit ligand polypeptide. A 6.5 kb mRNA was isolated with labelled probes.

[0029] **FIG. 10**. Derivation of cDNAs corresponding to the N-terminal amino acids 10-36 of KL by RT-PCR. One microgram of poly(A)$^+$ RNA from BALB/c 3T3 cells was used as template for cDNA synthesis and subsequent PCR amplification in combination with the two degenerate oligonucleotide primers. Electrophoretic analysis of the 101 bp PCR product in agarose is shown.

[0030] **FIG. 11**. Nucleotide Sequence and Predicted Amino Acid Sequence of the 1.4 kb KL cDNA clone. The predicted amino acid sequence of the long open reading frame is shown above and the nucleotide sequence using the single-letter amino acid code. The numbers at right refer to amino acids, with methionine (nucleotides 16-18) being number 1. The potential N-terminal signal sequence (SP)

and the transmembrane domain (TMS) are indicated with dashed lines above the sequence, and cysteine residues in the extracellular domain are circled. A schematic of the predicted protein structure is indicated below. N-linked glycosylation sites and the location of the N-terminal peptide sequence (Pep. Seq.) are indicated. The nucleic acid sequence is also identified as SEQ ID:NO: 1.

[0031] **FIG. 12**. Identification of KL-Specific RNA Transcripts in BALB/c 3T3 Cell RNA by Northern Blot Analysis. Poly(A)$^+$ RNA (4 μg) from BALB/c 3T3 cells was electrophoretically separated, transferred to nitrocellulose, and hybridized with ^{32}P. labeled 1.4 kb KL cDNA. The migration of 18S and 28S ribosomal RNAs is indicated.

[0032] **FIG. 13**. SDS-PAGE Analysis of KL.

[0033] A. Silver staining of KL.

[0034] B. Autoradiography of ^{125}I-KL.

[0035] **FIG. 14**. Binding of ^{125}I-K to Mast Cells and c-kit-Expressing ψ2 Cells.

[0036] A. NIH ψ2/c-kit cells containing the pLJ c-kit expression vector and expressing a high level of high c-kit protein.

[0037] B. Mast cells derived from bone marrow of +/+ or W/W^v adult mice or fetal liver cells of W/W or a normal littermate control (W/+ or +/+.

[0038] **FIG. 15**. Coprecipitation and Cross-Linking of ^{125}I-KL with the c-kit receptor on mast cells.

[0039] A. Coprecipitation of KL with normal rabbit serum (NRS) or two anti-c-kit rabbit antisera (α-v-kit and α-c-kit).

[0040] B. Cross-linking of KL to c-kit with di-succinimidyl substrate. SDS-PAGE analysis was on either 12% or 7.5% polyacrylamide gels. Cross-linked species are labeled "KL + cK".

[0041] **FIG. 16**. RFLP analysis of TaqI-digested DNA from Sl/+ and SIISI mice. The Sl allele from C3HeB/Fej a/a CaJ Sl Hm mice was introduced into a C57BL/6J background, and progeny of a C57BL/6J Sl^{C3H}×Sl^{C3H} cross were evaluated.

[0042] A. Hybridization of the 1.4 kb KL cDNA probe to DNA from two nonanemic (lanes SII+) and two anemic (lanes SIISI) mice. No hybridization to the DNA from the SIISI mice was detected.

[0043] B. Hybridization of the same blot to TIS Dra/SaI, a probe that is tightly linked to Sl (see Detailed Description, infra). This probe identifies a 4 kb C3HeB/FeJ-derived allele and a 2 kb C57BL/6J allele in the Sl^{c3H}l+heterozygotes and only the C3HeB/FeJ-derived allele in the Sl^{c3H}lSl^{c3H} homozygotes.

DETAILED DESCRIPTION OF THE INVENTION

[0044] The relationship of KL to the c-kit receptor has now been defined, and it is shown that KL is the ligand of c-kit based on binding and cross-linking experiments. N-terminal protein sequence of KL was used to derive KL-specific cDNA clones. These cDNA clones were used to investigate the relationship of the KL gene to the Sl locus, and it was demonstrated that KL is encoded by the Sl locus.

[0045] The hematopoietic growth factor was recently purified, i.e., KL, from conditioned medium of BALB/c 3T3 fibroblasts, and it has the biological properties expected of the c-kit ligand (Nocka et al, 1990b). KL was purified based on its ability to stimulate the proliferation of BMMC from normal mice but not from W mutant mice in the absence of IL-3. The purified factor stimulates the proliferation of BMMC and CTMC in the absence of IL-3 and therefore appears to play an important role in mature mast cells. In regard to the anticipated function of c-kit in erythropoiesis, KL was shown to facilitate the formation of erythroid bursts (day 7-14 BFU-E) in combination with erythropoietin. KL has a molecular mass of 30 kd and a pI of 3.8; it is not a disulfide-linked dimer, although the characteristics of KL upon gel filtration indicate the formation of noncovalently linked dimers under physiological conditions.

[0046] This invention provides an isolated nucleic acid molecule which encodes an amino acid sequence corresponding to a c-kit ligand (KL). The invention also encompasses nucleic acids molecules which differ from that of the nucleic acid molecule which encodes the amino acid sequence isolated by applicants, but which produce the same phenotypic effect. These altered, but phenotypically equivalent nucleic acid molecules are referred to as "equivalent nucleic acids". And this invention also encompasses nucleic acid molecules characterized by changes in non-coding regions that do not alter the phenotype of the polypeptide produced therefrom when compared to the nucleic acid molecule described hereinabove. This invention further encompasses nucleic acid molecules which hybridize to the nucleic acid molecule of the subject invention. As used herein, the term "nucleic acid" encompasses RNA as well as single and double-stranded DNA and cDNA. In addition, as used herein, the term "polypeptide" encompasses any naturally occurring allelic variant thereof as well as man-made recombinant forms.

[0047] For the purposes of this invention, the c-kit ligand (KL) is a human c-kit ligand (KL) or a murine c-kit ligand (KL).

[0048] Also provided by this invention is a vector which comprises the nucleic acid molecule which encodes an amino acid sequence corresponding to a c-kit ligand (KL). This vector may include, but is not limited to a plasmid, viral or cosmid vector.

[0049] This invention also provides the isolated nucleic acid molecule operatively linked to a promoter of RNA transcription, as well as other regulatory sequences. As used herein, the term "operatively linked" means positioned in such a manner that the promoter will direct the transcription of RNA off of the nucleic acid molecule. Examples of such promoters are SP6, T4 and T7. Vectors which contain both a promoter and a cloning site into which an inserted piece of DNA is operatively linked to that promoter are well known in the art. Preferable, these vectors are capable of transcribing RNA in vitro. Examples of such vectors are the pGEM series [Promega Biotec, Madison, Wis.].

[0050] A host vector system for the production the c-kit ligand (KL) polypeptide is further provided by this invention which comprises one of the vectors described hereinabove in

a suitable host. For the purposes of this invention, a suitable host may include, but is not limited to an eucaryotic cell, e.g., a mammalian cell, or an insect cell for baculovirus expression. The suitable host may also comprise a bacteria cell such as *E. coli*, or a yeast cell.

[0051] A purified c-kit ligand (KL) polypeptide as well as a fragment of the purified c-kit ligand (KL) polypeptide is further provided by this invention.

[0052] In one embodiment of this invention, the soluble, c-kit ligand (KL) polypeptide is conjugated to an imageable agent. Imageable agents are well known to those of ordinary skill in the art and may be, but are not limited to radioisotopes, dyes or enzymes such as peroxidase or alkaline phosphate. Suitable radioisotopes include, but are not limited to ^{125}I, ^{32}P and ^{35}S.

[0053] These conjugated polypeptides are useful to detect the presence of cells, in vitro or in vivo, which express the c-kit receptor protein. When the detection is performed in vitro, a sample of the cell or tissue to be tested is contacted with the conjugated polypeptide under suitable conditions such that the conjugated polypeptide binds to c-kit receptor present on the surface of the cell or tissue; then removing the unbound conjugated polypeptide, and detecting the presence of conjugated polypeptide, bound; thereby detecting cells or tissue which express the c-kit receptor protein.

[0054] Alternatively, the conjugated polypeptide may be administered to a patient, for example, by intravenous administration. A sufficient amount of the conjugated polypeptide must be administered, and generally such amounts will vary depending upon the size, weight, and other characteristics of the patient. Persons skilled in the art will readily be able to determine such amounts.

[0055] Subsequent to administration, the conjugated polypeptide which is bound to any c-kit receptor present on the surface of cells or tissue is detected by intracellular imaging.

[0056] In the method of this invention, the intracellular imaging may comprise any of the numerous methods of imaging, thus, the imaging may comprise detecting and visualizing radiation emitted by a radioactive isotope. For example, if the isotope is a radioactive isotope of iodine, e.g. ^{125}I, the detecting and visualizing of radiation may be effected using a gamma camera to detect gamma radiation emitted by the radioiodine.

[0057] In addition, the soluble, c-kit ligand (KL) polypeptide fragment may be conjugated to a therapeutic agent such as toxins, chemotherapeutic agents or radioisotopes. Thus, when administered to a patient in an effective amount, the conjugated molecule acts as a tissue specific delivery system to deliver the therapeutic agent to the cell expressing c-kit receptor.

[0058] A method for producing a c-kit ligand (KL) polypeptide is also provided which comprises growing the host vector system described hereinabove under suitable conditions permitting production of the c-kit ligand (KL) polypeptide and recovering the resulting c-kit ligand (KL) polypeptide.

[0059] This invention also provides the c-kit ligand (KL) polypeptide produced by this method.

[0060] A soluble, mutated c-kit ligand (KL) polypeptide is also provided, wherein this mutated polypeptide retains its ability to bind to the c-kit receptor, but that the biological response which is mediated by the binding of a functional ligand to the receptor is destroyed. Thus, these mutated c-kit ligand (KL) polypeptides act as antagonists to the biological function mediated by the ligand to the c-kit receptor by blocking the binding of normal, functioning ligands to the c-kit receptor.

[0061] A pharmaceutical composition which comprises the c-kit ligand (KL) purified by applicants or produced by applicants' recombinant methods and a pharmaceutically acceptable carrier is further provided. The c-kit ligand may comprise the isolated soluble c-kit ligand of this invention, a fragment thereof, or the soluble, mutated c-kit ligand (KL) polypeptide described hereinabove. As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents.

[0062] This invention further provides a substance capable of specifically forming a complex with the soluble, c-kit ligand (KL) polypeptide, or a fragment thereof, described hereinabove. This invention also provides a substance capable of specifically forming a complex with the c-kit ligand (KL) receptor protein. In one embodiment of this invention, the substance is a monoclonal antibody, e.g., a human monoclonal antibody.

[0063] A method of modifying a biological function associated with c-kit cellular activity is provided by this invention. This method comprises contacting cells, whose function is to be modified, with an effective amount of a pharmaceutical composition described hereinabove, effective to modify the biological function of the cell. Biological functions which may be modified by the practice of this method include, but are not limited to cell-cell interaction, propagation of a cell that expresses c-kit i.e., propagation of hemapoietic cells. In addition, in vitro fertilization may also be facilitated by this method. This method may be practiced in vitro or in vivo. When the method is practiced in vivo, an effective amount of the pharmaceutical composition described hereinabove is administered to a patient in an effective amount, effective to modify the biological function associated with c-kit function.

[0064] This invention also provides a method of stimulating the proliferation of mast cells in a patient which comprises administering to the patient the pharmaceutical composition described hereinabove in an amount which is effective to stimulate the proliferation of the mast cells in the patient. Methods of administration are well known to those of ordinary skill in the art and include, but are not limited to administration orally, intravenously or parenterally. Administration of the composition will be in such a dosage such that the proliferation of mast cells is stimulated. Administration may be effected continuously or intermittently such that the amount of the composition in the patient is effective to stimulate the proliferation of mast cells.

[0065] A method of inducing differentiation of mast cells or erythroid progenitors in a patient which comprises administering to the patient the pharmaceutical composition described hereinabove in an amount which is effective to

induce differentiation of the mast cells or erythroid progenitors is also provided by this invention. Methods of administration are well known to those of ordinary skill in the art and include, but are not limited to administration orally, intravenously or parenterally. Administration of the composition will be in such a dosage such that the differentiation of mast cells or erythroid progenitors is induced. Administration may be effected continuously or intermittently such that the amount of the composition in the patient is effective to induce the differentiation of mast cells or erythroid progenitors.

[0066] This invention also provides a method of facilitating bone marrow transplantation or treating leukemia in a patient which comprises administering to the patient an effective amount of the pharmaceutical composition described hereinabove in an amount which is effective to facilitate bone marrow transplantation or treat leukemia. Methods of administration are well known to those of ordinary skill in the art and include, but are not limited to administration orally, intravenously or parenterally. Administration of the composition will be in such a dosage such that bone marrow transplantation is facilitated or such that leukemia is treated. Administration may be effected continuously or intermittently such that the amount of the composition in the patient is effective. This method is particularly useful in the treatment of acute myelogenous leukemia and modifications of chronic myelogenous leukemia.

[0067] This invention also provides a method of treating melanoma in a patient which comprises administering to the patient an effective amount of a pharmaceutical composition described hereinabove in an amount which is effective to treat melanoma. Methods of administration are well known to those of ordinary skill in the art and include, but are not limited to administration orally, intravenously or parenterally. Administration of the composition will be in such a dosage such that melanoma is treated. Administration may be effected continuously or intermittently such that the amount of the composition in the patient is effective.

[0068] The soluble, c-kit ligand (KL) polypeptide may also be mutated such that the biological activity of c-kit is destroyed while retaining its ability to bind to c-kit. Thus, this invention provides a method of treating allergies in a patient which comprises administering to the patient an effective amount of the soluble, mutated c-kit ligand described hereinabove and a pharmaceutically acceptable carrier, in an amount which effective to treat the allergy. As is well known to those of ordinary skill in the art, the amount of the composition which is effective to treat the allergy will vary with each patient that is treated and with the allergy being treated. Administration may be effected continuously or intermittently such that the amount of the composition in the patient is effective.

[0069] Furthermore, this invention provides a method for measuring the biological activity of a c-kit (KL) polypeptide which comprises incubating normal bone-marrow mast cells with a sample of the c-kit ligand (KL) polypeptide under suitable conditions such that the proliferation of the normal bone-marrow mast cells are induced; incubating doubly mutant bone-marrow mast cells with a sample of the c-kit ligand (KL) polypeptide under suitable conditions; incubating each of the products thereof with ^{3}H-thymidine; determining the amount of thymidine incorporated into the DNA of the normal bone-marrow mast cells and the doubly mutant bone marrow mast cells; and comparing the amount of incorporation of thymidine into the normal bone-marrow mast cells against the amount of incorporation of thymidine into doubly mutant bone-marrow mast cells, thereby measuring the biological activity of c-kit ligand (KL) polypeptide.

[0070] Throughout this application, references to specific nucleotides in DNA molecules are to nucleotides present on the coding strand of the DNA. The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

C—cytosine	A—adenosine
T—thymidine	G—guanosine
U—uracil	

[0071] Experiment Number 1—Purification of c-kit Ligand

[0072] Experimental Methods

[0073] Mice and embryo identification

[0074] WBB6 +/+ and W/k^{V}, C57B16 W^{V}/+ and WB W/+mice were obtained from the Jackson Laboratory (Bar Harbor, Me.). Heterozygous W^{41}/+mice were kindly provided by Dr. J. Barker from the Jackson Laboratory and maintained in applicants' colony by brother sister mating. Livers were removed at day 14-15 of gestation from fetuses derived by mating W/+animals. W/W fetuses were identified by their pale color and small liver size relative to other W/+ and +/+fetuses in the litter. Their identity was confirmed by analysis of the c-kit protein in mast cells derived from each fetus (Nocka et al., 1990).

[0075] Mast Cell Cultures, Preparation of Peritoneal Mast Cell and Flow Cytometry

[0076] Mast cells were grown from bone marrow of adult mice and fetal liver cells of day 14-15 fetuses in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), conditioned medium from WEHI-3B cells, non-essential amino acids, sodium pyruvate, and 2-mercapto-ethanol (RPMI-Complete (C)) (Yung and Moore, 1982). Non-adherent cells were harvested, refed weekly and maintained at a cell density less than 7×10^{5} cells/ml. Mast cell content of cultures was determined weekly by staining cytospin preparations with 1% toluidine blue in methanol. After 4 weeks, cultures routinely contained greater than 95% mast cells and were used from proliferation assays. Peritoneal mast cells were obtained from C57B1/6 mice by lavage of the peritoneal cavity with 7-10 ml of RPMI-C. Mast cells were purified by density gradient centrifugation on 22% Metrizamide (Nycomed, Oslo, Norway) in PBS without Ca^{++} and Mg^{++}, essentially as previously described (Yurt et al, 1977). Mast cells were stained with 1% toluidine blue in methanol for 5 minutes and washed for 5 minutes in H_2O, and berberine sulfate by standard procedures (Enerback, 1974). Mast cells were labeled with c-kit specific rabbit antisera which recognizes extracellular determinants of c-kit as previously described and analyzed on a FACSCAN (Becton Dickinson) (Nocka et al. 1990).

[0077] Mast Cell Proliferation Assay

[0078] Mast cells were washed three times in RPMI to remove IL-3 and cultured at a concentration of 5×10^4 c/ml in RPMI-C in a volume of 0.2 ml in 96 well plates with two fold serial dilutions of test samples. Plates were incubated for 24 hours at 37° C., 2.5 μC of ^{3}H-TdR was added per well and incubation was continued for another 6 hours. Cells were harvested on glass fiber filters and thymidine incorporation into DNA was determined.

[0079] Preparation of Fibroblast Conditioned Medium

[0080] Balb/3T3 cells (Aaronson and Todaro, 1968) were grown to confluence in Dulbecco's Modified MEM (DME) supplemented with 10% calf serum (CS), penicillin and streptomycin in roller bottles. Medium was removed and cells washed two times with phosphate buffered saline (PBS). DME without CS was added and conditioned medium was collected after three days. Cells were refed with serum containing medium for one to two days, then washed free of serum, and refed with serum free medium and a second batch of conditioned medium was collected after three days. Conditioned medium (CM) was centrifuged at 2500 rpm for 15 minutes to remove cells, filtered through a 0.45 u filter and frozen at 4° C. The conditioned medium was then concentrated 100-200 fold with a Pellicon ultrafiltration apparatus followed by an Amicon stirred cell, both with membranes having a cut of f of 10,000 kD.

[0081] Column Chromatography

[0082] Blue Agarose chromatography (BRL, Gaithersburg, Md.) was performed by using column with a bed volume of 100 ml equilibrated with PBS. 50-80 ml of FCM concentrate was loaded onto the column and after equilibration for one hour the flow through which contained the active material was collected and concentrated to 15-20 ml in dialysis tubing with PEG 8000.

[0083] Gel filtration chromatography was performed on a ACA54 Ultrogel (LKB, Rockland, Md.) column (2.6×90 cm) which was equilibrated with PBS and calibrated with molecular weight markers; bovine serum albumin (Mr 68,000), chymotrypsinogen (Mr 25,700), and ribonuclease A (Mr 14,300), all obtained from Pharmacia, Piscataway, N.J. The concentrate from the Blue Agarose column was loaded onto the gel filtration column, the flow rate adjusted to 37.5 ml/hour and 7.5 ml fractions collected.

[0084] Anion Exchange and Reverse-Phase HPLC (RP-HPLC)

[0085] High performance liquid chromatography was performed using a Waters HPLC system (W600E Powerline controller, 490E programmable multiwavelength detector, and 810 Baseline Workstation, Waters, Bedford, Mass.). Active fractions from gel filtration were dialyzed in 0.05 M Tris-HCl pH 7.8 and loaded onto a Protein-Pak DEAE™ DEAE-5PW HPLC column (7.5 mm ×7.5 cm, Waters), equilibrated with 0.05 M Tris-HCl pH 7.8. Bound proteins were eluted with a linear gradient from 0 to 0.05 M Tris-HCl pH 7.8. Bound proteins were eluted with a linear gradient from 0 to 0.4M NaCl in 0.02 M Tris-HCl pH 7.8. The flow rate was 1 ml/minute and 2 ml fractions were collected.

[0086] RP-HPLC was performed using a semi-preparative and an analytical size C_{18} column from Vydac. For both columns buffer A was 100 mM ammonium acetate pH 6.0, and buffer B was 1-propanol. The biologically active fractions from anion exchange were pooled and loaded onto the semi-preparative C_{18} column. Bound proteins were eluted with a steep gradient of 0% -23% 1-propanol within the first 10 minutes and 23-33% 1-propanol in 70 minutes. The flow rate was adjusted to 2 ml/min and 2 ml fractions were collected. Biologically active fractions were pooled and diluted 1:1 with buffer A and loaded on the analytical C_{18} reverse phase column. Proteins were eluted with a steep gradient from 0% -26% 1-propanol in 10 minutes and then a shallow gradient from 26% -33% 1-propanol in 70 minutes. The flow rate was 1 ml/min and 1 ml fractions were collected. Separation on an analytical C4 reverse phase column was performed with a linear gradient of acetonitrile from 0-80% in aqueous 0.1% TFA.

[0087] Isolectric Focusing (IEF)

[0088] One ml of partially purified KL was supplemented with 20% glycerol (v/v) and 2% ampholine (v/v) at pH 3.5-10 (LKB, Gaithersburg, Md.). A 5 to 60% glycerol density gradient containing 2% ampholine (pH 3.5-10) was loaded onto an IEF column (LKB 8100). The sample was applied onto the isodense region of the gradient, followed by IEF (2000V, 24 h, 4° C.). Five ml fractions were collected and the pH determined in each fraction. The fractions were dialyzed against RPMI-C and then tested for biological activity.

[0089] Erythroid Progenitor Assays

[0090] Adult bone marrow, spleen and day 14 fetal liver cells were plated at 10^5, 10^6, and 10^7 cells/ml, respectively, in Iscove's modified Dulbecco's medium with 1.2% methylcellulose, 30% FCS, 100 uM 2-mercaptoethanol, human recombinant erythropoietin (2 units/ml, Amgen, Thousand Oaks, Calif.) (Iscove, 1978; Nocka and Pelus, 1987). Cultures were incubated for 7 days at 37° C. and hemoglobinized colonies and bursts scored under an inverted microscope. 0.1 mM hemin (Kodak) was added to cultures of bone marrow cells for optimum growth. Purified KL, IL-3 either as WEHI-3 CM (10%, vol/vol) or recombinant murine IL-3 (50 u/ml, Genzyme, Cambridge) was added where indicated.

[0091] Experimental Methods

[0092] Short Term Mast Cell Proliferation Assay Detects a Fibroblast Derived Activity

[0093] In order to identify and measure a fibroblast derived growth factor activity which facilitates the proliferation of normal but not W/W^V mast cells, BMMC were washed free of IL-3 containing medium, incubated with medium containing 20 fold concentrated fibroblast conditioned medium (FCM) or WEHI-3 CM (IL-3) and after 24 hours of incubation ^{3}H-thymidine incorporation was determined. The response of BMMC derived from normal +/+ and mutant W/W^V mice to IL-3 was similar (**FIG. 1**); in contrast, 20 fold concentrated fibroblast conditioned medium facilitated the proliferation of +/+mast cells, but little proliferation was seen with W/W^V mast cells. Concentrated FCM was also tested for its ability to stimulate the proliferation of other IL-3 dependent cells. The myeloid 32D cells are known to lack c-kit gene products (Nocka et al., 1989). No proliferation of the 32D cells was observed with FCM, although normal proliferation was obtained with WEHI-3 CM (not shown). Taken together these results and the known defects in c-kit for both the W and W^V alleles

(Nocka et al., 1990), suggested that FCM activity was dependent on the expression of a functional c-kit protein in mast cells (BMMC) and therefore might be the ligand of the c-kit receptor. In addition the FCM activity was distinct from IL-3. Therefore, normal and W mutant mast cells provide a simple, specific assay system for the purification of the putative c-kit ligand (KL) from fibroblast conditioned medium.

[0094] Purification of the Mast Cell Stimulating Activity KL

[0095] To purify KL, five liters of serum free conditioned medium from Balb/3T3 fibroblasts was concentrated 50 fold by ultrafiltration. The concentrate was passed through a Blue Agarose column equilibrated with PBS and the flow through, which contained the mast cell stimulating activity, was collected and concentrated with polyethylene glycol. In addition to the determination of the bio-activity by using normal mast cells, peak fractions throughout the purification were also tested with W/W^V mast cells where little activity was observed. The material from the Blue Agarose column was fractionated by gel filtration using a ACA 54 column (**FIG. 2A**). The biological activity eluted as a major and a minor peak corresponding to 55-70 kD and 30 kD, respectively. The fractions of the main peak were pooled, dialyzed and fractionated by FPLC chromatography on a DEAE-5PW column with a NaCl gradient (**FIG. 2B**). The activity eluted at 0.11 M NaCl from the FPLC column. Peak fractions were pooled and subjected to HPLC chromatography with a semi-preparative C18 column and an ammonium acetate/n-propanol gradient (**FIG. 2C**). The active material eluted at 30% n-propanol from the semi-preparative C18 column was diluted 1:1 with buffer A and rechromatographed by using an analytical C18 column (**FIG. 2D**). A single peak of activity eluted again at 30% n-propanol which corresponded to a major peak of absorbance (280 nm) in the eluant profile. Similar results were obtained by using a C4 column with H_2O and acetonitrile containing 0.1% TFA as solvents (**FIG. 3B**). SDS-PAGE analysis of the active fractions from the separations with both solvent systems and silver staining revealed one major band with a mobility corresponding to a molecular mass of 28-30 kD. The presence and magnitude of this band correlated well with the peak of biological activity (**FIG. 3**). There was no significant difference in the migration of this band under reduced and non-reduced conditions, indicating that KL was not a disulfide linked dimer (**FIG. 3C**). Three discrete species were observed on both reduced and non-reduced SDS-PAGE indicating size heterogeneity of the purified material. The total amount of protein estimated by absorbance at 280 nm correlated with the amount detected by silver stain relative to BSA as a reference standard. As indicated in Table 1, the purification of KL from conditioned medium of Balb/3T3 cells was more than 3000 fold and the recovery of the initial total activity 47%. Half maximal proliferation of +/+mast cells in applicants' assay volume of 0.2 ml is defined as 50 units of activity and corresponds to approximately 0.5 ng of protein. Isoelectric focusing of partially purified material (after ion exchange) revealed a major peak of activity in the pH range of 3.7-3.9 indicating an isoelectric point for KL of 3.7-3.9.

TABLE 1

Purification of KL from Balb/3T3 Conditioned Medium

Purification Step	Total Protein (mg)	Total Activity ($U \times 10^{-5}$)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
FCM (5L), 50X Concentrated	152	—	—	—	—
Blue Agarose	32	720	2.2×10^4	1	100
Gel Filtration	28	480	1.7×10^4	.77	67
DEAE-5PW	3	720	2.4×10^5	11	100
C18-Semiprep	.079	600	7.6×10^6	345	83
C18-Analytical	.004	340	8.5×10^7	3863	47

[0096] Proliferative Response to KL of Mast Cells with Different c-kit/W Mutations

[0097] Purified KL was tested for its ability to stimulate the proliferation of mast cells derived from wildtype animals as well as homozygotes and heterozygotes of W, W^V, and W^{41} alleles. The original W allele specifies a nonfunctional c-kit receptor and animals homozygous for the W allele die perinatally, are severely anemic and mast cells derived from W/W fetuses do not proliferate when co-cultured with Balb/3T3 fibroblasts (deAeberle, 1927; Nocka et al., 1990). The W^V and W^{41} alleles both specify a partially defective c-kit receptor and homozygous mutant animals are viable (Little and Cloudman, 1937; Geissler et al., 1981; Nocka et al., 1990). Homozygous W^V animals have severe macrocytic anemia and their mast cells display a minor response in the co-culture assay, and homozygotes for the less severe W^{41} allele have a moderate anemia and their mast cells show an intermediate response in the co-culture assay. Homozygous and heterozygous mutant and +/+mast cells were derived from the bone marrow for the W^V and W^{41} alleles and from day 14 fetal livers for the W allele as described previously (Nocka et al., 1990). Fetal liver derived W/W mast cells did not proliferate in response to KL whereas both heterozygous (W/+) and normal (+/+) mast cells displayed a similar proliferative response to KL (**FIG. 4**). Bone marrow derived mast cells from W^V/W^V mice were severely defective in their response to KL, although some proliferation, 10% of +/+values, was observed at 100 U/ml (**FIG. 4**). W^V/+mast cells in contrast to heterozygous W/+mast cells showed an intermediate response (40%) in agreement with the dominant characteristics of this mutation. W^{41}/W^{41} and W^{41}/+ mast cells were also defective in their ability to proliferate with KL, although less pronounced than mast carrying the W and the W^V alleles, which is consistent with the in vivo phenotype of this mutation (**FIG. 4**). These results indicate a correlation of the responsiveness of mast carrying the WI W^V and W^{41} alleles to KL with the severity and in vivo characteristics of these mutations. In contrast, the proliferative response of mutant mast cells to WEHI-3CM (IL-3) was not affected by the different W mutations.

[0098] KL Stimulates the Proliferation of Peritoneal Mast Cells

[0099] Mast cells of the peritoneal cavity (PMC) have been well characterized and in contrast to BMMC represent connective tissue-type mast cells (Stevens and Austin, 1989). PMC do not proliferate in response to IL-3 alone; however, their mature phenotype and viability can be main-

tained by co-culture with NIH/3T3 fibroblasts (Levi-Schaffer et al., 1985). Thus, it was of interest to determine whether KL could stimulate the proliferation of PMC. First, c-kit was examined to determine if it is expressed in PMC. Peritoneal mast cells were purified by sedimentation in a metrizamide gradient and c-kit expression on the cell surface analyzed by immunofluorescence with anti-c-kit sera or normal rabbit sera. The PMC preparation was 90-98% pure based on staining with toluidine blue and berberine sulfate. Berberine sulfate stains heparin proteoglycans in granules of connective tissue mast cells and in addition the dye is also known to stain DNA (**FIG. 5**) (Enerback, 1974). BMMC and mucosal mast cells contain predominantly chondroitin sulfate di-B/E proteoglycans rather than heparin proteoglycans (Stevens et al., 1986); berberine sulfate therefore did not stain the granules in BMMC (**FIG. 5A**). Analysis of c-kit expression by flow-cytometry indicated that virtually all PMC expressed c-kit at levels similar to those observed in BMMC (**FIG. 5B**). KL was then examined to determine if it would effect the survival or stimulate the proliferation of PMC (**FIG. 5C**). Culture of PMC in medium alone, or by the addition of WEHI-3CM at concentrations optimal for BMMC, results in loss of viability of PMC within 3-4 days although a few cells survived in WEHI-3CM for longer periods. Culture of PMC in the presence of KL sustained their viability and after two weeks the cell number had increased from 5000 to 60,000. A similar increase in the number of BMMC was observed in response to KL. In contrast to the lack of a proliferative response of PMC to WEHI-3CM, BMMC's proliferated with WEHI-3CM as expected. After one and two weeks in culture, cells were stained with toluidine blue and berberine sulfate. The mature phenotype of PMC was maintained in culture with 100% of cells staining with both dyes, although the staining with berberine sulfate was somewhat diminished when compared with freshly isolated PMC.

[**0100**] KL Stimulates the Formation of Erythroid Bursts (BFU-E)

[**0101**] An important aspect of W mutations is their effect on the erythroid cell lineage. The in vivo consequences of this defect are macrocytic anemia which is lethal for homozygotes of the most severe alleles (Russell, 1979; Geissler, et al., 1981). Analysis of erythroid progenitor populations in the bone marrow of W/WV mice indicates a slight decrease of BFU-E and CFU-E (Gregory and Eaves, 1978; Iscove, 1978b). In livers of W/W fetuses the number of BFU-E is not affected but a large decrease in the number of CFU-E is seen suggesting a role for c-kit at distinct stages of erythroid maturation presumably prior to the CFU-E stage (Nocka et al., 1989). In order to evaluate a role for KL in erythropoiesis and to further define its relationship to the c-kit receptor, the effect of KL on BFU-E formation was determined. Bone marrow, spleen and fetal liver cells were plated, by using standard culture conditions, in the presence and absence of KL, erythropoietin and WEHI-3 CM. BFU-E were then scored on day 7 of culture. In the absence of erythropoietin, no erythroid growth was observed with either WEHI-3 CM or KL. In the presence of erythropoietin, BFU-E from spleen cells were stimulated by KL in a dose dependent manner, from 12 BFU-E/10^6 cells with erythropoietin alone to 50 BFU-E/10^6 cells with maximal stimulation at 2.5 ng of KL/ml (**FIG. 6**). In addition to the effect on the number of BFU-E, the average size of the bursts was dramatically increased by KL. The number of BFU-E obtained by using spleen cells with KL+erythropoietin was similar to the number observed with WEHI-3 CM +erythropoietin. In contrast, KL +erythropoietin did not stimulate the proliferation of BFU-E from bone marrow cells, whereas WEHI-3 CM +erythropoietin induced the formation of 18 BFU-E from 10^5 bone marrow cells. The effect of KL on the day 14 fetal liver cells was also examined and similar results were observed as with spleen cells. A significant number of BFU-E from fetal liver cells were observed with erythropoietin alone to 50 BFU-E/10^6 cells with maximal stimulation at 2.5 ng of KL/ml (**FIG. 6**). In addition to the effect on the number of BFU-E, the average size of the bursts was dramatically increased by KL. The number of BFU-E obtained by using spleen cells with KL +erythropoietin was similar to the number observed with WEHI-3 CM +erythropoietin. In contrast, KL +erythropoietin did not stimulate the proliferation of BFU-E from bone marrow cells, whereas WEHI-3 CM +erythropoietin induced the formation of; 18 BFU-E from bone marrow cells, whereas WEHI-3 CM +erythropoietin induced the formation of 18 BFU-E from 10^5 bone marrow cells. The effect of KL on day 14 fetal liver, cells was also examined and similar results were observed as with spleen cells. In the presence of WEHI-3 CM +erythropoietin 18±3 BFU-E were observed with fetal liver cells. In the presence of erythropoietin, BFU-E from spleen cells were stimulated by KL in a dose dependent manner, from 12 BFU-E/10^6 with erythropoietin alone to 50 BFU-E/10^6 cells with maximal stimulation at 2.5 ng of KL/ml (**FIG. 6**). In addition to the effect on the number of BFU-E, the average size of the bursts was dramatically increased by KL. The number of BFU-E obtained by using spleen cells with KL +erythropoietin was similar to the number observed with WEHI-3 CM +erythropoietin. In contrast, KL +erythropoietin did not stimulate the proliferation of BFU-E from bone marrow cells, whereas WEHI-3 CM=erythropoietin induced the formation of 18 BFU-E from 10^5 bone marrow cells. The effect of KL on day 14 fetal liver cells was also examined and similar results were observed as with spleen cells. A significant number of BFU-E from fetal liver cells were observed with erythropoietin alone; however, this number increased from 6±2 to 20±5 with 2.5 ng/ml of KL. In the presence of WEHI-3 CM +erythropoietin 18±3 BFU-E were observed with fetal liver cell.

[**0102**] To further evaluate the relationship of KL to c-kit in the erythroid lineage, it was assessed whether KL facilitates the formation of erythroid bursts (BFU-E) from fetal liver cells of W/W mice. W/W and W/+ or +/+liver cells were prepared from fetuses at day 16.5 of gestation from mating w/+mice. The total number of nucleated cells was reduced eight fold in the liver of the W/W mutant embryo as compared to the healthy fetuses. The number of BFU-E from W/W and W/+ or +/+fetal liver was similar in cultures grown with IL-3 +erythropoietin and the low level of BFU-E in cultures grown with erythropoietin alone was comparable as well (**FIG. 7**). KL did not stimulate BFU-E above levels seen with erythropoietin alone for W/W fetal liver cells, whereas as the number of KL dependent BFU-E from W/+ or +/+liver cells were similar to those obtained with erythropoietin +IL-3. This result suggests that responsiveness of erythroid progenitors to KL is dependent on c-kit function.

[**0103**] Binding Studies with Purified KL

[**0104**] Purified KL was labelled with ^{125}I by the chloramine T method to a high specific activity, i.e., to 2.8×10^5

cpm/ng. Using the labelled KL, specific binding of KL to mast cells was detected. However, with W/W mast cells, no binding was detected and good binding to mast cells of littermates was seen. After binding to mast cells, KL coprecipitated with antisera to c-kit. In addition, binding of KL to W mutant mast cells correlates with c-kit expression on the cell surface, V, 37(+) versus W(−).

[0105] Determination of the Peptide Sequence of the c-kit Ligand

[0106] The c-kit receptor protein was isolated as described hereinabove and the sequence of the protein was determined by methods well known to those of ordinary skill in the art.

[0107] The single letter amino acid sequence of the protein from the N-teminal is:

K E I X G N P V T D N V K D I T K L V A N L P N D
Y M I T L N Y V A G M X V L P,

[0108] with:

[0109] K=lysine; E=glutamic acid; I=isoleucine; X=unknown; G=glycine; N=asparagine; P=proline; V=valine; T=threonine; D=aspartic acid; L=leucine; A=alanine; Y=tyrosine; and M=methionine.

[0110] Experimental Discussion

[0111] The finding that the W locus and the c-kit proto-oncogene are allelic revealed important information about the function of c-kit in developmental processes and in the adult animal. The knowledge of the function of the c-kit receptor in return provided important clues about tissues and cell types which produce the ligand of the c-kit receptor. In an attempt to identify the c-k ligand, a growth factor was purified, designated KL, from conditioned medium of Balb/3T3 fibroblasts, a cell type suspected to produce the c-kit ligand, which has biological properties expected of the c-kit ligand with regard to mast cell biology and erythropoiesis. KL has a molecular mass of 30 kD and an isoelectric point of 3.8. KL is not a disulfide linked dimer, in contrast to CSF-1, PDGF-A and PDGF-B which have this property (Das and Stanley, 1982; Betsholz et al., 1986). Although, the behavior of KL upon gel filtration in PBS indicated a size of 55 -70 kD which is consistent with the presence of non-covalently linked dimers under physiological conditions. KL is different from other hematopoietic growth factors with effects on mast cells, such as IL-3 and IL-4, based on its ability to stimulate the proliferation of BMMC and purified peritoneal mast cells (CTMC), but not BMMCs from W mutant mice. Balb/3T3 fibroblasts are a source for the hematopoietic growth factors G-CSF, GM-CSF, CSF-1, LIF and IL-6; however, none of these have the biological activities of KL (Nicola, 1989; Gough and Williams, 1989). Furthermore, preliminary results from the determination of the protein sequence of KL indicate that KL is different from the known protein sequences.

[0112] An essential role for c-kit and its ligand in the proliferation, differentiation, and/or survival of mast cells in vivo has been inferred because of the absence of mast cells in W mutant mice (Kitamura and Go, 1978; Kitamura and Fujita, 1989). The precise stage(s) at which c-kit function is required in mast cell differentiation are not known. Mast cells derived in vitro from bone marrow, fetal liver, or spleen with IL-3 resemble mucosal mast cells (MMC), although they may represent a precursor of both types of terminally differentiated mast cells, MMC and CTMC (Stevens and Austin, 1989). Apparently, c-kit is not required for the generation of BMMC from hematopoietic precursors since IL-3 dependent mast cells can be generated with comparable efficiency from bone marrow or fetal liver of both normal and W mutant mice (Yung et al., 1982). The demonstration of c-kit expression in BMMC and CTMC/PMC and the corresponding responsiveness of BMMC and mature CTMC/PMC to KL suggests a role for c-kit at multiple stages in mast cell differentiation. In addition to fibroblasts, it has been shown that the combination of IL-3 and IL-4, IL-3 and PMA, or crosslinking of IgE receptors can stimulate the proliferation of CTMC in vitro (Nakahata et al., 1986; Tsuji et al., 1990; Tsuji et al., 1990; Takagi et al., 1989; Hamguchi et al., 1987). In contrast to these biological response modifiers, which are mediators of allergic and inflammatory responses, KL by itself in the presence of FCS is capable of stimulating CTMC proliferation. Therefore, KL may be a mast cell proliferation and differentiation activity which is independent from these immune responses for its production and action on target cells.

[0113] The defect W mutations exert on erythropoiesis indicates an essential role for c-kit in the maturation of erythroid cells (Russell, 79; Gregory and Eaves, 1978; Iscove, 1978b). The analysis of erythroid progenitors in fetal livers of W/W fetuses compared with normal littermates suggested that in the absence c-kit function, maturation proceeds normally to the BFU-E stage, but that progression to the CFU-E stage is suppressed (Nocka et al., 1989). In vitro, this defect can be overcome by the inclusion of IL-3 in the culture system, which together with erythropoietin is sufficient to facilitate the maturation of BFU-E from W/W^V and +/+bone marrow (Iscove, 1978a). In vivo, a role for IL-3 in this process is not known and therefore c-kit may serve a critical function in the progression through this stage of erythroid differentiation. The ability of KL to stimulate the formation of erythroid bursts from spleen and fetal liver cells together with erythropoietin is consistent with c-kit functioning at this stage of erythroid differentiation. Furthermore, the ability of KL to stimulate W/W BFU-E suggest that c-kit function is required for KL mediated BFU-E formation and this is similar to the requirement of c-kit function for KL mediated mast cell proliferation. A burst promoting effect of Balb/3T3 cells on the differentiation of BFU-E from fetal liver cells had been described previously (Li and Johnson, 1985). It is likely that KL is responsible for the burst promoting activity of Balb/3T3 cells. An interesting finding of this study is the inability of KL to stimulate day 7 BFU-E from bone marrow cells. This result suggests that BFU-E in fetal liver, adult spleen and adult bone marrow differ in their growth requirements. Recent experiments indicate that KL may stimulate an earlier erythroid-multipotential precursor in bone marrow which appears at later times in culture (day 14-20). To demonstrate a direct effect of KL on BFU-E formation and to rule out the involvement of accessory cells or other endogenous growth factors, experiments with purified progenitor populations need to be performed.

[0114] In addition to the defects in erythropoiesis and mast cell development, W mutations are thought to affect the stem cell compartment of the hematopoietic system. The affected populations may include the spleen colony forming units

(CFU-S) which produce myeloid colonies in the spleen of lethally irradiated mice as well as cell with long term repopulation potential for the various cell lineages (McCulloch et al., 1964; Russell, 1970; Russell 1979; Harrison, 1980; Barker and McFarland, 1988). It will now be of interest to determine if there is an effect of KL in the self-renewal or the differentiation potential of hematopoietic stem cell populations, possibly in combination with other hematopoietic growth factors, in order to identify the stage(s) where the c-kit/W gene product functions in the stem cell compartment.

[0115] Mutations at the steel locus (Sl) of the mouse produce pleiotropic phenotypes in hematopoiesis, melanogenesis and gametogenesis similar to those of mice carrying W mutations (Russell, 79; Silvers, 79). However, in contrast to W mutations, Sl mutations affect the microenvironment of the cellular target of the mutation and are not cell autonomous (Russell, 1970). Because of the parallel and complementary effects of the W and the Sl mutations, it has been suggested that the Sl gene encode the ligand of the c-kit receptor or a gene product that is intimately linked to the production and/or function of this ligand (Chabot et al., 1988). In agreement with this conjecture Sl/Sl^d embryo fibroblasts or conditioned medium from Sl/Sl^d fibroblasts fail to support the proliferation of BMMC and mast cell progenitors, respectively, and presumably do not produce functional KL (Fujita et al., 1989 and Jarboe, et al., 1989). If KL is the ligand of the c-kit receptor, then molecular analysis will enable the determination of the identity of KL with the gene product of the Sl locus; in addition, one would predict that administration of KL to mice carrying Sl mutations would lead to the cure of at least some symptoms of this mutation.

[0116] Experiment Number 2—Isolation of the Nucleic Acid Sequence

[0117] Experimental Methods

[0118] Mice and Tissue Culture

[0119] WBB6+/+, C57BL/6J, C57BL/67 W^v/+, WB6W/+, C3HeB/FeJ a/a Ca^J Sl Hm, and *M. spretus* mice were obtained from The Jackson Laboratory (Bar Harbor, Me.). For the interspecific cross, female C57Bl/6J and male *M. spretus* mice were mated; progeny of this cross were scored for inheritance of C57BL/6J or M. spretus alleles as described infra. (C57BL/6J ×*M. spretus*) F1 female offspring were backcrossed with C57BL/6J males.

[0120] Mast cells were grown from the bone marrow of adult +/+, W^v/W^v and W/+mice and W/W fetal liver of day 14-15 fetuses in RPMI 1640 medium supplemented with 10% fetal cell serum (FCS), conditioned medium from WEHI-3B cells, nonessential amino acids, sodium pyruvate, and 2-mercaptoethanol (RPMI-Complete) (Nocka et al., 1990a; Yung and Moore, 1982). BALB/c 3T3 cells (Aaronson and Todaro, 1968) were obtained from Paul O'Donnell (Sloan-Kettering Institute, New York, New York) and were grown in Dulbecco's modified MEM supplemented with 10% calf serum, penicillin, and streptomycin.

[0121] Purification and Amino Acid Sequence Determination of KL

[0122] KL was purified from conditioned medium of BALB/c 3T3 cells by using a mast cell proliferation assay as described elsewhere (Nocka et al., 1990b). Conditioned medium was then concentrated 100- to 200-fold with a Pellicon ultrafiltration apparatus followed by an Amicon stirred cell. The concentrate was then chromatographed on Blue Agarose (Bethesda Research Laboratories, Gaithersburg, Md.), and the flow-through, which contained the active material, was concentrated in dialysis tubing with polyethylene glycol 8000 and then fractionated by gel filtration chromatography on an ACA54 Ultrogel (LKB, Rockland, Md.) column. The biological activity eluted as a major and a minor peak, corresponding to 55-70 kd and 30 kd, respectively. The fractions of the main peak were pooled, dialyzed, and fractionated by FPLC on a DEAE-5PW column with an NaCl gradient. The activity eluted at 0.11 M NaCl from the FPLC column. Peak fractions were pooled and subjected to HPLC with a semi-preparative C18 column and an ammonium acetate-n-propanol gradient. The active material eluted at 30% n-propanol from the semipreparative C18 column was diluted 1:1 and re-chromatographed by using an analytical C18 column. A single peak of activity eluted again at 30% n-propanol, which corresponded to a major peak of absorbance (280 nm) in the eluant profile. Similar results were obtained by using a C4 column with H_2O and acetonitrile containing 0.1% TFA as solvents. N-terminal amino acid sequence was determined on an Applied Biosystems 477A on-line PTH amino acid analyzer (Hewick et al., 1961).

[0123] Iodination

[0124] KL was iodinated with chloramine T with modifications of the method of Stanley and Guilbert (1981). Briefly, the labeling reaction contained 200 ng of KL, 2 nmol of chloramine T, 10% dimethyl sulfoxide, and 0.02% polyethylene glycol 8000, in a total volume of 25 μl in 0.25 M phosphate buffer (pH 6.5). The reaction was carried out for 2 min. at 4° C. and stopped by the addition of 2 nmol of cysteine and 4 μM KI. KL was then separated from free NaI by gel filtration on a PD10 column (Pharmacia). Iodinated KL was stored for up to 2 weeks at 4° C.

[0125] Binding Assay

[0126] Binding buffer contained RPMI 1640 medium, 5% BSA (Sigma), 20 mM HEPES (pH 7.5) and NaN_3. Binding experiments with nonadherent cells were carried out in 96-well tissue culture dishes with 2×10^5 cells per well in a volume of 100 μl. Binding experiments with ψ2 cells were carried out in 24-well well dishes in a volume of 300 μl. Cells were equilibrated in binding buffer 15 minutes prior to the addition of competitor or labeled KL. To determine nonspecific binding, unlabeled KL or anti-c-kit rabbit serum was added in a 10-fold excess 30 minutes prior to the addition of ^{125}I-KL. Cells were incubated with ^{125}1-*KL for* 90 minutes, and nonadherent cells were pelleted through 150 μl of FCS. Cell pellets were frozen and counted.

[0127] Immunoprecipitation and Cross-Linking

[0128] BMMC were incubated with ^{125}I-KL under standard binding conditions and washed in FCS and then in PBS at 40° C. Cells were lysed as previously described (Nocka et al., 1989) in 1% Triton X-100, 20 mM Tris (pH 7.4), 150 mM NaCl, 20 mM EDTA, 10% glycerol, and protease inhibitors phenylmethylsufonyl fluoride (1 mM) and leupeptin (20 μg/ml). Lysates were immunoprecipitated with normal rabbit serum, or c-kit specific sera raised by immuni-

zation of rabbits with a fragment of the v-kit tyrosine kinase domain (Majumder et al., 1988); or the murine c-kit expressed from a cDNA in a recombinant vaccinia virus (Nocka et al., 1990a). For coprecipitation experiments, immunoprecipitates were washed three times with wash A (0.1% Triton X-100, 20 mM Tris [pH 7.4], 150 mM NaCl, 10% glycerol), solubilized in SDS sample buffer, and analyzed by SDS-PAGE and autoradiography. For cross-linking experiments, cells were incubated with disuccinimidyl substrate (0.25 mg/ml) in PBS for 30 minutes at 4° C., washed in PBS, and lysed as described above. Washing conditions following precipitation were as follows: one time in wash B (50 mM Tris, 500 mM NaCl, 5 mM EDTA, 0.2% Triton X-100), three times in wash C (50 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 5mM EDTA), and one time in wash D (10 mM Tris, 0.1% Triton X-100).

[0129] cDNA Synthesis, PCR Amplification (RT-PCR), and Sequence Determination

[0130] The RT-PCR amplification was carried out essentially as described (Tan et al., 1990). For cDNA synthesis, 1 μg of poly(A)-RNA from confluent BALB/c 3T3 cells in 25 μl of 0.05 M Tris-HCl (pH 8.3), 0.075 M KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 μM dNTPs and 25 U of RNAsin (Promega) was incubated with 50 pmol of antisense primer and 50 U of Moloney murine leukemia virus reverse transcriptase at 40° C. for 30 minutes. Another 50 U of reverse transcriptase was added, and incubation was continued for another 30 minutes. The cDNA was amplified by bringing up the reaction volume to 50 μl with 25 μl of 50 mM KCl, 10 mM Tris-HCl(pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, and 200 μM dNTPs, adding 50 pmol of sense primer and 2.5 U of Tag DNA polymerase, and amplifying for 25-30 cycles in an automated thermal cycler (Perkin-Elmer Cetus). The amplified fragments were purified by agarose gel electrophoresis, digested with the appropriate restriction enzymes, and subcloned into M13 mp18 and M13 mp19 for sequence analysis (Sanger et al., 1977).

[0131] cDNA Isolation and Sequencing

[0132] A mouse 3T3 fibroblast lambda g11 cDNA library obtained from Clontech was used in this work. Screening in duplicate was done with Escherichia coli Y1090 as a host bacterium (Sambrook et al, 1989); 5' end-labeled oligonucleotide was used as a probe. Hybridization was in 6×SSC at 63° C., and the final wash of the filters was in 2×SSC, 0.2% SDS at 63° C. Recombinant phage were digested with EcoRI and the inserts subcloned into M13 for sequence analysis. The nucleotide sequence of these cDNAs was determined, on both strands and with overlaps, by the dideoxy chain termination method of Sanger et al. (1977) by using synthetic oligodeoxynucleotides (17-mers) as primers.

[0133] DNA and RNA Analysis

[0134] Genomic DNA was prepared from tail fragments, digested with restriction enzymes, electrophoretically fractionated, and transferred to nylon membranes as described elsewhere (D.R.B. and P.L., submitted). For hybridization, the 1.4 kb KL cDNA and TIS Dra/SaI (a probe derived from the transgene insertion site in the transgenic line TG.EB (Schmidt et al., 1988]) were used as probes.

[0135] BALB/c 3T3 cells were homogenized in guanidinium isothiocyanate, and RNA was isolated according the method of Chirgwin et al. (1979). Total cellular RNA (10 μg) and poly(A)$^+$ RNA were fractionated in 1% agarose-formaldehyde gels and transferred to nylon membranes (Nytran, Schleicher & Schuell); prehybridization and hybridization were performed as previously described (Lehrach et al., 1978; Nocka et al., 1989). The 1.4 kb KL cDNA labeled with [^{32}P)phosphate was used as a probe for hybridization (Feinberg and Vogelstein, 1983).

[0136] Preparation of c-kit and c-kit Ligand Monoclonal Antibodies

[0137] For the isolation of human monoclonal antibodies, eight week old Balb/c mice are injected intraperitoneally with 50 micrograms of a purified human soluble c-kit ligand (KL) polypeptide, or a soluble fragment thereof, of the present invention (prepared as described above) in complete Freund's adjuvant, 1:1 by volume. Mice are then boosted, at monthly intervals, with the soluble ligand polypeptide or soluble ligand polypeptide fragment, mixed with incomplete Freund's adjuvant, and bled through the tail vein. On days 4, 3, and 2 prior to fusion, mice are boosted intravenously with 50 micrograms of polypeptide or fragment in saline. Splenocytes are then fused with non-secreting myeloma cells according to procedures which have been described and are known in the art to which this invention pertains. Two weeks later, hybridoma supernatant are screened for binding activity against c-kit receptor protein as described hereinabove. Positive clones are then isolated and propagated.

[0138] Alternatively, to produce the monoclonal antibodies against the c-kit receptor, the above method is followed except that the method is followed with the injection and boosting of the mice with c-kit receptor protein.

[0139] Alternatively, for the isolation of murine monoclonal antibodies, Sprague-Dawley rats or Louis rats are injected with murine derived polypeptide and the resulting splenocydes are fused to rat myeloma (y3-Ag 1.2.3) cells.

[0140] Experimental Results

[0141] Isolation and Characterization of Murine cDNAs Encoding the Hematopoietic Growth Factor KL

[0142] The KL protein was purified from conditioned medium from BALB/c 3T3 cells by a series of chromatographic steps including anion exchange and reverse-phase HPLC as described hereinabove (Nocka et al, 1990b). As previously noted, the sequence of the N-terminal 40 amino acids of KL was determined to be:

K E I X G N P V T D N V K D I T K L V A N L P N D
Y M I T L N Y V A G M X V L P.

[0143] To derive a nondegenerate homologous hybridization probe, fully degenerate oligonucleotide primers corresponding to amino acids 10-16 (sense primer) and 31-36 (antisense is primer) provided with endonuclease recognition sequences at their 5' ends were synthesized as indicated in **FIG. 8**. A cDNA corresponding to the KL mRNA sequences that specify amino acids 10-36 of KL was obtained by using the reverse transcriptase modification of the polymerase chain reaction (RT-PCR). Poly (A)$^+$ RNA from BALB/c 3T3 cells was used as template for cDNA synthesis and PCR amplification in combination with the degenerate oligonucleotide primers.

[0144] The amplified DNA fragment was subcloned into M13, and the sequences for three inserts were determined. The sequence in between the primers was found to be unique and to specify the correct amino acid sequence (**FIG. 8**). An oligonucleotide (49 nucleotides) corresponding to the unique sequence of the PCR products was then used to screen a λ gt11 mouse fibroblast library. A 1.4 kb clone was obtained that, in its 3' half, specifies an open reading frame that extends to the 3' end of the clone and encodes 270 amino acids (**FIG. 11**). The first 25 amino acids of the KL amino acid sequence have the characteristics of a signal sequence. The N-terminal peptide sequence that had been derived from the purified protein (amino acids 26-65) follows the signal sequence. A hydrophobic sequence of 21 amino acids (residues 217-237) followed at its carboxyl end by positively charged amino acids has the features of a transmembrane segment. In the sequence between the signal peptide and the transmembrane domain, four potential N-linked glycosylation sites and four irregularly spaced cysteines are found. A C-terminal segment of 33 amino acids follows the transmembrane segment without reaching a termination signal (end of clone). The KL amino acid sequence therefore has the features of a transmembrane protein: an N-terminal signal peptide, an extracellular domain, a transmembrane domain, and a C-terminal intracellular segment.

[0145] RNA blot analysis was performed to identify KL-specific RNA transcripts in BALB/c 3T3 cells (**FIG. 12**). A major transcript of 6.5 kb and two minor transcripts of 4.6 and 3.5 kb were identified on a blot containing poly(A)$^+$ RNA by using the 1.4 kb KL cDNA as a probe. Identical transcripts were detected by using an end-labeled oligonucleotide derived from the N-terminal protein sequence. This result then indicates that KL is encoded by a large mRNA that is abundantly expressed in BALB/c 3T3 cells.

[0146] The Soluble form of KL is a Ligand of the c-kit Receptor

[0147] The fibroblast-derived hematopoietic growth factor KL had been shown to facilitate the proliferation of primary bone marrow mast cells and peritoneal mast cells and to display erythroid burst-promoting activity. To determine if KL is the ligand of the c-kit receptor, it was first thought to demonstrate specific binding of KL to cells that express high levels of the c-kit protein: mast cells (BMMC) and NIH ψ2 cells expressing the c-kit cDNA. KL was labeled to high specific activity with ^{125}I by using the modified chloramine T method (Stanley and Guilbert, 1981). Analysis of the labeled material by SDS-PAGE showed a single band of 28-30 kd (**FIG. 13**), and mast cell proliferation assays indicated that the labeled material had retained its biological activity. Binding of increasing concentrations of ^{125}I-KL to NIH ψ2 cells expressing the c-kit cDNA, NIH ψ2 control cells, normal BMMC, and W/W, W/+, and W^v/W^v BMMC at 4° C. was measured. The results shown in **FIG. 14** indicate binding of labeled KL to NIH ψ2 c-kit cells and to +/+, W/+, and W^v/W^v mast cells, but not to NIH ψ2 control cells or W/W mast cells. The W^v mutation is the result of a missense mutation in the kinase domain of c-kit that impairs the in vitro kinase activity but does not affect the expression of the c-kit protein on the cell surface (Nocka et al., 1990a). By contrast, W results from a deletion due to a splicing defect that removes the transmembrane domain of the c-kit protein; the protein therefore is not expressed on the cell surface (Nocka et al., 1990a). Furthermore, binding of ^{125}I-KL could be completed with unlabeled KL and with two different anti-c-kit antisera. These results indicated binding of ^{125}I-labeled KL cells that express c-kit on their cell surface.

[0148] To obtain more direct evidence that KL is the ligand of the c-kit receptor, it was determined if receptor-ligand complexes could be purified by immunoprecipitation with c-kit antisera. This experiment requires that a KL-c-kit complex be stable and not be affected by the detergents used for the solubilization of the c-kit receptor. Precedent for such properties of receptor-ligand complexes derives from the closely related macrophage colony-stimulating factor (CSF-1) receptor and PDGF receptor systems (Sherr et al, 1985; Heldin et al., 1989). ^{125}I-KL was bound to receptors on BMMC by incubation at 4° C. Upon washing to remove free ^{125}I-KL, the cells were solubilized by using the Triton X-100 lysis procedure and precipitated with anti-v-kit and anti-c-kit rabbit sera conjugated to protein A-Sepharose. ^{125}I-KL was retained in immunoprecipitates obtained by incubation with anti-kit sera but not with nonimmune controls, as shown by the analysis of the immune complexes by SDS-PAGE (**FIG. 15A**), where recovery of intact ^{125}I-KL was demonstrated from the samples containing the immune complexes prepared with anti-kit sera.

[0149] To further characterize the c-kit-KL receptor-ligand complexes, it was determined whether KL could be cross-linked to c-kit. BMMC were incubated with ^{125}I-KL, washed and treated with the cross-linked disucciminidyl substrate. Cell lysates were then immunoprecipitated with anti-v-kit antiserum and analyzed by SDS-PAGE. Autoradiography indicated three species: one at approximately 30 kd, representing KL coprecipitated by not cross-linked to c-kit; one at 180-190 kd, corresponding to a covalently linked c-kit-KL monomer-monomer complex; and a high molecular weight structure that is at the interface between the separating and stacking gels (**FIG. 15B**). Molecular structures of similar size were observed if the cell lysates were separated directly on -SDS-PAGE without prior immunoprecipitation. Following precipitation with nonimmune serum, no ^{125}I-labeled molecules were observed. The formation of the high molecular weight structures was dependant on the incubation of KL with mast cells and was not observed by cross-linked KL with itself. Taken together, these results provide evidence that KL specifically binds to the c-kit receptor and is a ligand of c-kit.

[0150] Mapping of KL to the Sl Locus

[0151] To test whether KL is encoded at the Sl locus, recombination analysis was used to determine the map position of KL with respect to a locus that is tightly linked to Sl. This locus is the site of the transgene insertion in the transgenic line TG.EB (Schmidt et al., 1988). It was determined that genomic sequences cloned from the insertion site map 0.8±0.8 cM from Sl (D.R.B. and P.L., submitted). This therefore represents the closest known marker to Sl.

[0152] To map KL with respect to the transgene insertion site, interspecific mapping analysis was employed utilizing crosses of C57BL/6J mice with mice of the species *Mus spretus*. This strategy exploits the observation that restriction fragment length polymorphism (RFLPs) for cloned DNA are observed much more frequently between mice of different species than between different inbred laboratory strains (Avner et al., 1988). Linkage between the 1.4 kb KL

cDNA probe and TIS Dra/SaI, a probe from the transgene insertion site, was assessed by scoring for concordance of inheritance of their respective C57BL/6J or *M. spretus* alleles. These could be easily distinguished by analyzing RFLPs that are revealed by TaqI restriction digests. The results of this linkage analysis are shown in Table 2. Only one recombinant was found in 53 progeny. This corresponds to a recombination percentage of 1.9±1.9. Since this value is very close to the genetic distance measured between the transgene insertion site and Sl, this result is consistent with the notion that KL maps to the Sl locus.

TABLE 2

Mapping of the Position of the KL Gene by Linkage Analysis Using an Interspecific Cross

	Progeny			
Probe	Nonrecominant		Recombinant	
1.4 kb KL cDNA	B6	Sp	B6	Sp
TIS Dra/SaI	B6	Sp	Sp	B6
	32	20	0	1

n = 53
% recombination = 1.9 ± 1.9
The concordance of inheritance of C57Bl/6J (B6) or *M. spretus* (Sp) alleles in progeny of an interspecific cross (see Experimental Procedures) was determined by scoring for TaqI RFLPs of the KL 1.4 kb cDNA probe and TIS Dra/SaI (a probed from a transgene insertion site that is tightly linked to Sl; see Results). Percent recombination was calculated according to Green (1981).

[0153] The locus identified by KL was also examined in mice that carry the original Sl mutation (Sarvella and Russell, 1956). For this purpose, the observation that the transgene insertion site locus is polymorphic in inbred strains was taken advantage of, and was utilized to determine the genotype at Sl during fetal development (D.R.B. and P.L., submitted). C57BL/6J mice that carry the S1 mutation maintained in the C3HeB/FeJ strain were generated by mating, and F1 progeny carrying the Sl allele were intercrossed (C57BL/6J Sl_{3CH}/+Sl^{C3H}/+). Homozygous SIISI progeny from this mating are anemic and are homozygous for a C3HeB/FeJ-derived RFLP at the transgene integration site **(FIG. 16)**. Nonanemic mice are either heterozygous SII+ or wild type, and are heterozygous for the C3HeB/FeJ- and C57BL/6J-derived polymorphism or are homozygous for the C57BL/6J polymorphism, respectively. When genomic DNA from SII+ and SIISI mice was analyzed using the 1.4 kb KL cDNA probe, no hybridization to the homozygous SIISI DNA was observed **(FIG. 16)**. It thus appears that the locus that encodes the KL protein is deleted in the Sl mutation. This finding further supports the notion that KL is the product of the Sl gene.

[0154] Experimental Discussion

[0155] The discovery of allelism between the c-kit proto-oncogene and the murine W locus revealed the pleiotropic functions of the c-kit receptor in development and in the adult animal. Furthermore, it provided the first genetic system of a transmembrane tyrosine kinase receptor in a mammal. Mutations at the Sl locus and at the c-kit/W locus affect the same cellular targets. Because of the complementary and parallel properties of these mutations, it was proposed that the ligand of the c-kit receptor is encoded by the Sl locus.

[0156] The experiments reported herein provide evidence that the Sl gene encodes the ligand of the c-kit receptor. The evidence for this conclusion is a follows. Based on the knowledge of the function of the c-kit receptor designated KL, a putative ligand of the c-kit receptor designated KL was identified and purified (Nocka et al., 1990b). It was also demonstrated that specific binding of KL to the c-kit receptor, as evidenced by the binding of KL to cells expressing a functional c-kit receptor and the formation of a stable complex between KL and the c-kit protein. KL-specific cDNA clones were derived and it was shown that KL maps to the Sl locus on mouse chromosome 10. In addition, it was also demonstrated that KL sequences are deleted in the genome of the Sl mouse. Taken together, these results suggest that KL is encoded by the Sl locus and is the ligand of the c-kit receptor, thus providing a molecular basis for the Sl defect.

[0157] The amino acid sequence predicted from the nucleotide sequence of the KL cDNA clone suggests that KL is synthesized as an integral transmembrane protein. The structural features of the primary translation product of KL therefore are akin to those of CSF-1. CSF-1 is synthesized as a transmembrane molecule, which is processed by proteolytic cleavage to form a soluble product that is secreted (Kawasaki et al., 1985; Rettenmier and Roussel, 1988). Presumable, like CSF-1, KL is also synthesized as a cell surface molecule that may be processed to form a soluble protein. The protein purified from conditioned medium of BALB/c 3T3 cells then would represent the soluble form of KL that was released from the cell membrane form by proteolytic cleavage. Although the post-translational processing and expression of the KL protein have not yet been characterized, a cell surface-bound form of KL may mediate the cell-cell interactions proposed for the proliferative and migratory functions of the c-kit/W receptor system. In agreement with the notion of a cell membrane-associated form of KL, a soluble c-kit receptor-alkaline phosphatase fusion protein has been shown to bind to the cell surface of BALB/c 3T3 cells but not to fibroblasts derived from SIISI mice (Flanagan and Leder, 1990).

[0158] A most significant aspect of the identification of the ligand of the c-kit receptor lies in the fact that it will facilitate the investigation of the pleiotropic functions of c-kit. In the hematopoietic system c-kit/W mutations affect the erythroid and mast cell lineages, and an effect on the stem cell compartment has been inferred as well. In erythroid cell maturation c-kit/KL plays an essential role, and this is best seen by the anemia of mutant animals. Furthermore, the number of CFU-E in fetal livers from W/W and $SIISI^{d}$ animals is repressed, whereas the number of BFU-E remains normal, suggesting that c-kit/KL facilitates the progression from the BFU-E to the CFU-E stage of differentiation (Chui et al., 1978; Nocka et al., 1989). In this regard, KL has been shown to stimulate the proliferation and differentiation of BFU-E (day 7) as well as earlier erythroid multipotential precursors in bone marrow, which appear at later times in culture (day 14-20) (Nocka et al., 1990b).

[0159] An essential role for c-kit/KL in the proliferation, differentiation, and/or survival of mast cells in vivo has been inferred because of the absence of mast cells in W and Sl mutant mice (Kitamura et al., 1978; Kitamura and Go, 1978; Kitamura and Fujita, 1989). The precise stage(s) at which c-kit/KL function is required in mast cell differentiation is

not known. The in vitro derivation of BMMC from bone marrow or fetal liver does not require c-kit-KL function since BMMC can be generated with comparable efficiency from both normal and W mutant mice (Yung and Moore, 1982). Applicants' demonstration of proliferation of BMMC and connective tissue-type mast cells in response to KL indicates a role for c-kit/KL at multiple stages in mast cell proliferation and differentiation independent of IL-3 and IL-4, which are thought to be mediators of allergic and inflammatory responses (Stevens and Austen, 1989). In the stem cell compartment the affected populations possibly include the spleen colony-forming units (CFU-S), which produce myeloid colonies in the spleen of lethally irradiated mice, as well as cells with long-term repopulation potential for the various cell lineages (McCulloch et al., 1964; Russell, 1979; Harrison, 1980; Barker and McFarland, 1988). It will now be of interest to determine the effect of KL on the self-renewal or the differentiation potential of hematopoietic stem cell populations in vitro, possibly in combination with other hematopoietic growth factors, in order to identify the stage(s) where c-kit/KL functions in stem cells. Another possible function for c-k might be to facilitate the transition from noncycling to cycling cells (McCulloch, 1970). The increased radiation sensitivity of $SIISI^d$ and of W/W^v mice might suggest such a role in stem cell dynamics; furthermore, the related PDGF receptor is known to promote entry into the cell cycle.

[0160] In gametogenesis the W and Sl mutations affect the proliferation and the survival of primordial germ cells, and their migration from the yolk sac splanchnopleure to the genital ridges during early development. In postnatal gametogenesis c-kit expression has been detected in immature and mature oocytes and in spermatogonia A and B as well as in interstitial tissue (Orr-Urtreger et al., 1990). In melanogenesis c-kit/KL presumable functions in the proliferation and migration of melanoblast from the neural crest to the periphery in early development as well as in mature melanocytes. The availability of KL may now facilitate in vitro studies of the function of the c-kit receptor in these cell systems.

[0161] The microenvironment in which c-kit-expressing cells function is defective in Sl mutant mice and is the presumed site where the c-kit ligand is produced. Because of the extrinsic nature of the mutation, the precise identity of the cell types that produce KL in vivo is not known. in vitro systems that reproduce the genetic defect of the W and the Sl mutations, however, have shed some light on this question. In the long-term bone marrow culture system, $SIISI^d$ adherent cells are defective but the nonadherent hematopoietic cells are not, and in the mast cell-fibroblast coculture system $SIISI^d$ fibroblasts are defective but the mast cells are not (Dexter and Moore, 1977; Fujita et al., 1989). The results from these in vitro systems then would suggest that hematopoietic stromal cells and embryonic and connective tissue fibroblasts produce KL. The BALB/c 3T3 cell line, which is of embryonic origin, expresses significant levels of KL and was the source for its purification. Knowledge of KL-expressing cell types may help to evaluate if there is a function for c-kit in the digestive tract, the nervous system, the placenta, and certain craniofacial structures, sites where c-kit expression has been documented (Nocka et al., 1989; Orr-Urtreger et al., 1990). No Sl or W phenotypes are known to be associated with these cell systems.

[0162] Interspecific backcrosses were used to establish close linkage between the KL gene, the Sl locus, and the transgene insertion locus Tg.EB on mouse chromosome 10. A similar approach had previously been used to map the Tg.EB locus in the vicinity of Sl (D.R.B. and P.L. submitted). The finding that the KL coding sequences are deleted in the original Sl allele, however, supports the identity of the Sl locus with the KL gene. The size of the deletion in the Sl allele at this time is not known. It will be important to determine whether it affects neighboring genes as well.

[0163] The lack of KL coding sequences in the Sl allele indicates that this allele is a KL null mutation. When homozygous for the Sl allele, most mice die perinatally of macrocytic anemia, and rare survivors lack coat pigmentation and are devoid of germ cells (Bennett, 1956). This phenotype closely parallels that of severe c-kit/W loss-of-function mutations, in agreement with the ligand-receptor relationship of KL and c-kit. Although differences exist between SIISI and W/W homozygotes, e.g., in germ cell development, Sl may have a more pronounced effect, and in hematopoiesis Sl may cause a more severe anemia; however, it is not known if these differences are a result of different strain backgrounds or are possibly effects of the Sl deletion on neighboring genes (Bennett, 1956).

[0164] The original W mutation is an example of a c-kit null mutation (Nocka et al., 1990a). When heterozygous with the normal allele, WI+mice typically have a ventral spot but no coat dilution and no effects on hematopoiesis and gametogenesis. The weak heterozygous phenotype of WI^+ mice is in contrast to the phenotype of heterozygous SII^+ mice, which have moderate macrocytic anemia and a diluted coat pigment in addition to a ventral spot and gonads that are reduced in size. Thus 50% gene dosage of KL is limiting and is not sufficient for normal function of the c-kit receptor, yet 50% dosage of the c-kit receptor does not appear to be limiting in most situations.

[0165] The c-kit receptor system functions in immature progenitor cell populations as well as in more mature cell types in hematopoiesis, gametogenesis, and melanogenesis. Severe Sl or W mutations may block the development of these cell W mutations in which c-kit/KL function is only partially impaired often reveal effects in more mature cell populations. Numerous weak Si alleles are known. Their phenotypes, e.g., in gametogenesis and melanogenesis, will be of great value in the elucidation of the pleiotropic functions of the c-kit receptor system.

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SEQUENCE LISTING

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(iii) NUMBER OF SEQUENCES: 2

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(A) LENGTH: 825 base pairs
(B) TYPE: nucleic acid

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        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

  (iii) HYPOTHETICAL: N

   (iv) ANTI-SENSE: Y

    (v) FRAGMENT TYPE: N-terminal

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        (H) CELL LINE: Swiss 3T3 fibroblasts

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        (B) CLONE: 1.4 KL

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        (D) OTHER INFORMATION: /product= "extracellular domain"
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        (B) LOCATION: 282..291
        (D) OTHER INFORMATION:

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        (A) NAME/KEY: misc_signal
        (B) LOCATION: 304..312
        (D) OTHER INFORMATION:

   (ix) FEATURE:
        (A) NAME/KEY: misc_signal
        (B) LOCATION: 448..457
        (D) OTHER INFORMATION:

   (ix) FEATURE:
        (A) NAME/KEY: misc_signal
        (B) LOCATION: 598..606
        (D) OTHER INFORMATION:

    (x) PUBLICATION INFORMATION:
        (A) AUTHORS: Huang , Eric
             Nocka, Karl
             Beier, David R.
             Chu, Yang-Yuan
             Buck, Jochen
             Lahm, Hans-Werner
             Wellner, Daniel
             Leder, Phillip
             Besmer, Peter
        (B) TITLE: The Hematopoietic Growth Factor KL Is Encoded By
             The Sl Locus And Is The Ligand Of The c-kit Receptor, The
             Gene Product Of The W Locus
        (C) JOURNAL: Cell
        (D) VOLUME: 63
        (G) DATE: 5-OCT-1990-1990
        (K) RELEVANT RESIDUES IN SEQ ID NO:1: FROM 1 TO 825
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AGTAATTACT CCATCATAGA CAAACTTGGG AAAATAGTGG ATGACCTCGT GTTATGCATG 360

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ACTCCTGAAG AATTCTTTAG TATTTTCAAT AGATCCATTG ATGCCTTTAA GGACTTTATG 480

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(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(v) FRAGMENT TYPE: N-terminal

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Huang , Eric
Nocka, Karl
Beier, David R.
Chu, Tang-Yuan
Buck, Jochen
Lahm, Hans-Werner
Wellner, Daniel
Leder, Phillip
Besmer, Peter
(B) TITLE: The Hematopoietic Growth Factor KL Is Encoded By
The Sl Locus And Is The Ligand Of The c-kit Receptor, The
Gene Product Of The W Locus
(C) JOURNAL: Cell
(D) VOLUME: 63
(G) DATE: 05-OCT-1990

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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20 25 30

Val Ala Gly Met Xaa Val Leu Pro
35 40

What is claimed is:

1. An isolated nucleic acid molecule which encodes an amino acid sequence corresponding to a c-kit ligand (KL).

2. An isolated nucleic acid molecule of claim 1, wherein the c-kit ligand (KL) is a human c-kit ligand (KL).

3. An isolates nucleic acid molecule of claim 1, wherein the c-kit ligand (KL) is a murine c-kit ligand (KL).

4. An isolated nucleic acid molecule of claim 1, wherein the isolated nucleic acid molecule is a DNA molecule.

5. The DNA molecule claim 4, wherein the DNA molecule is a cDNA molecule.

6. An isolated nucleic acid molecule of claim 1, wherein the isolated nucleic acid molecule is an RNA molecule.

7. The isolated nucleic acid molecule of claim 4 wherein the isolated nucleic acid molecule is operatively linked to a promoter of RNA transcription.

8. A vector which comprises the isolated nucleic acid molecule of claim 1.

9. A vector of claim 8 which comprises a plasmid.

10. A vector of claim 8 which comprises a virus.

11. A host vector system for the production of an amino acid sequence which is the c-kit ligand which comprises the plasmid of claim 9 in a suitable host.

12. A host vector system of claim 11, wherein the suitable host is a eucaryotic cell.

13. A host vector system of claim 13, wherein the eucaryotic cell is a mammalian cell.

14. A host vector system of claim 13, wherein the eucaryotic cell is an insect cell.

15. A host vector system of claim 13, wherein the eucaryotic cell is a yeast cell.

16. A host vector system of claim 11, wherein the suitable host is a procaryotic cell.

17. An isolated c-kit ligand (KL) polypeptide.

18. A soluble c-kit ligand (KL) polypeptide wherein the soluble, c-kit ligand (KL) polypeptide comprises a fragment of the isolated c-kit ligand (KL) polypeptide of claim 17.

19. A soluble, mutated, c-kit ligand (KL) polypeptide wherein the biological activity mediated by the binding of the ligand to the receptor is destroyed.

20. A substance capable of specifically forming a complex with the soluble, c-kit ligand (KL) polypeptide of claim 18.

21. The substance of claim 20, wherein the substance is a monoclonal antibody.

22. The substance of claim 21, wherein the monoclonal antibody is a human monoclonal antibody.

23. The soluble, c-kit ligand (KL) polypeptide of claim 17 conjugated to an imageable agent.

24. The soluble, c-kit ligand (KL) polypeptide of claim 23, wherein imageable agent is selected from the group consisting of radioisotopes, dyes or enzymes.

25. The soluble, c-kit ligand (KL) polypeptide of claim 18 conjugated to a therapeutic agent.

26. The soluble, c-Kit ligand (KL) polypeptide of claim 25, wherein the therapeutic agent is selected from the group consisting of toxins, chemotherapeutic agents or radioisotopes.

27. A method for producing a c-kit ligand (KL) polypeptide which comprises growing the host vector system of claim 11 under suitable conditions permitting production of the c-kit ligand (KL) polypeptide and recovering the resulting c-kit ligand (KL) polypeptide.

28. The c-kit ligand (KL) produced by the method of claim 27.

29. A Pharmaceutical composition which comprises the soluble, mutated c-kit ligand of claim 19 and a pharmaceutically acceptable carrier.

30. A pharmaceutical composition which comprises the soluble, c-kit ligand (KL) of claim 18 and a pharmaceutically acceptable carrier.

31. A method of modifying a biological function associated with c-kit cellular activity which comprises contacting cells, whose function is to be modified, with an effective amount of the pharmaceutical composition of claim 30, effective to modify the biological function of the cells.

32. The method of claim 31, wherein the biological function is the propagation of a cell that expresses c-kit.

33. The method of claim 32, wherein the cell which expresses c-kit is a hematopoietic cell.

34. The method of claim 31, wherein the biological function is in vitro fertilization.

35. A method of modifying a biological function associated with c-kit cellular activity in a patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 30, effective to modify the biological function associated with c-kit function.

36. A method of stimulating the proliferation of mast cells in a patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 30, effective to stimulate the proliferation of the mast cells in the patient.

37. A method of inducing differentiation of mast cells in a patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 30, which is effective to induce differentiation of the mast cells.

38. A method of inducing differentiation of erythroid progenitors in a patient which comprises administering the patient an effective amount of the pharmaceutical composition of claim 30, which is effective to induce differentiation of the erythroid progenitors.

39. A method of facilitating bone marrow transplantation in a patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 30, effective to facilitate bone marrow transplantation.

40. A method of treating leukemia in a patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 30, effective to treat the leukemia.

41. A method of treating leukemia according to claim 40, wherein the leukemia is acute myelogenous leukemia.

42. A method of treating leukemia according to claim 40, wherein the leukemia is chronic myelogenous leukemia.

43. A method of treating allergies in a patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 29, effective to treat the allergy.

44. A method of treating melanoma in a patient, which comprises administering to the patient an effective amount of the composition of claim 30, effective to treat the melanoma.

45. A method for measuring the biological activity of a c-kit (KL) polypeptide which comprises:

a) incubating normal bone-marrow mast cells with a sample of the c-kit ligand (KL) polypeptide under suitable conditions such that the proliferation of the normal bone-marrow mast cells are induce ;

b) incubating doubly mutant bone-marrow mast cells with a sample of the c-kit ligand (KL) polypeptide under suitable conditions;

c) incubating a. and b. with ^{3}H-thymidine;

d) determining the amount of thymidine incorporated into the DNA of the normal bone-marrow mast cells and the doubly mutant bone-marrow mast cells; and

e) comparing the amount of incorporation of thymidine into the normal bone-marrow mast cells against the amount of corporation of thymidine into doubly mutant bone-marrow mast cells, thereby measuring the bioligical activity of c-kit ligand (KL) polypeptide.

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