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<p>(54) Title: LIGAND FOR THE c-KIT RECEPTOR AND METHODS OF USE THEREOF</p> <p>(57) Abstract</p> <p>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.</p> <p style="text-align: right;">60</p> <p style="text-align: right;">120</p> <p style="text-align: right;">5</p> <p style="text-align: right;">180</p> <p style="text-align: right;">25</p> <p style="text-align: right;">240</p> <p style="text-align: right;">45</p> <p style="text-align: right;">300</p> <p style="text-align: right;">65</p> <p style="text-align: right;">360</p> <p style="text-align: right;">85</p> <p style="text-align: right;">420</p> <p style="text-align: right;">105</p> <p style="text-align: right;">480</p> <p style="text-align: right;">125</p> <p style="text-align: right;">540</p> <p style="text-align: right;">145</p> <p style="text-align: right;">600</p> <p style="text-align: right;">165</p> <p style="text-align: right;">660</p> <p style="text-align: right;">185</p> <p style="text-align: right;">720</p> <p style="text-align: right;">205</p> <p style="text-align: right;">780</p> <p style="text-align: right;">225</p> <p style="text-align: right;">235</p> <p style="text-align: right;">265</p> <p style="text-align: right;">840</p> <p style="text-align: right;">245</p> <p style="text-align: right;">900</p> <p style="text-align: right;">265</p> <p style="text-align: right;">81^d</p> <p style="text-align: right;">780</p> <p style="text-align: right;">273</p> <p style="text-align: right;">81^d</p> <p style="text-align: right;">1020</p> <p style="text-align: right;">1080</p> <p style="text-align: right;">1140</p> <p style="text-align: right;">1200</p> <p style="text-align: right;">1260</p> <p style="text-align: right;">1320</p> <p style="text-align: right;">1344</p>			

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

5 This invention is a continuation-in-part application of U.S. Serial No. 549,306, filed October 5, 1990, which in turn is a continuation-in-part of U.S. Serial No. 573,483, filed August 27, 1990, the contents of which are hereby incorporated by reference into the present application.

10 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 15 rights in this invention.

Background of the Invention

20 Throughout this application various publications are referred by arabic numerals 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 25 application to more fully describe the state of the art to which this invention pertains.

30 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 (7, 41, 57, 23). c-kit was recently shown to be allelic with the white-spotting (W) locus of the mouse (9, 17, 35). Mutations at the W locus affect proliferation and/or 35 migration and differentiation of germ cells, pigment cells and distinct cell populations of the hematopoietic system during development and in adult life (47, 51). The effects

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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 (46), and a complete absence of connective tissue and mucosal mast cells (72). W mutations exert their effects in a cell autonomous manner (28, 46), and in agreement with this property, c-kit RNA transcripts were shown to be expressed in targets of W mutations (35). High levels of c-kit RNA transcripts were found in primary bone marrow 5 derived mast cells and mast cell lines. Somewhat lower 10 levels were found in melanocytes and erythroid cell lines.

The identification of the ligand for c-kit is of great significance and interest because of the pleiotropic effects 15 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 20 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 25 interleukin 3 (IL-3) and resemble mast cells found in the gastrointestinal mucosa (MMC) (92, 93). Connective tissue mast cells derived from the peritoneal cavity (CTMC) in vitro require both IL-3 and IL-4 for proliferation (79, 75). The interleukins IL-3 and IL-4 are well characterized 30 hematopoietic growth factors which are produced by activated T-cells and by activated mast cells (92, 94, 95, 96, 97). An additional mast cell growth factor has been predicted which is produced by fibroblasts (47). In the absence of IL-3, BMMC and CTMC derived from the peritoneal cavity can be maintained by co-culture with 3T3 fibroblasts (98). 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 (99, 100, 38). 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 5 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 (84). A short term mast cell proliferation assay was developed which means 10 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 15 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 20 evidence for c-kit RNA expression in cellular targets of W mutations (35, 39). The recent characterization of the molecular lesions of several mutant alleles indicated that 25 they are loss-of-function mutations that disrupt the normal activity or expression of the c-kit receptor (35, 100, 101, 36).

Mutations at the steel locus (S1) on chromosome 10 of the mouse result in phenotypic characteristics that are very similar to those seen in mice carrying W mutations, i.e., 30 they affect hematopoiesis, gametogenesis, and melanogenesis (5, 47, 51). Many alleles are known at the S1 locus; they are semidominant mutations, and the different alleles vary in their effects on the different cell lineages and their degree of severity (47, 51). The original S1 allele is a

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severe mutation. SIIISI homozygotes are deficient in germ cells, are devoid of coat pigment, and die perinatally of macrocytic anemia (5, 50). Mice homozygous for the S1 allele, although viable, have severe macrocytic anemia, 5 lack coat pigment, and are sterile. Both SII⁺ and S1^{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, S1 mutations are not cell autonomous and are thought to be caused by a 10 defect in the micro-environment of the targets of these mutations (28, 30, 12). Because of the parallel and complementary characteristics of mice carrying S1 and W mutations, we and others had previously hypothesized that 15 the S1 gene product is the ligand of the c-kit receptor (51, 9).

The proto-oncogene c-kit is the normal cellular counterpart of the oncogene v-kit of the HZ4 - feline sarcoma virus (7). c-kit encodes a transmembrane tyrosine kinase receptor which 20 is a member of the platelet derived growth factor receptor subfamily and is the gene product of the murine white spotting locus (9, 17, 23, 35, 41, 57). The demonstration of identity of c-kit with the W locus implies a function for 25 the c-kit receptor system in various aspects of melanogenesis, gametogenesis and hematopoiesis during embryogenesis and in the adult animal (47,51). In agreement with these predicted functions c-kit mRNA is expressed in cellular targets of W mutations (3, 24, 25, 35, 39).

30 The ligand of the c-kit receptor, KL, has recently been identified and characterized, based on the known function of c-kit/W in mast cells (2, 14, 37, 38, 56, 58, 59). In agreement with the anticipated functions of the c-kit receptor in hematopoiesis KL stimulates the proliferation of

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bone marrow derived and connective tissue mast cells and in erythropoiesis, in combination with erythropoietin, KL promotes the formation of erythroid bursts (day 7-14 BFU-E). Furthermore, recent in vitro experiments with KL have demonstrated enhancement of the proliferation and differentiation of erythroid, myeloid and lymphoid progenitors when used in combination with erythropoietin, GM-CSF, GCSF and IL7 respectively suggesting that there is a role for the c-kit receptor system in progenitors of several hematopoietic cell lineages (27, 37).

Mutations at the steel locus 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 (5, 47, 51). The ligand of the c-kit receptor, KL, was recently shown to be allelic with the murine steel locus based on the observation that KL sequences were found to be deleted in several severe S1 alleles (11, 38, 59). In agreement with the ligand receptor relationship between KL and c-kit, S1 mutations affect the same cellular targets as W mutations, however, in contrast to W mutations, S1 mutations are not cell autonomous and they affect the microenvironment of the c-kit receptor (12, 28, 30). Mutations at the steel locus are semidominant mutations and the different alleles vary in their effects on the different cell lineages and their degree of severity (47, 51). The original S1 allele is an example of a severe S1 mutation. S1/S1 homozygotes are deficient in germ cells, are devoid of coat pigment and they die perinatally of macrocytic anemia (5, 50). Mice homozygous for the S1^d allele, although viable, have severe macrocytic anemia, lack coat pigment and are sterile (6). Both S1/+ and S1^d/+ heterozygotes have a diluted coat color and a moderate macrocytic anemia, but they are fertile,

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although their gonads are reduced in size. Southern blot analysis of Sld/+ DNA by using a KL cDNA as a probe indicated an EcoR1 polymorphism, suggesting that this mutation results from a deletion, point mutation or DNA rearrangement of the KL gene (11).
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Summary of Invention

This invention provides a nucleic acid molecule which encodes an amino acid sequence corresponding to a c-kit 5 ligand (KL) and a purified c-kit ligand (KL) polypeptide.

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 10 treating patients which comprise administering to the patient the pharmaceutical composition of this invention.

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Brief Description of the Figures

Figure 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 (20X concentrated), or medium alone. Incorporation of ³H-thymidine was determined from 24-30 hours of culture.

Figure 2. Chromatographic profiles of the purification of KL.

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.

B. Anion exchange FPLC on a DEAE-5PW column. The NaCl gradient is indicated by a dotted line.

C. Separation on semi-preparative C18 column. The 1-propanol gradient is indicated by a dotted line.

D. Separation on analytical C18 column.

Figure 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

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fractions is shown below.

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

10 B. Analysis of 0.5 ml fractions from analytical C4 column eluted with aqueous .1% TFA and absence of 2-mercapto-ethanol.

15 Figure 4. Proliferation of W* mutant mast cells in response to KL. Mast cells were derived from individual fetal livers from W/+ X W/+ mating, or bone marrow of wildtype, W^V and W⁴¹ heterozygotes and homozygotes. 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/+.

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25 Figure 5. Comparison of c-kit expression and growth factor responsiveness in BMMC and peritoneal mast cells (CTMC/PMC).

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

 B. Determination of c-kit cell surface

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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.

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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.

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Figure 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.

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Figure 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.

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Figure 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 Figure 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.

Figure 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.

Figure 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.

Figure 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

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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.

15 Figure 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 ³²P-labeled 1.4 kb KL cDNA. The migration of 18S and 28S ribosomal RNAs is indicated.

20 Figure 13. SDS-PAGE Analysis of KL.

25 A. Silver staining of KL.

B. Autoradiography of ¹²⁵I-KL.

30 Figure 14. Binding of ¹²⁵I-KL to Mast Cells and c-kit-Expressing ψ 2 Cells.

A. NIH ψ 2/c-kit cells containing the pLJ c-kit expression vector and expressing a high

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level of high c-kit protein.

5 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 +/+).

10 Figure 15. Coprecipitation and Cross-Linking of ¹²⁵I-KL with the c-kit receptor on mast cells.

15 A. Coprecipitation of KL with normal rabbit serum (NRS) or two anti-c-kit rabbit antisera (α -c-kit).

20 B. Cross-linking of KL to c-kit with disuccinimidyl substrate. SDS-page analysis was on either 12% or 7.5% polyacrylamide gels. Cross-linked species are labeled "KL + cK".

25 Figure 16. RFLP analysis of Taql-digested DNA from S1/+ and SIISI mice. The S1 allele from C3HeB/Fej a/a CaJ S1 Hm mice was introduced into a C57BL/6J S1 Hm mice was introduced into a C57BL/6J background, and progeny of a C57BL/6J S1^{C3H} x S1^{C3H} cross were evaluated.

30 A. Hybridazation 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.

B. Hybridization of the same blot to TIS Dra/SaI, a probe that is tightly linked to

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S1 (see Detailed Description, *infra*). This probe identifies a 4 kB C3HeB/FeJ-derived allele and a 2 kb C57BL/6J allele in the $SI^{c3H}1SI^{c3H}$ homozygotes.

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Figure 17. Nucleotide and predicted amino acid sequence of KL-1, KL-2 and KL-S1^d cDNAs. The nucleotide sequence of the KL cDNA obtained from the Balb3T3 cell plasmid cDNA library is shown. The RT-PCT products from different tissues and S1^d/+ total RNA, KL-1, KL-2 and KL-S1^d, were subcloned and subjected to sequence analysis. Open triangles indicate the 5' and 3' boundaries of the exon which is spliced out in KL-2; the closed triangles indicate the deletion endpoints in the S1^d cDNA. The 67 nucleotide inset sequence of the S1^d cDNA is shown above the KL cDNA sequence. Arrows indicate the putative proteolytic cleavage sites in the extracellular region of KL-1. The signal peptide (SP) and transmembrane segment (TMS) are indicated with overlying lines.

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Figure 18. Panels A and B. Identification by RT-PCR cloning of KL cDNAs from normal tissues and S1^d mutant fibroblasts. Total RNA was obtained from different tissues of C57BI6/J mice and S1^d/+ fibroblasts. RT-PCR reactions with RNA (10 μ g) from normal tissues and Balb 3T3 cells were done using primers #1 and #2 and reactions with RNA from +/+ and S1^d/+ fibroblasts were done by

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5 using the primer combinations #1, + #2, #1 + #3 and #1 + #4. The reaction products were analyzed by electrophoresis in 1% NuSieve agarose gels in the presence of 0.25 μ g/ml ethidium bromide. The migration of ϕ X174 Hae III DNA markers is indicated.

10 Figure 19. Topology of different KL protein products. Shaded areas delineate N-terminal signal peptides, solid black areas transmembrane domains and Y N-linked glycosylation sites. Dotted lines indicate the exon boundaries of the alternatively spliced exon and corresponding amino acid numbers are indicated. Arrows indicate the presumed proteolytic cleavage sites. The shaded region at the C-terminus of KL-S1^d indicates 15 amino acids that are not encoded by KL. KL-S designates the soluble form of KL produced by proteolytic cleavage or the C-terminal 20 truncation mutation of KL.

25 Figure 20. Identification of KL-1 and KL-2 transcripts in different tissues by RNase protection assays. ³²P-labelled antisense riboprobe (625 nt.) was hybridized with 20 μ g total cell RNA from tissues and fibroblasts except for lung and heart where 10 μ g was used. Upon RNase digestion, reaction mixtures were 30 analyzed by electrophoresis in a 4% polyacrylamide/urea gel. For KL-1 and KL-2 protected fragments of 575 nts. and 449 nts., are obtained respectively. Autoradiographic exposures were for 48 or 72

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hours, except for the 3T3 fibroblast RNA, which was for 6 hours.

Figure 21.

Panels A-C. Biosynthetic characteristics of KL-1 and KL-2 protein products in COS cells. COS-1 cells were transfected with 5 µg of the KL-1 and KL-2 expression plasmids, using the DEAE-dextran method. After 72 hours the cells were labelled with 35 S-Met for 30 minutes and then chased with complete medium. Supernatants and cell lysates were immunoprecipitated with anti-KL rabbit serum. Immunoprecipitates were analyzed by SDS-PAGE (12%). Migration of molecular weight markers is indicated in kilo daltons (kD).

Figure 22.

Panels A-C. PMA induced cleavage of the KL-1 and KL-2 protein products. COS-1 cells were transfected with 5 μ g of the KL-1 and KL-2 expression plasmids and after 72 hours the cells were labelled with 35 S-Met for 30 minutes and then chased with medium a) in the absence of serum; b) containing the phorbol ester PMA (1 μ M and c) containing the calcium ionophore A23187 (1 μ M). Supernatants and cell lysates were immunoprecipitated with anti-KL rabbit serum. Immunoprecipitates were analyzed by SDS-PAGE (12%). Migration of molecular weight markers is indicated in kilo daltons (kD).

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Figure 23. Panels A and B. Biosynthetic characteristics of KL-S1^d and KL-S protein products in COS cells.

5 Figure 24. Determination of biological activity in COS cell supernatants. Supernatants from COS cells transfected with the KL-1, KL-2, KL-S1^d and KL-S expression plasmids were assayed for activity in the mast cell proliferation assay. Serial dilutions of supernatant were incubated with BMMCs and incorporation of ³H-thymidine was determined from 24-30 hours of culture.

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Detailed Description of the Invention

5 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 S1 locus, and it was demonstrated that KL is encoded by the S1 locus.

10 The hematopoietic growth factor KL was recently purified from conditioned medium of BALB/c 3T3 fibroblasts, and it has the biological properties expected of the c-kit ligand (37). 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. The soluble form of KL, which has been isolated from the conditioned medium of Balb/3T3 cells 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.

20 The predicted amino acid sequence of KL, deduced from the nucleic acid sequence cDNAs, indicates that KL is synthesized as a transmembrane protein, rather than as a secreted protein. The soluble form of KL then may be generated by proteolytic cleavage of the membrane-associated form of KL. The ligand of the CSF-1 receptor, the closest

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relative of c-kit, shares the topological characteristics of KL and has been shown to be proteolytically cleaved to produce the soluble growth factor (44, 45). A recent analysis of the presumed structural characteristics of KL, furthermore indicates a relationship of KL and CSF-1 based on amino acid homology, secondary structure and exon arrangements indicating an evolutionary relationship of the two factors and thus strengthening the notion that the two receptor systems evolved from each other (4).

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Alternatively spliced KL mRNAs which encode two different forms of the KL protein, i.e., KL-1 and KL-2, have recently been described (15). The KL encoded protein products have been defined and characterized in COS cells transfected with the KL cDNAs and extended the findings of Flanagan et al. in several ways. As noted hereinabove, KL is synthesized as a transmembrane protein which is proteolytically cleaved to produce the soluble form of KL. The protein product of the alternatively spliced transcript of KL, KL-2, which lacks the exon that encodes the presumptive proteolytic cleavage site was shown to display turnover characteristics that are distinct from those of KL-1. In addition, the proteolytic cleavage of both KL-1 and KL-2 can be regulated by agents such as PMA and the calcium ionophore A23187. The relative abundance of KL-1 and KL-2 has been determined in a wide variety of different mouse tissues. This indicates that the expression of KL-1 and KL-2 is controlled in a tissue specific manner.

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The gene products of the Sl^d allele have also been defined (15). Sl^d results from a deletion within KL which includes the sequences encoding the transmembrane and cytoplasmic domains of the protein resulting in a biologically active, secreted mutant KL protein. The respective roles of the

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soluble and cell-associated forms of KL in the proliferative and migratory functions of c-kit are discussed in the light of these results.

5 This invention provides a purified mammalian protein corresponding to a ligand for the c-kit which comprises a homodimer of two polypeptides, each polypeptide having a molecular weight of about 30 kD and an isoelectric point of about 3.8. As used herein, the term "c-kit ligand" is to
10 mean a polypeptide or protein which has also been defined as stem cell factor, mast cell factor and steel factor. As used herein, c-kit ligand protein and polypeptide encompasses both naturally occurring and recombinant forms, i.e., non-naturally occurring forms of the protein and the
15 polypeptide which are sufficiently identically to naturally occurring c-kit to allow possession of similar biological activity. Examples of such polypeptides includes the polypeptides designated KL-1.4 and S-KL, but are not limited to them. Such protein and polypeptides include derivatives
20 and analogs. In one embodiment of this invention, the purified mammalian protein is a murine protein. In another embodiment of this invention, the purified mammalian protein is a human protein.
25 Also provided by this invention is a purified mammalian protein corresponding to a c-kit ligand, wherein the purified protein is glycosolated. However, this invention also encompasses unglycosylated forms of the protein. This invention also encompasses purified mammalian proteins
30 containing glycosolation sufficiently similar to that of naturally occurring purified mammalian protein corresponding to c-kit ligand. This protein may be produced by the introduction of a cysteine cross-link between the two homodimer polypeptides described hereinabove by methods

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known to those of skill in the art.

5 Also provided by this invention is a pharmaceutical composition which comprises an effective amount of the purified mammalian protein corresponding to c-kit ligand described hereinabove and a pharmaceutically acceptable carrier.

10 Further provided is a pharmaceutical composition for the treatment of leucopenia in a mammal comprising an effective amount of the above mentioned pharmaceutical composition and an effective amount of a hemopoietic factor, wherein the factor is selected from the group consisting of GCSF, GMCSF and IL-3, effective to treat leucopenia in a mammal.

15 Also provided by this invention is a pharmaceutical composition for the treatment of anemia in a mammal, which comprises an effective amount of the pharmaceutical composition described hereinabove and an effective amount of EPO (erythropoietin) or IL-3, effective to treat anemia in a mammal. Anemia encompasses, but is not limited to Diamond Black fan anemia and aplastic anemia. However, for the treatment of Black fan anemia and aplastic anemia, a pharmaceutical composition comprising an effective amount of the composition described hereinabove and an effective amount of G-CSF and GM-CSF, effective to treat anemia is preferred. A method of treating anemia in mammals by administering to the mammals the above composition is further provided by this invention. A pharmaceutical composition effective for enhancing bone marrow during transplantation in a mammal which comprises an effective amount of the pharmaceutical composition described hereinabove, and an effective amount of IL-1 or IL-6, effective to enhance engraftment of bone marrow during

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transplantation in the mammal is also provided. A pharmaceutical composition for enhancing bone marrow recovery in the treatment of radiation, chemical or chemotherapeutic induced bone marrow, aplasia or myelosuppression is provided by this invention which comprises an effective amount of the pharmaceutical composition described hereinabove and an effective amount of IL-1, effective to enhance bone marrow recovery in the mammal. Also provided by this invention is a pharmaceutical composition for treating acquired immune deficiency syndrome (AIDS) in a patient which comprises an effective amount of the pharmaceutical composition described hereinabove and an effective amount of AZT or G-CSF, effective to treat AIDS in the patient.

15

A composition for treating nerve damage is provided by this invention which comprises an effective amount of the pharmaceutical composition described hereinabove in an amount effective to treat nerve damage in a mammal.

20

Also provided is a composition for treating infants exhibiting symptoms of defective lung development which comprises an effective amount of the purified mammalian protein and a pharmaceutically acceptable carrier, effective to treat infants exhibiting symptoms of defective lung development.

25

Further provided is a composition for the prevention of hair loss in a subject which comprises an effective amount of the purified mammalian protein corresponding to c-kit ligand and a pharmaceutically acceptable carrier, effective to prevent the loss of hair in the subject. Also provided by this invention is a pharmaceutical composition for inhibiting the loss of pigment in a subject's hair which comprises an

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effective amount of the purified mammalian protein corresponding to c-kit ligand and a pharmaceutically acceptable carrier, effective to inhibit the loss of pigment in the subject's hair.

5

Methods of treating the above-listed disorders by the administration of the effective composition, in an amount effective to treat that disorder, also is provided.

10

As used herein, the terms "subject" shall mean, but is not limited to, a mammal, animal, human, mouse or a rat. "Mammal" shall mean, but is not limited to meaning a mouse (murine) or human.

15

This invention provides an isolated nucleic acid molecule which encodes an amino acid sequence corresponding to a c-kit ligand (KL). Examples of such nucleic acids include, but are not limited to the nucleic acids designated KL 1.4, KL-1, KL-2 or S-KL. The invention also encompasses nucleic acids molecules which differ from that of the nucleic acid molecule which encode these amino acid sequences, 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

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variant thereof as well as man-made recombinant forms.

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).

5

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

10

cosmid vector.

This invention also provides the isolated nucleic acid molecule of this invention operatively linked to a promoter of RNA transcription, as well as other regulatory sequences.

15

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

20

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, WI].

25

A host vector system for the production of 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.

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To recover the protein when expressed in E. coli, E. coli cells are transfected with the claimed nucleic acids to express the c-kit ligand protein. The E. coli are grown in one (1) liter cultures in two different media, LB or TB and pelleted. Each bacterial pellet is homogenized using two 5 passages through a French pressure cell at 20'000 lb/in² in 20 ml of breaking buffer (below). After a high speed spin 120k rpm x 20 minutes) the supernatants were transferred into a second tube. The c-kit protein or polypeptide is located in the particulate fraction. This may be 10 solubilized using 6M guanidium-HCl or with 8M urea followed by dialysis or dilution.

Breaking Buffer

15

50 mM Hepes, pH 8.0

20% glycerol

150 mM NaCl

1 mM Mg So₄

20

2 mM DTT

5mM EGTA

20 µg/ml DNase I.

25

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

30

In one embodiment of this invention, the c-kit ligand polypeptide corresponds to amino acids 1 to 164. In other embodiments of this invention, the c-kit ligand polypeptide corresponds to amino acids 1 to about 148, or fusion polypeptides corresponding to amino acids 1 to about 148 fused to amino acids from about 165 to about 202 or 205, as well as a fusion polypeptide corresponding to amino acids 1

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to about 164 fused to amino acids 177 to about amino acid 202 or about amino acid 205.

5 In another embodiment of this invention, the c-kit ligand polypeptide may comprise a polypeptide corresponding to amino acids 1 to about 164 linked to a biologically active binding site. Such biological active binding sites may comprise, but are not limited to an amino acids corresponding to an attachment site for binding stromal 10 cells, the extracellular matrix, a heparin binding domain, a hemonectin binding site or cell attachment activity. For example, see U.S. Patent Nos. 4,578,079, 4,614,517 and 4,792,525, issued March 25, 1986; September 30, 1986 and December 20, 1988, respectively.

15 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, 20 dyes or enzymes such as peroxidase or alkaline phosphate. Suitable radioisotopes include, but are not limited to ^{125}I , ^{32}P , and ^{35}S .

25 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; 30 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.

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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.

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.

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.

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.

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.

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

5 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 10 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.

15 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 20 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 25 various types of wetting agents.

30 This invention further provides a substance capable of specifically forming a complex with the c-kit ligand protein, 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.

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A method of modifying a biological function associated with c-kit cellular activity is provided by this invention. This method comprises contacting a sample of the cell, 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 and in vitro fertilization. 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.

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.

A method of inducing differentiation of mast cells or erythroid progenitors in a patient which comprises administering to the patient the pharmaceutical composition

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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.

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.

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

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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.

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.

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 (KL) polypeptide which comprises incubating normal bone-marrow mast cells with 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 ^3H -thymidine; determining the amount of thymidine

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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.

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:

15	C - cytosine	A - adenosine
	T - thymidine	G - guanosine
	U - uracil	

EXPERIMENT NUMBER 1 - PURIFICATION OF C-KIT LIGAND

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Experimental Materials

Mice and embryo identification

25 WBB6 $+/+$ and W/W^V, C57B16 W^V/+ and WB W/+ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Heterozygous W⁴¹/+ 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 (38).

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Mast cell cultures, preparation of peritoneal mast cell and
flow cytometry

Mast cells were grown from bone marrow of adult mice and
5 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)) (60).
Non-adherent cells were harvested, refed weekly and
10 maintained at a cell density less than 7×10^5 cells/ml.
Mast cell content of cultures was determined weekly by
staining cytopspin preparations with 1% toluidine blue in
methanol. After 4 weeks, cultures routinely contained
15 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
20 centrifugation on 22% Metrizamide (Nycomed, Oslo, Norway) in
PBS without Ca^{++} and Mg^{++} , essentially as previously
described (61). Mast cells were stained with 1% toluidine
blue in methanol for 5 minutes and washed for 5 minutes in
25 H_2O , and berberine sulfate by standard procedures (62).
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) (38).

Mast cell proliferation assay

30 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 .2 ml in 96 well plates with two fold serial
dilutions of test samples. Plates were incubated for 24
hours at 37°C , $2.5 \mu\text{C}$ of ^3H -TdR was added per well and

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incubation was continued for another 6 hours. Cells were harvested on glass fiber filters and thymidine incorporation into DNA was determined.

5

Preparation of fibroblast conditioned medium

Balb/3T3 cells (1) were grown to confluence in Dulbecco's Modified MEM (DME) supplemented with 10% calf serum (CS), penicillin and streptomycin in roller bottles. Medium was 10 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 15 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 .45 u filter and frozen at 4°C. The conditioned medium was then concentrated 100-200 fold with a Pellicon ultrafiltration 20 apparatus followed by an Amicon stirred cell, both with membranes having a cut off of 10,000 kD.

Column chromatography

25

Blue Agarose chromatography (BRL, Gaithersburg, MD) was performed by using column with a bed volume of 100ml 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 30 collected and concentrated to 15-20 ml in dialysis tubing with PEG 8000.

Gel filtration chromatography was performed on a ACA54 Ultrogel (LKB, Rockland, MD) column (2.6 X 90 cm) which was

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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, NJ. The concentrate from the 5 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.

Anion exchange and reverse-phase HPLC (RP-HPLC)

10 High performance liquid chromatography was performed using a Waters HPLC system (W600E Powerline controller, 490E programmable multiwavelength detector, and 810 Baseline Workstation, Waters, Bedford, MA). Active fractions from 15 gel filtration were dialyzed in 0.05 M Tris-HCl pH 7.8 and loaded onto a Protein-Pak™ DEAE-5PW HPLC column (7.5 mm X 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 20 linear gradient from 0 to 0.4M NaCl in .02 M Tris-HCl pH 7.8. The flow rate was 1 ml/minute and 2 ml fractions were collected.

25 RP-HPLC was performed using a semi-preparative and an analytical size C₁₈ 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 30 anion exchange were pooled and loaded onto the semi-preparative C₁₈ 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 C₁₈ reverse phase

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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.

Isoelectric focusing (IEF)

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.

Erythroid progenitor assays

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, CA) (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.

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Experimental Methods

Short term mast cell proliferation assay detects a fibroblast derived activity

5 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
10 WEHI-3 CM (IL-3) and after 24 hours of incubation ³H-thymidine incorporation was determined. The response of BMMC derived from normal +/+ and mutant W/W^V mice to IL-3 was similar (Figure 1); in contrast, 20 fold concentrated
15 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 (35). No proliferation of the 32D cells
20 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 (38), suggested that FCM activity was dependent on
25 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
30 putative c-kit ligand (KL) from fibroblast conditioned medium.

Purification of the mast cell stimulating activity KL

To purify KL, five liters of serum free conditioned medium from Balb/3T3 fibroblasts was concentrated 50 fold by 5 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 10 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 (Figure 2A). The biological activity eluted 15 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 (Figure 2B). The activity eluted at 0.11 M NaCl from the FPLC column. Peak 20 fractions were pooled and subjected to HPLC chromatography with a semi-preparative C18 column and an ammonium acetate/n-propanol gradient (Figure 2C). The active material eluted at 30% n-propanol from the semi-preparative C18 column was diluted 1:1 with buffer A and 25 rechromatographed by using an analytical C18 column (Figure 2D). A single peak of activity eluted again at 30% n-propanol which corresponded to a major peak of absorbance (280nm) in the eluant profile. Similar results were obtained by using a C4 column with H₂O and acetonitrile 30 containing .1% TFA as solvents (Figure 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

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correlated well with the peak of biological activity (Figure 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 (Figure 5 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 10 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' 15 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.

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TABLE 1

Purification of KL from Balb/3T3 Conditioned Medium

Purification Step	Total Protein (mg)	Total Activity (U x 10 ⁻⁵)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
FCM (5L), 50X Concentrated	152	-	-	-	-
Blue Agarose	32	720	2.2x10 ⁴	1	100
Gel Filtration	28	480	1.7x10 ⁴	.77	67
DEAE-5PW	3	720	2.4x10 ⁵	11	100
C18-Semiprep	.079	600	7.6x10 ⁶	345	83
C18-Analytical	.004	340	8.5x10 ⁷	3863	47

Proliferative response to KL of mast cells with different c-kit/W mutations

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 W⁴¹ 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 (63, 38). The W^V and W⁴¹ alleles both specify a partially defective c-kit receptor and homozygous mutant animals are viable (64, 65, 38). Homozygous W^V animals have severe macrocytic anemia and their mast cells

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display a minor response in the co-culture assay, and homozygotes for the less severe W⁴¹ 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 W⁴¹ alleles and from day 14 fetal livers for the W allele as described previously (38). 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 (Figure 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 (Figure 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. W⁴¹/W⁴¹ and W⁴¹/+ 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 (Figure 4). These results indicate a correlation of the responsiveness of mast carrying the W, W^V and W⁴¹ 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.

KL stimulates the proliferation of peritoneal mast cells

Mast cells of the peritoneal cavity (PMC) have been well characterized and in contrast to BMMC represent connective tissue-type mast cells (66). PMC do not proliferate in response to IL-3 alone; however, their mature phenotype and viability can be maintained by co-culture with NIH/3T3

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fibroblasts (67). 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 (Figure 5) (62). BMMC and mucosal mast cells contain predominantly chondroitin sulfate di-B/E proteoglycans rather than heparin proteoglycans (67); berberine sulfate therefore did not stain the granules in BMMC (Figure 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 (Figure 5B). KL was then examined to determine if it would effect the survival or stimulate the proliferation of PMC (Figure 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.

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KL stimulates the formation of erythroid bursts (BFU-E)

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 (47, 65). Analysis of erythroid progenitor populations in the bone marrow of W/W^V mice indicates a slight decrease of BFU-E and CFU-E (68,69). 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 (35). 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/10⁶ cells with erythropoietin alone to 50 BFU-E/10⁶ cells with maximal stimulation at 2.5 ng of KL/ml (Figure 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 10⁵ bone marrow cells. The effect of KL on day 14 fetal liver cells was also examined and similar results were observed as with

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5 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 cells.

10 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 15 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 (Figure 7). KL did not stimulate BFU-E above levels seen 20 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.

25

Binding studies with purified KL

30 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

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of KL to W mutant mast cells correlates with c-kit expression on the cell surface, V, 37(+) versus W(-).

Determination of the peptide sequence of the c-kit ligand

5

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.

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The single letter amino acid sequence of the protein from the N-terminal is:

5 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.

with:

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.

Experimental Discussion

15 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-kit 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 (70, 71). 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

-47-

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 (35, 71). Furthermore, preliminary results from the determination of the protein sequence of KL indicate that KL is different from the known protein sequences.

10 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 (72, 73). 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 (66). 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 (60). 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 (74, 75, 76, 77, 78). 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 have a mast cell

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proliferation and differentiation activity which is independent from these immune responses for its production and action on target cells.

5 The defect W mutations exert on erythropoiesis indicates an essential role for c-kit in the maturation of erythroid cells (80, 68, 69). 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 (35). 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 and +/+ bone marrow (78). 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 (79). 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

-49-

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.

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 (81, 46, 47, 81, 82). 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.

Mutations at the steel locus (S1) of the mouse produce pleiotropic phenotypes in hematopoiesis, melanogenesis and gametogenesis similar to those of mice carrying W mutations (47, 51). However, in contrast to W mutations, S1 mutations affect the microenvironment of the cellular target of the mutation and are not cell autonomous (46). Because of the parallel and complementary effects of the W and the S1 mutations, it has been suggested that the S1 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 (9). In agreement with this conjecture S1/S1^d embryo fibroblasts or conditioned medium from S1/S1^d fibroblasts

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fail to support the proliferation of BMMC and mast cell progenitors, respectively, and presumably do not produce functional KL (16,84). 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.

10 The 1.4 kb cDNA clone is used to screen a human fibroblast or a human placenta library using the methods disclosed hereinabove. Upon isolating the gene which encodes the human c-kit ligand, the gene will be characterized using the methods disclosed hereinabove.

15

EXPERIMENT NUMBER 2 - ISOLATION OF THE NUCLEIC ACID SEQUENCE

Experimental Materials

20 Mice and tissue culture

25 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 x M. spretus) F1 female offspring were backcrossed with C57BL/6J males.

30 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-

-51-

Complete) (36,60). BALB/c 3T3 cells (1) 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.

5

Purification and amino acid sequence determination of KL

KL was purified from conditioned medium of BALB/c 3T3 cells by using a mast cell proliferation assay as described 10 elsewhere (37). 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 15 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, 20 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 25 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 30 (280nm) in the eluant profile. Similar results were obtained by using a C4 column with H₂O 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).

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Iodination

KL was iodinated with chloramine T with modifications of the method of Stanley and Gilbert (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 μ l 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 μ M 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.

Binding assay

Binding buffer contained RPMI 1640 medium, 5% BSA (Sigma), 20 mM HEPES (pH 7.5) and NaN₃. 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 μ l. Binding experiments with $\#2$ cells were carried out in 24-well dishes in a volume of 300 μ l. 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 ¹²⁵I-KL. Cells were incubated with ¹²⁵I-KL for 90 minutes, and nonadherent cells were pelleted through 150 μ l of FCS. Cell pellets were frozen and counted.

Immunoprecipitation and cross-linking

BMMC were incubated with ¹²⁵I-KL under standard binding conditions and washed in FCS and then in PBS at 4°C. Cells were lysed as previously described (35) in 1% Triton X-100,

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20 mM Tris (pH 7.4), 150 mM NaCl, 20 mM EDTA, 10% glycerol, and protease inhibitors phenylmethylsulfonyl fluoride (1mM) and leupeptin (20 μ g/ml). Lysates were immunoprecipitated with normal rabbit serum, or c-kit specific sera raised by immunization of rabbits with a fragment of the v-kit tyrosine kinase domain (23); or the murine c-kit expressed from a cDNA in a recombinant vaccinia virus (36). 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).

20

CDNA synthesis, PCR amplification (RT-PCR), and sequence determination

25 The RT-PCR amplification was carried out essentially as described (53). For cDNA synthesis, 1 μ g of poly(A)⁺ RNA from confluent BALB/c 3T3 cells in 25 μ l of 0.05 M Tris-HCl (pH 8.3), 0.075 M KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 200 μ M dNTPs and 25 U of RNAsin (Promega) was incubated with 50 pmol of antisense primer and 50 U of Moloney murine leukemia 30 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 μ l with 25 μ l of 50 mM KCl, 10mM Tris-HCl(pH 8.3), 1.5 mM MgCl₂, 0.01% (w/v)

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5 gelatin, and 200 μ M dNTPs, adding 50 pmol of sense primer and 2.5 U of Taq 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 M13mp18 and M13mp19 for sequence analysis (49).

10 cDNA isolation and sequencing

15 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 (48); 5' end-labeled oligonucleotide was used as a probe. Hybridization was in 6X SSC at 63°C, and the final wash of the filters was in 2X 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. (49) by using synthetic oligodeoxynucleotides (17-mers) as primers.

20 DNA and RNA analysis

25 Genomic DNA was prepared from tail fragments, digested with restriction enzymes, electrophoretically fractionated, and transferred to nylon membranes. 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 (85) were used as probes.

BALB/c 3T3 cells were homogenized in guanidinium isothiocyanate, and RNA was isolated according the method of

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Chirgwin et al. (10). Total cellular RNA (10 μ g) and poly(A)⁺ RNA were fractionated in 1% agarose-formaldehyde gels and transferred to nylon membranes (Nytran, Schleicher & Schuell); prehybridization and hybridization were 5 performed as previously described (86, 35). The 1.4 kb KL cDNA labeled with [³²P]phosphate was used as a probe for hybridization (87).

Preparation of c-kit and c-kit ligand monoclonal antibodies

10

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 15 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 20 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 25 weeks later, hybridoma supernatants are screened for binding activity against c-kit receptor protein as described hereinabove. Positive clones are then isolated and propagated.

30

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.

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

5

Experimental Results

Isolation and characterization of murine cDNAs encoding the hematopoietic growth factor KL

10

15

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 (37). 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.

20

25

30

To derive a nondegenerate homologous hybridization probe, fully degenerate oligonucleotide primers corresponding to amino acids 10-16 (sense primer) and 31-36 (antisense primer) provided with endonuclease recognition sequences at their 5' ends were synthesized as indicated in Figure 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.

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

-57-

the correct amino acid sequence (Figure 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
5 that, in its 3' half, specifies an open reading frame that extends to the 3' end of the clone and encodes 270 amino acids (Figure 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
10 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
15 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
20 protein: an N-terminal signal peptide, an extracellular domain, a transmembrane domain, and a C-terminal intracellular segment.

25 RNA blot analysis was performed to identify KL-specific RNA transcripts in BALB/c 3T3 cells (Figure 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
30 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.

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The soluble form of KL is a ligand of the c-kit receptor

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 $\#2$ cells expressing the c-kit cDNA. KL was labeled to high specific activity with ^{125}I by using the modified chloramine T method (88). Analysis of the labeled material by SDS-PAGE showed a single band of 28-30 kd (Figure 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 $\#2$ cells expressing the c-kit cDNA, NIH $\#2$ control cells, normal BMMC, and W/W, W/+, and W^v/W^v BMMC at 4°C was measured. The results shown in Figure 14 indicate binding of labeled KL to NIH $\#2$ c-kit cells and to +/+, W/+, and W^v/W^v mast cells, but not to NIH $\#2$ 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 (36). 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 (36). 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.

To obtain more direct evidence that KL is the ligand of the

-59-

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 (89). 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 (Figure 15A), where recovery of intact 125 I-KL was demonstrated from the samples containing the immune complexes prepared with anti-kit sera.

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 disuccinimidyl 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 but 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 (Figure 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

-60-

5 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.

Mapping of KL to the S1 locus

10 To test whether KL is encoded at the S1 locus, recombination analysis was used to determine the map position of KL with respect to a locus that is tightly linked to S1. This locus is the site of the transgene insertion in the transgenic line TG.EB (85). It was determined that genomic sequences cloned from the insertion site map 0.8 ± 0.8 cM from S1. This therefore represents the closest known marker to S1.

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 spreitus*. 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 (90). 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. spreitus* 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

-61-

insertion site and S1, this result is consistent with the notion that KL maps to the S1 locus.

5

Table 2. Mapping of the Position of the KL Gene by Linkage Analysis Using an Interspecific Cross

10

Progeny

15

Probe	Nonrecombinant	Recombinant	
1.4 kb KL cDNA	B6	Sp	B6
TIS Dra/SaI	B6	Sp	Sp
	32	20	0

20

1

n = 53

% recombination = 1.9 ± 1.9

25

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 probe from a transgene insertion site that is tightly linked to S1; see Results). Percent recombination was calculated according to Green (1981).

35

The locus identified by KL was also examined in mice that carry the original S1 mutation (50). 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 S1 during fetal development. C57BL/6J mice that carry the S1 mutation maintained in the C3HeB/FeJ strain were generated by mating, and F1 progeny carrying the S1 allele were intercrossed (C57BL/6J S1^{3CH/+} S1^{C3H/+}). Homozygous SIISI progeny from

40

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5 this mating are anemic and are homozygous for a C3HeB/FeJ-derived RFLP at the transgene integration site (Figure 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 10 SIISI mice was analyzed using the 1.4 kb KL cDNA probe, no hybridization to the homozygous SIISI DNA was observed (Figure 16). It thus appears that the locus that encodes the KL protein is deleted in the S1 mutation. This finding 15 further supports the notion that KL is the product of the S1 gene.

Experimental Discussion

15 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 20 transmembrane tyrosine kinase receptor in a mammal. Mutations at the S1 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 S1 locus.

25 The experiments reported herein provide evidence that the S1 gene encodes the ligand of the c-kit receptor. The evidence for this conclusion is as follows. Based on the knowledge of the function of the c-kit receptor designated KL, a putative 30 ligand of the c-kit receptor designated KL was identified and purified (37). 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

-63-

and the c-kit protein. KL-specific cDNA clones were derived and it was shown that KL maps to the S1 locus on mouse chromosome 10. In addition, it was also demonstrated that KL sequences are deleted in the genome of the S1 mouse. 5 Taken together, these results suggest that KL is encoded by the S1 locus and is the ligand of the c-kit receptor, thus providing a molecular basis for the S1 defect.

10 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 15 proteolytic cleavage to form a soluble product that is secreted (91, 44). Presumably, 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 20 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 25 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 30 fibroblasts derived from SII/SI mice (14).

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

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5 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 SII^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 (90, 35). 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) (37).

10 15 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 S1 mutant mice (72, 73). 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 (60). 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 (66). 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 (80,

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81, 82, 83). 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 5 factors, in order to identify the stage(s) where c-kit/KL functions in stem cells. Another possible function for c-kit might be to facilitate the transition from noncycling to cycling cells (31). The increased radiation sensitivity of 10 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.

In gametogenesis the W and S1 mutations affect the proliferation and the survival of primordial germ cells, and 15 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 (39). In melanogenesis c-kit/KL 20 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.

25 The microenvironment in which c-kit-expressing cells function is defective in S1 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 30 the cell types that produce KL *in vivo* is not known. *In vitro* systems that reproduce the genetic defect of the W and the S1 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

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hematopoietic cells are not, and in the mast cell-fibroblast coculture system, SIISI^d fibroblasts are defective but the mast cells are not (12, 16). 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 (35, 39). No S1 or W phenotypes are known to be associated with these cell systems.

Interspecific backcrosses were used to establish close linkage between the KL gene, the S1 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 S1. The finding that the KL coding sequences are deleted in the original S1 allele, however, supports the identity of the S1 locus with the KL gene. The size of the deletion in the S1 allele at this time is not known. It will be important to determine whether it affects neighboring genes as well.

The lack of KL coding sequences in the S1 allele indicates that this allele is a KL null mutation. When homozygous for the S1 allele, most mice die perinatally of macrocytic anemia, and rare survivors lack coat pigmentation and are devoid of germ cells (5). 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

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homozygotes, e.g., in germ cell development, S1 may have a more pronounced effect, and in hematopoiesis S1 may cause a more severe anemia; however, it is not known if these differences are a result of different strain backgrounds or 5 are possibly effects of the S1 deletion on neighboring genes (5).

The original W mutation is an example of a c-kit null mutation (36). When heterozygous with the normal allele, 10 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 15 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.

20 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 S1 or W mutations may block the development of these cell 25 lineages, and therefore a function for the c-kit receptor in more mature cell populations would not be evident. S1 and W mutations in which c-kit/KL function is only partially impaired often reveal effects in more mature cell populations. Numerous weak S1 alleles are known. Their 30 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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EXPERIMENT NUMBER 3 - KL-1 AND KL-2

Experimental Materials

5 Mice and tissue culture

WBB6 +/+, C57BL/6J and 129/Sv-S1^d/+ mice were obtained from the Jackson Laboratory (Bar Harbor, ME) (52). 129/Sv-S1^d/+ male and female mice were mated and day 14 fetuses were obtained and used for the derivation of embryonic fibroblasts according to the method of Todaro and Green (54). Mast cells were grown from bone marrow of adult +/+ mice 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)) (36). Balb/3T3 cells (1) were grown in Dulbecco's Modified MEM (DME) supplemented with 10% calf serum (CS), penicillin and streptomycin. COS-1 cells (18) were obtained from Dr. Jerrard Hurwitz (SKI) and were grown in DME supplemented with 10% fetal bovine serum, glutamine, penicillin and streptomycin.

Production of anti-KL antibodies

25 Murine KL was purified from conditioned medium of Balb3T3 cells by using a mast cell proliferation assay as described elsewhere (37). In order to obtain anti-KL antibodies one rabbit was immunized subcutaneously with 1 μ g of KL in complete Freund's adjuvant. Three weeks later the rabbit was boosted intradermally with 1 μ g in incomplete Freunds adjuvant. Serum was collected one week later and then biweekly thereafter. The ¹²⁵I-labelled KL used for this purpose was iodinated with chloramine T with modifications of the method of Stanley and Gilbert as described previously

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(38).

cDNA Library Screening

5 Poly(A) RNA was prepared by oligo(dT)-cellulose chromatography from total RNA of Balb/c 3T3 fibroblast. A custom made plasmid cDNA library was then prepared by Invitrogen Inc. Essentially, double-stranded cDNA was synthesized by oligo dT and random priming. Non-palindromic
10 BstXI linkers were ligated to blunt-ended cDNA and upon digestion with BstXI the cDNA was subcloned into the expression plasmid pcDNAI (Invitrogen). The ligation reaction mixture then was used to transform E. coli MC1061/P3 by the electroporation method to generate the
15 plasmid library. The initial size of the library was approximately 10^7 independent colonies. For screening of the plasmid library an end-labelled oligonucleotide probe described previously was used (38). Hybridization was done
20 in 6X SSC at 63°C and the final wash of the filters was in 2X SSC and 0.2% SDS at 63°C. The inserts of recombinant plasmids were released by digestion with HindIII and XbaI and then subcloned into the phage M13mp18 for sequence analysis.

25 PCR amplification (RT-PCR) and sequence determination

30 Total RNA from tissues and cell lines was prepared by the guanidium isothiocyanate/CsCl centrifugation method of Chirgwin (10). The RT-PCR amplification was carried out essentially as described previously (38). The following primers were used for RT-PCR:

Primer #1: 5'-GCCCAAGCTTCGGTGCCTTCCTTATG-3' (nt. 94-107);

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Primer #2: 5'-AGTATCTCTAGAATTTACACCTCTTGAAATTCTCT-3' (nt. 907-929);

5 Primer #3: 5'-CATTATCTAGAAAACATGAACTGTTACCAGCC-3' (nt. 963-978);

Primer #4: 5'-ACCCCTCGAGGGCTGAAATCTACTTG-3' (nt. 1317-1333).

For cDNA synthesis, 10 μ g of total RNA from cell lines or tissues in 50 μ l of 0.05 mM Tris-HCl (pH 8.3), 0.75 M KCl, 3 mM MgCl₂, 10 mM DTT, 200 μ M dNTP's and 25 U of RNasin (BRL) was incubated with 50 pmole of antisense primer and 400 U of Moloney murine leukemia virus reverse transcriptase (BRL) at 37°C for 1 hour. The cDNA was precipitated by adding 1/10 volume of 3 M NaOAc (pH 7.0) and 2.5 volume of absolute ethanol and resuspended in 50 μ l of ddH₂O. PCR was carried out for 30 cycles in 100 μ l of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 200 μ M dNTP's, 500 pmole of both sense and antisense primers and 2.5 U of Taq polymerase (Perkin-Elmer-Cetus). HindIII sites and XbaI sites were placed within the sense - and antisense primers respectively. The amplified DNA fragments were purified by agarose gel electrophoresis, digested with the appropriate restriction enzymes, and subcloned into M13mp18 and M13mp19 for sequence analysis (49). The KL-1, KL-2, KL-S and KL-S1^d PCR products were digested with HindIII and XbaI and subcloned into the expression plasmids pCDM8 or pCDNA1 (Invitrogen). Miniprep plasmid DNA was prepared by the alkaline-lysis method (48) followed by phenol-chloroform extraction and ethanol precipitation. Maxiprep plasmid DNA used for the transfection of COS-1 cells was prepared by using the "Qiagen" chromatography column procedure.

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RNase Protection Assay

A riboprobe for RNase protection assays was prepared by linearizing the KL-1 containing pcDNA1 plasmid with SpeI. 5 The antisense riboprobe was then synthesized by using SP6 polymerase according to the Promega Gemini kit. Riboprobe labelled to high specific activity was then hybridized to 10 or 20 μ g of total RNA in the presence of 80% formamide at 45°C overnight. The hybridization mixture was digested with 10 RNase A and T1 (Boehringer-Mannheim) and treated with proteinase K (48) and the protected labelled RNA fragments were analyzed on a 4% urea/polyacrylamide gel. Autoradiograms of RNase protection assay were analyzed by densitometry and parts of the films were reconstructed on a 15 PhosphoImage analyzer (Molecular Dynamics) for better resolution.

Transient expression of "KL" cDNAs in COS-1 cells

20 For transient expression of KL cDNAs COS-1 cells were transfected with the DEAE-dextran method described previously (20) with minor modifications. Briefly, COS-1 cells were grown to subconfluence one day before use and were trypsinized and reseeded on 150mm petri dishes at a 25 density of 6×10^6 cells per dish. After 24 hours, the cells had reached about 70% confluence and were transfected with 5 μ g of plasmid DNA in the presence of 10% DEAE-dextran (Sigma) for 6 to 12 hours. Medium containing plasmid DNA was removed and the cells were chemically shocked with 10% 30 DMSO/PBS⁺⁺ for exactly 1 minute. Residual DMSO was removed by washing the cells with PBS⁺⁺ twice. Transfected COS-1 cells were grown in DME plus 10% fetal calf serum, 100 mg/ml L-glutamine, and antibiotics.

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Pulse chase and immunoprecipitation analysis of "KL" proteins

Transfected COS-1 cells were used for pulse-chase experiments 72 hours after the transfection. Cells were incubated with methionine-free DME containing 10% dialyzed fetal calf serum for 30 minutes and labelled with ^{35}S -methionine (NEN) at 0.5 mCi/ml. At the end of the labelling period, the labelling medium was replaced with regular medium containing an excess amount of methionine. In order to determine the effect of phorbol 12-myristate 13-acetate (PMA) and A23187 on the proteolytic cleavage of KL, 1 μM PMA or 1 μM A23187 was added to the transfected cells at the end of the labelling period after replacement of the labelling medium with regular medium. The cells and supernatants were collected individually at the indicated times for immunoprecipitation analysis. Cell lysates were prepared as described previously (35) in 1% Triton -100, 20 mM Tris (pH 7.5), 150 mM NaCl, 20 mM EDTA, 10% glycerol and protease inhibitors phenylmethyl sulfonyl chloride (1 mM) and leupeptin (20 $\mu\text{g}/\text{ml}$). For the immunoprecipitation analysis of KL protein products the anti-mouse KL rabbit antiserum was used. The anti-KL serum was conjugated to protein-A Sepharose (Pharmacia) and washed 3 times with Wash A (0.1% Triton X-100, 20 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol). Anti-KL serum-protein A sepharose conjugate was incubated with supernatant and cell lysate at 4°C for at least 2 hours. The immunoprecipitates then were washed once in Wash B (50 mM Tris, 500 mM NaCl, 5 mM EDTA, 0.2% Triton X-100), 3 times in Wash C (50 mM Tris, 500 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 5 mM EDTA) and once in Wash D (10 mM Tris, 0.1% Triton X-100). For gel analysis immunoprecipitates were solubilized in SDS sample buffer by boiling for 5 minutes, and analyzed by SDS-PAGE (12%) and

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autoradiography.

Determination of biological activity of soluble KL

5 Mast cells were grown from bone marrow of adult WBB6 +/- mice in RPMI-1640 medium supplemented with 10% fetal calf serum, conditioned medium from WEHI-3B cells, non-essential amino acids, sodium pyruvate and 2-mercaptoethanol (RPMI-Complete) as described previously (37). Non-adherent cells
10 were harvested by centrifugation and refed weekly and maintained at a cell density of $<7 \times 10^5$ cells/ml. The mast cell content of cultures was determined weekly by staining cytocentrifuge preparations with 1% toluidine blue in methanol. After 4 weeks, cultures routinely contained $>95\%$ mast cells
15 and were used for proliferation assay. Supernatants from transfected COS-1 cells were collected from 48 to 72 hours after transfection. The biological activity of soluble KL in the supernatants was assessed by culturing BMMCs with different dilutions of COS-1 cell supernatants in the absence of IL-3. BMMCs were washed three times with complete RPMI and grown in 0.2% IL-3. The following day, cells were harvested and suspended in complete RPMI (minus IL-3) and 10^4 BMMCs in 100 μ l/well were seeded in a 96-well plate.
20 Equal volume of diluted supernatant was added to each well and cultures were incubated for 24 hours at 37°C, 2.5 μ Ci of [3 H]-thymidine/well was then added and incubation was continued for another 6 hours. Cells were harvested on glass fiber filters (GF/C Whatman) and thymidine incorporation was determined in a scintillation counter.
25 Assays were performed in triplicate and the mean value is shown. Standard deviations of measurements typically did not exceed 10% of the mean values.
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Experimental ResultsAlternatively spliced transcript of KL encodes a truncated transmembrane form of the KL protein

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A cDNA clone, which had been isolated from a mouse 3T3 fibroblast library and contained most of the KL coding sequences (267 amino acids), has been described herein. In an attempt to obtain the complete cDNA sequences corresponding to the 6.5 kb KL mRNA, a plasmid cDNA library was constructed by using polyA⁺ RNA from Balb/c3T3 fibroblasts. The plasmid vector pcDNAI which was used for this purpose is a mammalian expression vector in which cDNA inserts are expressed from a CMV promoter and contains an SV40 origin of replication for transient expression in COS cells (Invitrogen). The library was screened with oligonucleotide probes corresponding to N-terminal and C-terminal KL coding sequences as described herein. A cDNA clone which contains the complete KL coding sequences as well as 5' and 3' untranslated sequences was obtained. The nucleotide sequence of this clone (Figure 17) is in agreement with the previously published sequences except for a single base change at position 664 which results in the substitution of serine 206 to alanine (2,38).

The analysis of murine KL cDNA clones by Anderson and collaborators indicated a spliced cDNA with an inframe deletion of 48 nucleotides suggesting the presence of alternatively spliced KL RNA transcripts in KL expressing cells (2). To identify alternatively spliced KL RNA transcripts in RNA from tissues and cell lines, the RT-PCR method was used. The primers used corresponded to the 5' and 3' untranslated regions of the KL cDNA and were modified to contain unique restriction sites. Electrophoretic

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analysis of the RT-PCR reaction products shown in Figure 18 indicates a single fragment of approximately 870 bp in the samples from Balb3T3 cells and brain, whereas in the samples from spleen, testis and lung two fragments were seen, approximately 870 and 750 bp in size. For further analysis the two PCR reaction products were subcloned into the mammalian expression vector pCDM8. DNA sequence analysis first indicated that the larger PCR product corresponds to the known KL cDNA sequence, subsequently referred to as KL-1. In the smaller PCR product, however, a segment of 84 nucleotides of the KL coding sequences was lacking, generating an inframe deletion. The deletion endpoints corresponded to exon boundaries in the rat and the human KL genes and it is quite likely that these boundaries are also conserved in the mouse gene (27). Therefore, the smaller PCR product appeared to correspond to an alternatively spliced KL RNA transcript, designated KL-2. The exon missing in KL-2 precedes the transmembrane domain; it contains one of the four N-linked glycosylation sites and includes the known C-terminus (Ala-166 and Ala-167) of the soluble form of KL (58). KL-2 therefore is predicted to encode a truncated version of KL-1 which is presumably synthesized as a transmembrane protein (Figures 17 and 19).

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KL-2 Is Expressed In A Tissue Specific Manner

30 The alternatively spliced transcript KL-2 had been detected in spleen, testis and lung RNA, but not in fibroblasts and brain RNA, suggesting that the expression of KL-2 may be controlled in a tissue specific manner. In order to address this question in more detail the steady state levels of KL-1 and KL-2 RNA transcripts in RNA were determined from a wide variety of tissues by using an RNase protection assay.

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pcDNA1 plasmid containing the KL-1 cDNA was linearized with SpeI in order to generate an RNA hybridization probe of 625 nucleotides by using SP6 RNA polymerase. The probe was hybridized with 20 μ g of total RNA from Balb/c 3T3 fibroblasts, brain, spleen and testis of a 40 days old mouse, as well as from brain, bone marrow, cerebellum, heart, lung, liver, spleen and kidney of an adult mouse and placenta (14 days p.c.). The samples then were digested with RNase and the reaction products analyzed by electrophoresis in a 4% urea/polyacrylamide gel. In these experiments KL-1 mRNA protected a single fragment of 575 bases, while KL-2 mRNA protected fragments of 449 and 42 nucleotides. As shown in Figure 20, in Balb/c3T3 fibroblasts, KL-1 is the predominant transcript whereas the KL-2 is barely detectable. In brain and thymus KL-1 is the predominant transcript, but in spleen, testis, placenta, heart and cerebellum both KL-1 and KL-2 transcripts are seen in variable ratios. The ratio of the KL-1 to KL-2 in tissues determined by densitometry in brain is 26:1, in bone marrow 3:1, in spleen 1.5:1 and in testis (40 days p.n.) 1:2.6. These results suggest that the expression of KL-1 and KL-2 is regulated in a tissue-specific manner.

Biosynthetic characteristics of KL protein products in cos cells

Although KL was purified from conditioned medium of Balb/c 3T3 cells and is a soluble protein, the predicted amino acid sequences for KL-1 and KL-2 suggest that these proteins are membrane-associated. In order to investigate the relationship of KL-S with the KL-1 and KL-2 protein products their biosynthetic characteristics were determined. The KL-1 and KL-2 cDNAs, prepared by RT-PCR, were subcloned into the HindIII and XbaI sites of the expression vectors pcDNA1

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or pCDM8 for transient expression in COS-1 cells. To facilitate transient expression of the KL-1 and KL-2 protein products COS-1 cells were transfected with the KL-1 and KL-2 plasmids by using the DEAE-dextran/DMSO protocol as described herein. KL protein synthesis in the COS-1 cells was shown to be maximal between 72 to 96 hours subsequent to the transfection. In order to determine the biosynthetic characteristics of the KL-1 and KL-2 proteins pulse-chase experiments were carried out. 72 hours subsequent to transfection, cultures were labeled with ^{35}S -methionine (0.5mCi/ml) for 30 minutes and then chased with regular medium. The cell lysate and supernatants then were collected at the indicated times and processed for immunoprecipitation with anti-KL antiserum, prepared by immunizing rabbits with purified murine KL, and analysis by SDS-PAGE (12%). In cells transfected with the KL-1 plasmid, at the end of the labelling period, KL specific protein products of 24, 35, 40 and 45 kD are found (Figure 21). These proteins presumably represent the primary translation product and processed KL protein products which are progressively modified by glycosylation. Increasingly longer chase times reveal the 45 kD form as the mature KL protein product and it is quite likely that this protein represents the cell membrane form of KL. In the supernatant beginning at 30 minutes a 28 kD KL protein product is seen which, with increasing time, increases in amount. Two minor products of 38 and 24 kD were also found with increasing time. These results are consistent with the notion that KL-1 is first synthesized as a membrane-bound protein and then released into the medium probably through proteolytic cleavage.

A pulse-chase experiment of COS-1 cells transfected with the KL-2 plasmid is shown in Figure 20. The KL-2 protein

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products are processed efficiently to produce products of 32 kD and 28 kD which likely include the presumed cell membrane form of KL-2. The cell membrane form of KL-2 is more stable than the corresponding KL-1 protein with a half-life of more 5 than 5 hours. In the cell supernatant, after 3 hours, a soluble form of KL-2 of approximately 20 kD is seen. The appearance and accumulation of the soluble form of KL-2 in the cell supernatant is delayed compared with that of KL-1 in agreement with less efficient proteolytic processing of 10 the KL-2 protein product. In KL-2, as a result of alternative splicing, sequences which include the known C-terminus of the soluble form of KL and thus the presumed cleavage site of KL-1 is missing. Proteolytic cleavage of 15 KL-2, therefore, presumably involves a secondary cleavage site which is present in both KL-1 and KL-2, either on the N-terminal or C-terminal side of the sequences encoded by the deleted exon. A 38 kD KL-1 protein product seen in the supernatant may represent a cleavage product which involves 20 a cleavage site near the transmembrane domain (Figure 19).

Proteolytic processing of KL-1 And KL-2 in COS cells is modulated by PMA and the calcium ionophore A23187

The protein kinase C inducer PMA is known to facilitate 25 proteolytic cleavage of cell membrane proteins to produce soluble forms of the extra-cellular domain of these proteins as shown with the examples of the CSF-1 receptor, the c-kit receptor and TGF- α (13,4). The effect of PMA treatment on the biosynthetic characteristics of KL-1 and KL-2 in COS-1 30 cells has been determined. The pulse-chase experiments shown in Figure 22B indicate that PMA induces the rapid cleavage of both KL-1 and KL-2 with similar kinetics and that the released KL-1 and KL-2 protein products are indistinguishable from those obtained in the absence of

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inducer. These results suggest that the proteolytic cleavage machinery for both KL-1 and KL-2 is activated similarly by PMA. On one hand this may mean that two distinct proteases, specific for KL-1 and KL-2 respectively, 5 are activated by PMA or alternatively, that there is one protease which is activated to a very high level which cleaves both KL-1 and KL-2 but with different rates. The major cleavage site in KL-1 based on the known C-terminal amino acid sequence of rat KL, includes amino acids PPVA A 10 SSL (186-193) and may involve an elastase like enzyme (22,34). The recognition sequence in KL-2, based on the arguments presented above, presumably lies C-terminal of the deleted exon and therefore might include amino acids RKAAGA 15 (202-207) and thus could involve an enzyme with a specificity similar to the KL-1 protease, alternatively, it could be a trypsin-like protease. The effect of the calcium ionophore A23187 on KL cleavage has been determined. Both KL-1 and KL-2 cleavage is accelerated by this reagent 20 indicating that mechanisms that do not involve the activation of protein kinase C can mediate proteolytic cleavage of both KL-1 and KL-2 (Figure 22C).

Biological activity of the released KL protein products

25 To test the biological activity of the released KL protein products, the supernatants of transfected COS-1 cells were collected 72 hours after transfection and assayed for activity in the mast cell proliferation assay. Bone marrow derived mast cells (BMMC) were incubated for 24 hours with 30 different dilutions of the collected supernatants and assayed for 3 H-thymidine incorporation as described previously (Figure 23). Supernatants from KL-1 transfectants produced 3 to 5 times more activity than KL-2 transfectants in agreement with the differential release of

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soluble KL from KL-1 and KL-2. Importantly the proteins released from both the KL-1 and the KL-2 transfectants appeared to display similar specific activities in the mast cell proliferation assay.

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The Steel dickie allele results from a deletion of c-terminal KL coding sequences including the transmembrane and the cytoplasmic domains

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Mice homozygous for the $S1^d$ allele are viable, in contrast to mice homozygous for the $S1$ allele, although they lack coat pigment, are sterile and have macrocytic anemia. The c-kit receptor system in these mice, therefore, appears to display some residual activity. The $S1^d$ mutation affects the three cell lineages to similar degrees suggesting that the mutation affects an intrinsic property of KL. Thus, to investigate the molecular basis of $S1^d$, the KL coding sequences were first characterized in this allele by using PCR cloning technology. Primary embryo fibroblasts from an $S1^d/+$ embryo were derived by standard procedures. RNA prepared from $S1^d/+$ embryo fibroblasts and different primers then were used to amplify the $S1^d$ KL coding region paying attention to the possibility that $S1^d$ is a deletion mutation. RT-PCR amplification by using $S1^d/+$ total RNA and primers 1 and 2 produced one DNA fragment that migrated with a mobility identical to that of the product obtained from $+/+$ fibroblast RNA and sequence determination showed it to be indistinguishable from the known KL sequence. This fragment therefore presumably represented the normal allele. When primers 1 and 3 or 1 and 4 were used a faster migrating DNA fragment was amplified was well (Figure 18). Both the 850 and 1070 bp DNA fragments obtained with primers 1 + 3 and 1 + 4 were subcloned into pCDM8 and then sequenced. In the KL- $S1^d$ cDNA the segment from nucleotides 660 to 902 of

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the wild-type sequence is deleted, instead, a sequence of 67 bp was found to be inserted (Figure 17). The deletion insertion results in a termination codon three amino acids from the 5' deletion endpoint. The predicted amino acid sequence of KL-S1^d cDNA consists of amino acids 1 - 205 of the known KL sequence plus 3 additional amino acids (Figures 17 and 19). The KL-S1^d amino acid sequence includes all four N-linked glycosylation sites and all sequences contained in the soluble form of KL, while the transmembrane and the cytoplasmic domains of wild-type KL-1 are deleted. Consequently, the KL-S1^d protein product is a secreted protein, which displays biological activity.

Biosynthetic Characteristics And Biological Activity Of The
KL-S1^d and KL-S Protein Products

For comparison with the KL-S1^d protein product, a truncated version of KL-1 was made, designated KL-S, in which a termination codon was inserted at amino acid position 191 which is the presumed C-terminus of the soluble KL protein. COS-1 cells were transfected with the KL-S1^d and the KL-S plasmids and pulse-chase experiments were carried out to determine the biosynthetic characteristics of the two protein products. The KL-S1^d protein product is rapidly processed, presumably by glycosylation and then secreted into the medium, where the major 30 kD species is found after as early as 30 minutes of chase time and then increases in amount thereafter (Figure 24). The biosynthetic characteristics of the KL-S protein products are very similar to those of KL-S1^d (Figure 24). Again, with increasing time increasing amounts of secreted material are detected in the medium, conversely the cell associated KL-S protein products decrease with time.

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To assess the biological activity of the secreted KL-S1^d and KL-S protein products, mast cell proliferation assays were performed. The medium from transfected COS-1 cells was collected 72 hours after transfection and then different dilutions were used to assess proliferative potential conferred on BMMC in the absence of IL-3. Both samples contained significant biological activity that exceeded that of KL-1 to some degree (Figure 23). Taken together, these results demonstrate convincingly, that the KL-S1^d protein products are secreted and are biologically active.

Experimental Discussion

The demonstration of allelism between c-kit and the murine W locus brought to light the pleiotropic functions of the c-kit receptor in development and in the adult animal and facilitated the identification of its ligand KL. The recent discovery of allelism between KL and the murine steel locus, furthermore provided a molecular notion of the relationship between the W and the S1 mutations which had been anticipated by mouse geneticists based on the parallel and complementary phenotypes of these mutations. The predicted transmembrane structure of KL implicated that, both, membrane-associated and soluble forms of KL play significant roles in c-kit function. In this application, experimental evidence for this conjecture is provided.

First, it is shown that the soluble form of KL is generated by efficient proteolytic cleavage from a transmembrane precursor, KL-1. Second, an alternatively spliced version of KL-1, KL-2, in which the major proteolytic cleavage site is removed by splicing, is shown to produce a soluble biologically active form of KL as well, although, with somewhat diminished efficiency. Third, cleavage of KL-1 and

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KL-2 in COS-1 cells is a process that can be modulated. Fourth, KL-1 and KL-2 are expressed in a tissue-specific manner. Furthermore, the viable S1^d mutation was shown to be the result of a deletion that includes the C-terminus of 5 the KL coding sequence including the transmembrane domain generating a biologically active secreted form of KL. The phenotype of mice carrying the S1^d allele provides further support for the concept for a role for both the secreted and the cell membrane- associated forms of KL in c-kit function.

10 Because of the close evolutionary relationship of c-kit with CSF-1R it was reasonable to predict a relationship between the corresponding growth factors, KL and CSF-1, in regards to both structural and topological aspects. Alternatively 15 spliced forms of CSF-1 mRNAs are known to encode protein products which differ in sequences N-terminal of the transmembrane domain, a spacer segment of 298 amino acids located in between the ligand portion and the transmembrane domain of the protein (43). In addition, alternatively 20 spliced CSF-1 RNA transcripts differ in their 3' untranslated regions (21). Analysis of KL RNA transcripts in several tissues identified an alternatively spliced KL RNA in which, similar to the situation in CSF-1, the spacer between the presumed ligand portion and the transmembrane 25 domain is deleted. Interestingly, the expression of this alternatively spliced RNA product is controlled in a tissue specific manner. A recent comparative analysis of the ligand portions of KL and CSF-1 indicates structural homology between the two proteins based on limited amino 30 acid homology and the comparison of corresponding exons and matching of "exon-encoded secondary structure" (4). Furthermore, the super position of 4 α -helical domains and cysteine residues which form intra-molecular disulfide bonds implies related tertiary structures for the ligand domains

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of KL and CSF-1; and the homology seen in the N-terminal signal peptides, the transmembrane domains and the intracellular domains of the two proteins may indicate that these domains fulfill important related functions in the two 5 proteins. These results strengthen the notion of an evolutionary relationship and structural homology between KL and CSF-1.

10 A unique feature of KL is its predicted tripartite structure as a transmembrane protein. Both forms of KL, KL-1 and KL-2, are synthesized as transmembrane proteins which are processed by proteolytic cleavage to release a soluble biologically active form of KL; although, the processing step in the two forms follows differing kinetics, as 15 determined in the COS cell system. Proteolytic cleavage of the KL-1 protein is very efficient, in contrast, the KL-2 protein is more stable or resistant to proteolytic cleavage. The sequences encoded by the deleted exon, amino acids 174-201 include the C-terminus of the soluble KL protein and the 20 presumed proteolytic cleavage site (27). A secondary or alternate proteolytic cleavage site is therefore presumably being used to generate the soluble KL-2 protein and this cleavage might involve another protease. The induction of 25 proteolytic cleavage of KL-1 and KL-2 in COS-1 cells by the protein kinase C activator PMA and by the calcium ionophore A23187 suggests that in different cell types this process may be subject to differential regulation. Interestingly, the soluble KL-2 protein displays normal biological activity 30 indicating that the sequences encoded by the deleted exon are not essential for this activity.

On one hand, KL-1 and KL-2 in their membrane associated versions may function to mediate their signal by cell-cell contact or, alternatively, they might function as cell

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adhesion molecules (19, 26). On the other hand, the soluble forms of KL are diffusible factors which may reach the target cell and its receptor over a relatively short or longer distances. But the soluble forms of KL might also 5 become associated with, or sequestered in the extracellular matrix, in an analogous fashion to FGF, LIF or int-1, and thus function over a short distance similar to the membrane-associated form (8,33,42). When cell membrane-associated, KL may be able to provide or sustain high concentrations of 10 a localized signal for interaction with receptor-carrying target cells. In turn the soluble form of KL may provide a signal at lower and variable concentrations. c-kit is thought to facilitate cell proliferation, cell migration, cell survival and post-mitotic functions in various cell 15 systems. By analogy with the CSF-1 receptor system, the cell survival function and cell migration might require lower concentrations of the factor than the cell proliferation function (55). The cell membrane-associated and the soluble forms of KL then may serve different aspects 20 of c-kit function. Both the CSF-1 receptor and c-kit can be down-regulated by protein kinase C mediated proteolytic release of the respective extracellular domains (13). The functional significance of this process is not known but it has been hypothesized that the released extracellular domain 25 of these receptors may neutralize CSF-1 and KL, respectively, in order to modulate these signals. In some ways proteolytic cleavage of KL results in a down modulation of c-kit function and the processes, therefore, may be considered as complementary or analogous. In summary, the 30 synthesis of variant cell membrane-associated KL molecules and their proteolytic cleavage to generate soluble forms of KL provide means to control and modulate c-kit function in various cell types during development and in the adult animal.

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A unique opportunity to evaluate the role of the soluble form of KL during development and in adult animals was provided through the characterization of the molecular basis of the S1^d mutation. The S1^d allele encodes a secreted version of the KL protein and no membrane associated forms as a result of a deletion which includes the transmembrane domain and the C-terminus of KL. The biological characteristics of S1^d/S1^d and S1/S1^d mice, therefore should give clues about the role of the soluble and the membrane-associated forms of KL. S1/S1^d mice produce only the S1^d protein, since the S1 allele is a KL null-mutation (11,38). These mice are viable and are characterized by a severe macrocytic anemia, lack of tissue mast cells, lack of coat pigmentation and infertility. In most aspects of their mutant phenotype, these mice resemble W/W^v mice (47,51). However some significant differences exist. The anemia of S1/S1^d mice appear to be more sensitive to hypoxia than W/W^v mice (46, 47). In regards to gametogenesis in W/W^v mice primordial germ cells do not proliferate and their migration is retarded (32). In S1/S1^d embryos primordial germ cells similar to W/W^v embryos do not proliferate, however the remaining cells appear to migrate properly and they reach the gonadal ridges at the appropriate time of development (29,51). From these experiments one might hypothesize that the S1^d KL protein product is able to sustain cell migration but not cell proliferation and consequently the cell membrane form of KL therefore may play a critical role in the proliferative response of c-kit. Furthermore, S1/S1^d fibroblasts do not support the proliferation and maintenance of bone marrow mast cells in the absence of IL-3, in contrast to normal embryo fibroblasts which have this property (16). Provided that the S1/S1^d fibroblast indeed synthesize the S1^d protein products, the inability of the S1/S1^d fibroblasts to support the proliferation of mast

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cells, on one hand, may indicate that the amount of soluble KL-S1^d protein which is released by these cells is not sufficient to facilitate proliferation; on the other hand, these results may suggest that there is a critical role for
5 the cell membrane associated form of KL in this process.

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What is claimed is:

1. A purified mammalian protein corresponding to a c-kit ligand which comprises a homodimer of two polypeptides, each polypeptide having a molecular weight of about 30 kilodaltons and an isoelectric point of 3.8.
5
2. A purified mammalian protein of claim 1, wherein the mammalian protein is a murine protein.
10
3. A purified mammalian protein of claim 1, wherein the mammalian protein is a human protein.
15
4. A purified mammalian protein corresponding to a c-kit ligand which comprises a homodimer of two polypeptides, each polypeptide having a molecular weight of about 30 kilodaltons, an isoelectric point of 3.8, and wherein the two polypeptides are linked by a disulfide bond.
20
5. A pharmaceutical composition comprising the purified mammalian protein of claims 1 or 4 and a pharmaceutically acceptable carrier.
25
6. A pharmaceutical composition for the treatment of leucopenia in a mammal, which comprises an effective amount of the pharmaceutical composition of claim 5 and an effective amount of a factor selected from the group consisting of G-CSF, GM-CSF and IL-3, effective to treat leucopenia in a mammal.
30
7. A pharmaceutical composition for the treatment of

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anemia in a mammal, which comprises an effective amount of the pharmaceutical composition of claim 5, and an effective amount of EPO or IL-3, effective to treat anemia in a mammal.

5

8. A pharmaceutical composition for enhancing engraftment of bone marrow during transplantation in a mammal, which comprises an effective amount of the pharmaceutical composition of claim 5 and an effective amount of IL-1 or IL-6, effective to enhance a graftment of bone marrow during transplantation in a mammal.

10

9. A composition for enhancing bone marrow recovery in treatment of radiation, chemical or chemotherapeutic induced bone marrow aplasia or myelosuppression which comprises an effective amount of the pharmaceutical composition of claim 5 and an effective amount of IL-1, effective to enhance bone marrow recovery in a mammal.

15

10. A composition for treating acquired immune deficiency syndrome (AIDS) in a patient which comprises an effective amount of the pharmaceutical composition of claim 5 and an effective amount of AZT or G-CSF, effective to treat AIDS in a patient.

20

11. A pharmaceutical composition for treating nerve damage in a mammal, which comprises an effective amount of the pharmaceutical composition of claim 5, effective to treat nerve damage in a mammal.

25

12. A composition for treating infants exhibiting

30

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symptoms of defective lung development which comprises an effective amount of the composition of claim 5, effective to treat infants exhibiting symptoms of defective lung development.

5

13. A composition for the prevention of hair loss in a subject which comprises an effective amount of the pharmaceutical composition of claim 5, effective to prevent hair loss in the subject.

10

14. A composition for inhibiting the loss of pigment in a subject's hair, which comprises an effective amount of the pharmaceutical composition of claim 5, effective to prevent the loss of pigment in the subject's hair.

15

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

20

16. An isolated nucleic acid molecule of claim 15, wherein the c-kit ligand (KL) is a human c-kit ligand (KL).

25

17. An isolated nucleic acid molecule of claim 15, wherein the c-kit ligand (KL) is a murine c-kit ligand (KL).

30

18. An isolated nucleic acid molecule of claim 15, wherein the nucleic acid molecule is a DNA molecule.

19. The DNA molecule of claim 18, wherein the DNA molecule is a cDNA molecule.

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20. A nucleic acid molecule of claim 15, wherein the nucleic acid molecule is an RNA molecule.
- 5 21. An isolated nucleic acid molecule of claim 15, encoding a soluble c-kit ligand (KL).
- 10 22. An isolated nucleic acid molecule of claim 21, wherein the soluble c-kit ligand is a soluble human c-kit ligand.
- 15 23. An isolated nucleic acid molecule of claim 21, wherein the soluble c-kit ligand is murine.
- 20 24. An isolated nucleic acid molecule of claim 21, wherein the nucleic acid molecule is a DNA molecule.
25. The DNA molecule of claim 24, wherein the DNA molecule is a cDNA molecule.
26. An isolated nucleic acid molecule of claim 21, wherein the isolated nucleic acid molecule is an RNA molecule.
- 25 27. The isolated nucleic acid molecule of claim 15 or 21 wherein the isolated nucleic acid molecule is operatively linked to a promoter of RNA transcription.
- 30 28. A vector which comprises the isolated nucleic acid molecule of claim 15 or 21.
29. A vector of claim 28 which comprises a plasmid.

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30. A vector of claim 28 which comprises a virus.
31. A host vector system for the production of an amino acid sequence which is the c-kit ligand which comprises the plasmid of claim 29 in a suitable host.
5
32. A host vector system of claim 31, wherein the suitable host is a eucaryotic cell.
10
33. A host vector system of claim 32, wherein the eucaryotic cell is a mammalian cell.
34. A host vector system of claim 32, wherein the eucaryotic cell is an insect cell.
15
35. A host vector system of claim 32, wherein the eucaryotic cell is a yeast cell.
36. A host vector system of claim 31, wherein the suitable host is a procaryotic cell.
20
37. A c-kit ligand (KL) polypeptide wherein the c-kit ligand (KL) polypeptide comprises a fragment of the protein of claim 1.
25
38. A mutated c-kit ligand (KL) polypeptide wherein the biological activity mediated by the binding of the ligand to the receptor is destroyed.
30
39. A substance capable of specifically forming a complex with the c-kit ligand (KL) polypeptide of claim 1 or 37.

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40. The substance of claim 39, wherein the substance is a monoclonal antibody.
- 5 41. The substance of claim 40, wherein the monoclonal antibody is a human monoclonal antibody.
42. The c-kit ligand (KL) polypeptide of claim 37 conjugated to an imageable agent.
- 10 43. The c-kit ligand (KL) polypeptide of claim 42, wherein the imageable agent is selected from the group consisting of radioisotopes, dyes or enzymes.
- 15 44. The c-kit ligand (KL) polypeptide of claim 1 or 38 conjugated to a therapeutic agent.
- 20 45. The c-kit ligand (KL) polypeptide of claim 44, wherein the therapeutic agent is selected from the group consisting of toxins, chemotherapeutic agents or radioisotopes.
- 25 46. A method for producing a c-kit ligand (KL) polypeptide which comprises growing the host vector system of claim 43 under suitable conditions permitting production of the c-kit ligand (KL) polypeptide and recovering the resulting c-kit ligand (KL) polypeptide.
- 30 47. The c-kit ligand (KL) produced by the method of claim 46.
48. A pharmaceutical composition which comprises the mutated c-kit ligand of claim 38 and a

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pharmaceutically acceptable carrier.

5 49. A method of modifying a biological function associated with c-kit cellular activity which comprises contacting a cell, whose function is to be modified, with an effective amount of the pharmaceutical composition of claim 5, effective to modify the biological function of the cell.

10 50. The method of claim 49, wherein the biological function is the propagation of a cell that expresses c-kit.

15 51. The method of claim 50, wherein the cell which expresses c-kit is a hematopoietic cell.

20 52. The method of claim 49, wherein the biological function is in vitro fertilization.

25 53. 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 5, effective to modify the biological function associated with c-kit function.

30 54. 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 5, effective to stimulate the proliferation of the mast cells in the patient.

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5 55. 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 5, which is effective to induce differentiation of the mast cells.

10 56. A method of inducing differentiation of erythroid progenitors in a patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 5, which is effective to induce differentiation of the erythroid progenitors.

15 57. A pharmaceutical composition for the treatment of leukemia in a mammal which comprises an effective amount of the pharmaceutical composition of claim 5 and an effective amount of GM-CSF, effective to treat leukemia in a mammal.

20 58. A method of treating leukemia in a mammal which comprises administering to the mammal an effective amount of the pharmaceutical composition of claim 54, effective to treat the leukemia.

25 59. A method of treating leukemia according to claim 58, wherein the leukemia is acute myelogenous leukemia.

30 60. A method of treating leukemia according to claim 58, wherein the leukemia chronic myelogenous leukemia.

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5 61. A method of treating allergies in a patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 5, effective to treat the allergy.

10 62. A method of treating melanoma in a patient, which comprises administering to the patient an effective amount of the composition of claim 5, effective to treat the melanoma.

15 63. A method for the treatment of leucopenia in a patient which comprises administering an effective amount of the composition of claim 6.

20 64. A method for the treatment of anemia in a patient which comprises administering an effective amount of the composition of claim 7.

25 65. A method for enhancing engraftment of bone marrow during transplantation in a patient which comprises administering an effective amount of the composition of claim 8.

30 66. A method of enhancing bone marrow recovery in treatment of radiation, chemical, or chemotherapeutic induced bone marrow aplasia or myelosuppression which comprises treating patients with therapeutic effective doses of the composition of claim 9.

67. A method of treating acquired immune deficiency in a patient which comprises administering to the patient a therapeutically effective amount of the

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composition of claim 10.

5 68. A method for enhancing transfection of early hematopoietic progenitor cells with a gene which comprises:

a) contacting early hematopoietic cells with the polypeptide of claim 1;

10 b) and transfecting the cultured cells of step (a) with the gene.

69. The method of claim 61, wherein the gene encodes for antisense RNA.

15 70. A method of transferring a gene to a mammal which comprises:

20 a) contacting early hematopoietic progenitor cells with the polypeptide of claim 1;

b) transfecting the cells of (a) with the gene; and

25 c) administering the transfected cells of (b) to the mammal.

71. The method of claim 70, wherein the gene encodes for antisense RNA.

30

72. A method of treating nerve damage in a subject which comprises administering to the patient therapeutically an effective amount of the polypeptide of claim 11.

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73. A method of treating infants exhibiting symptoms of defective lung development which comprises administering to the infant a therapeutically effective amount of the polypeptide of claim 12.

5

74. A method of preventing the loss of hair in a patient which comprises administering to the patient an effective amount of the composition of claim 13.

10

75. A method of inhibiting the loss of pigment in a subject's hair which comprises administering to the subject an effective amount of the composition of claim 14.

15

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

20

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 induced;

25

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

30

c) incubating a. and b. with ^3H -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

-110-

5

- e) 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.

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CPM X 10-3

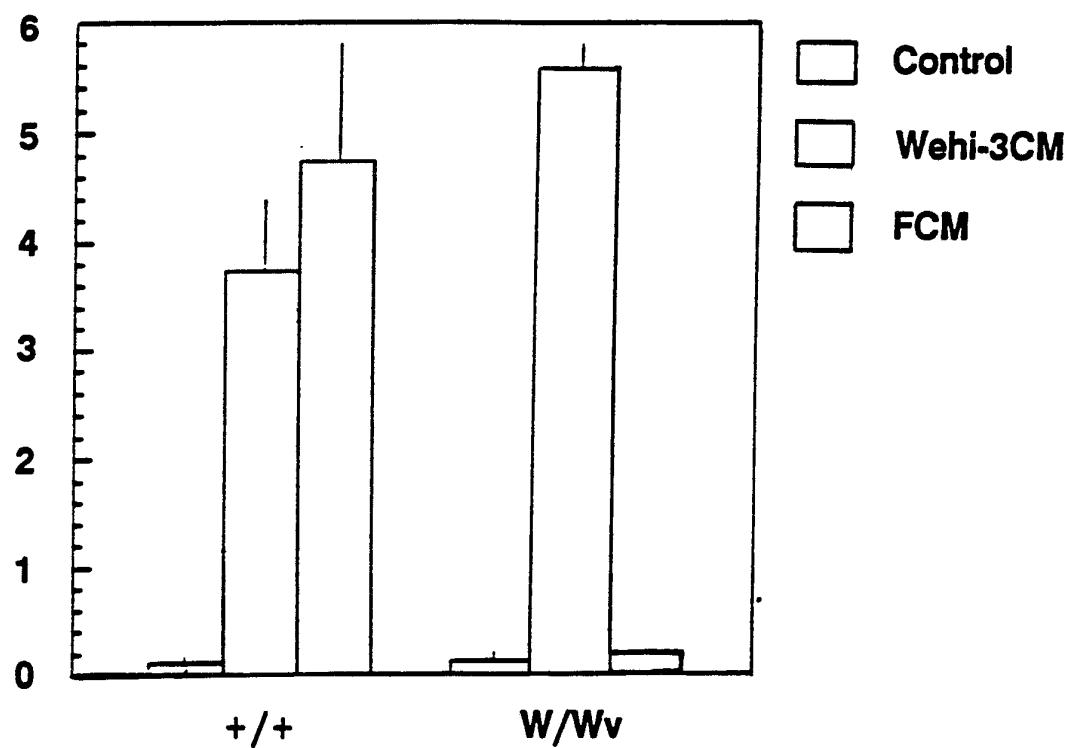
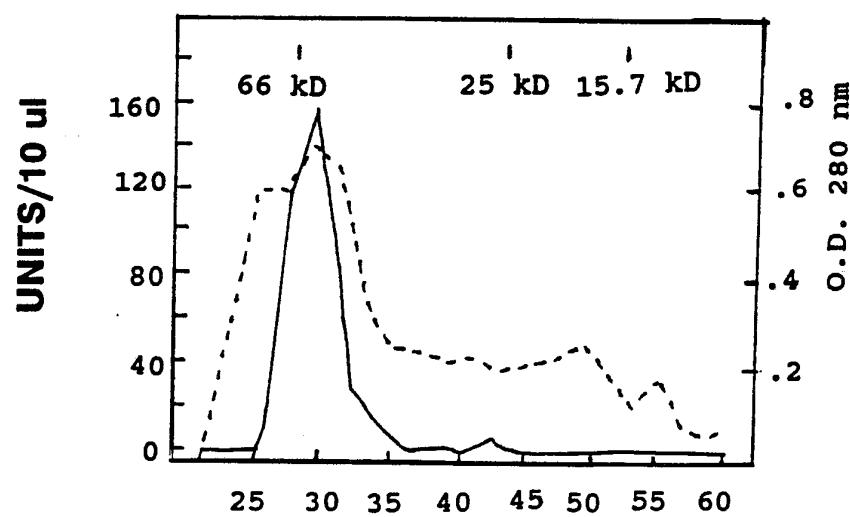


FIGURE 1

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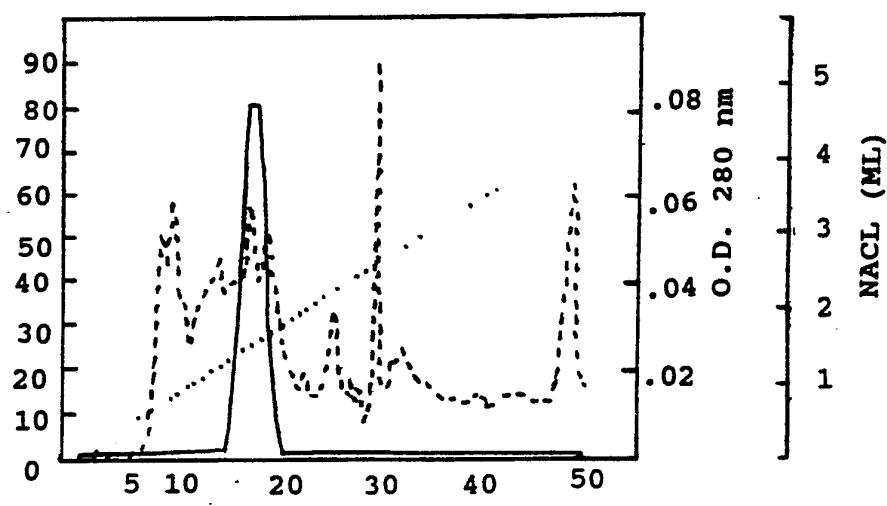
2/35

FIGURE 2 A**ACA 54****FRACTION #**

-3/35

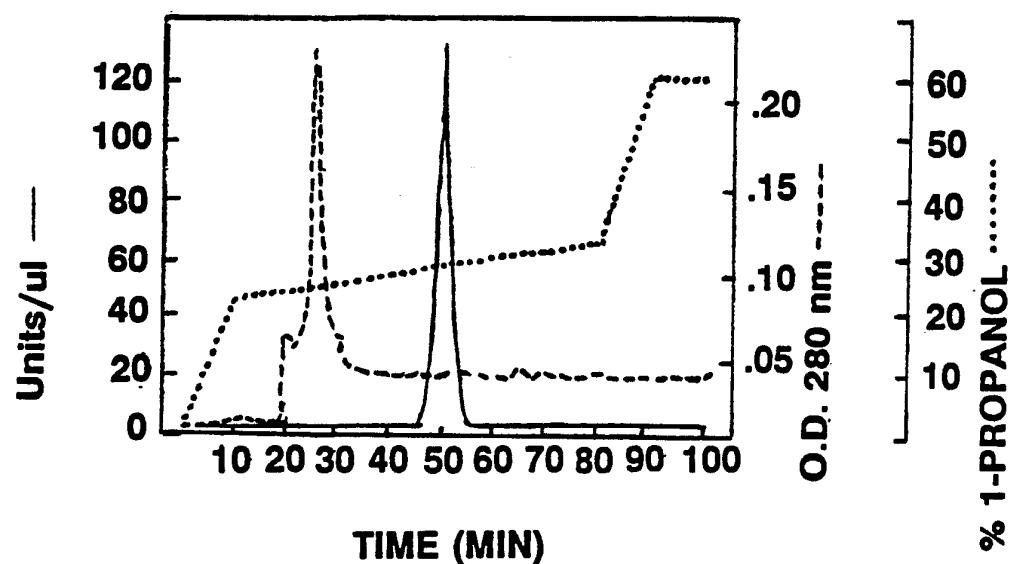
FIGURE 2 B

DEAU-5PW



FRACTION #

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FIGURE 2 C**SEMI-PREPARATIVE C 18**

5/35

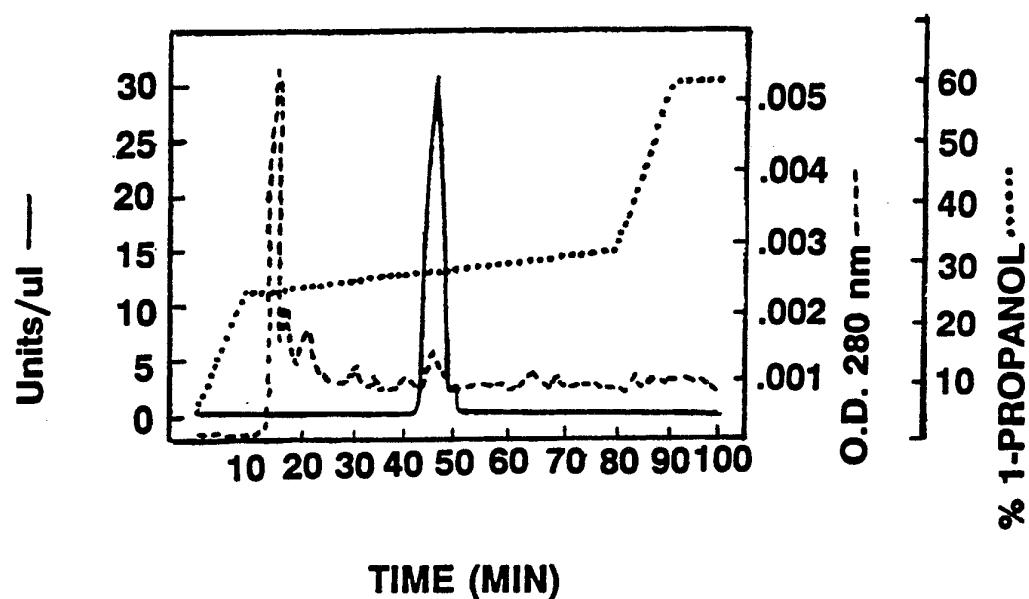
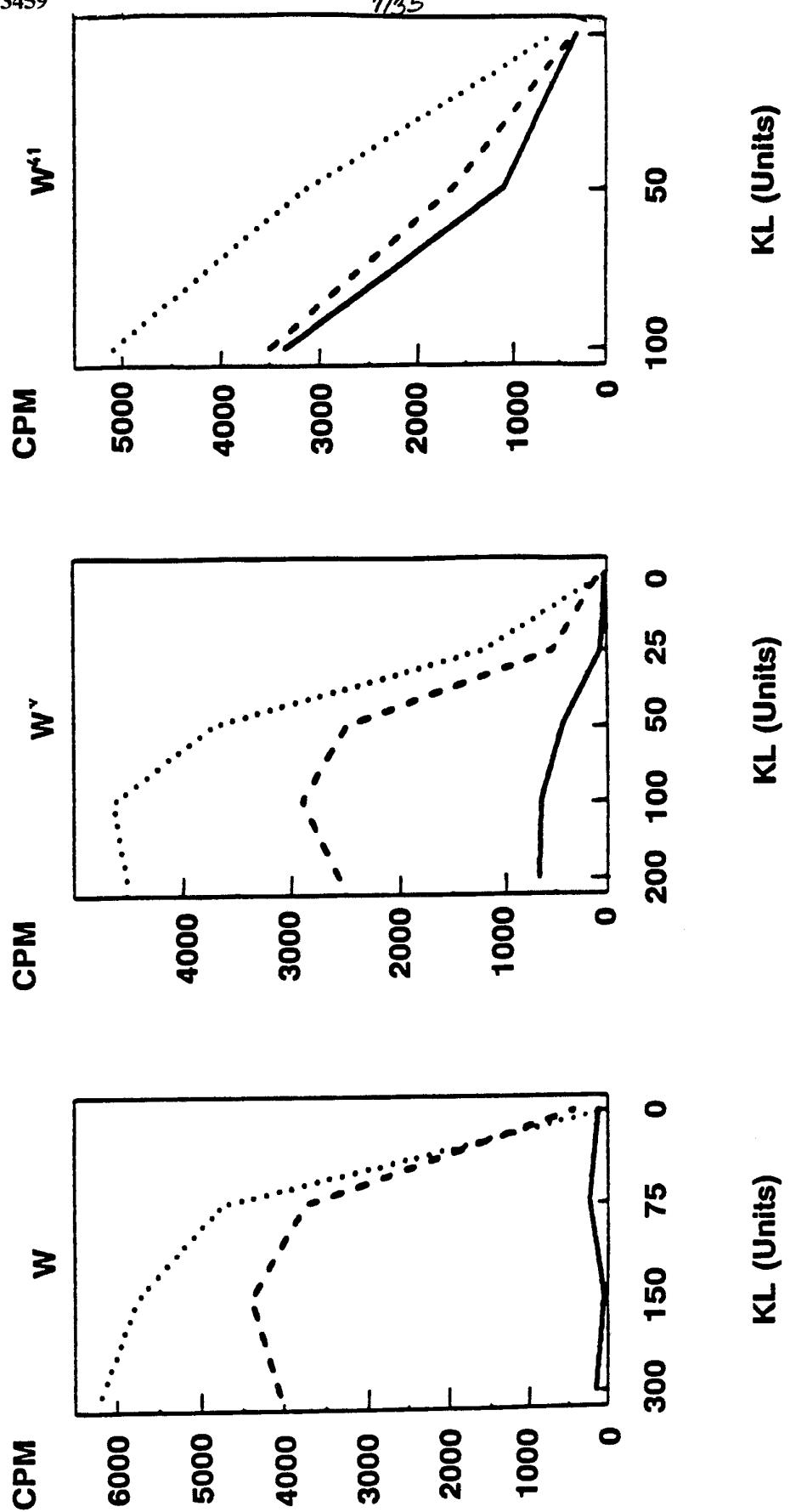
FIGURE 2 D**ANALYTICAL C 18**



FIGURE 3

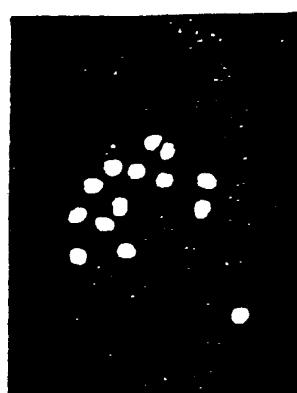
FIGURE 4



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FIGURE 5 A

**BONE MARROW
DERIVED
MAST
CELLS**



**PERITONEAL MAST
CELLS**



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C-KIT SURFACE EXPRESSION

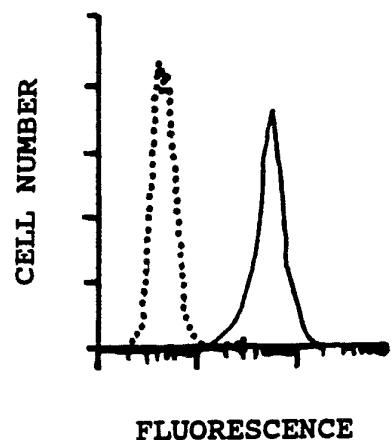
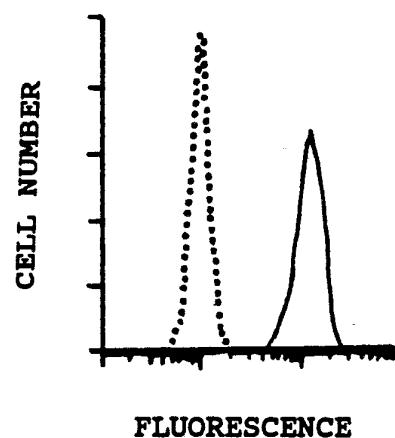
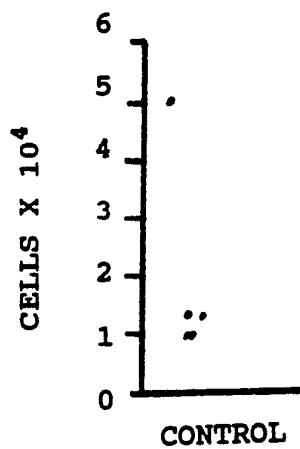
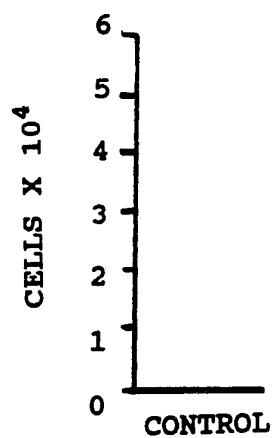


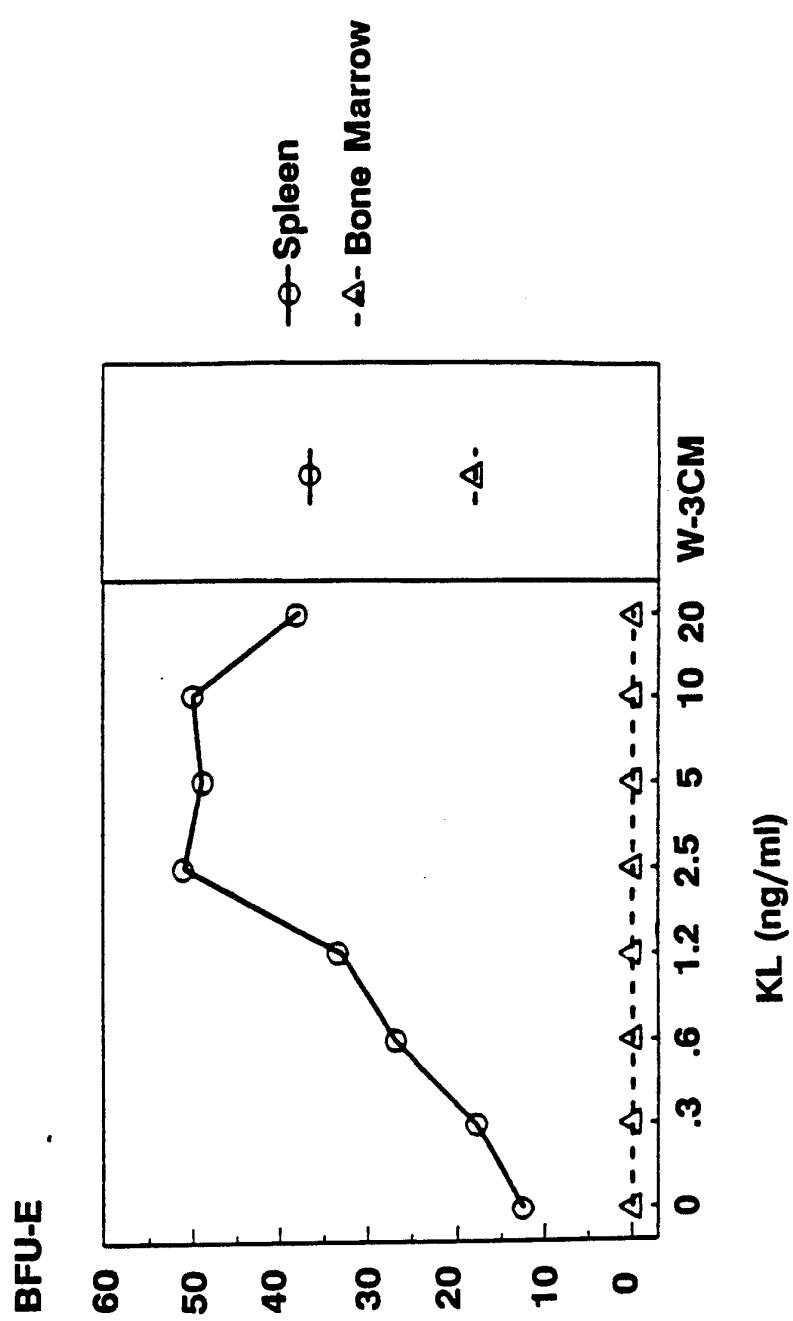
FIGURE 5 B

10/35

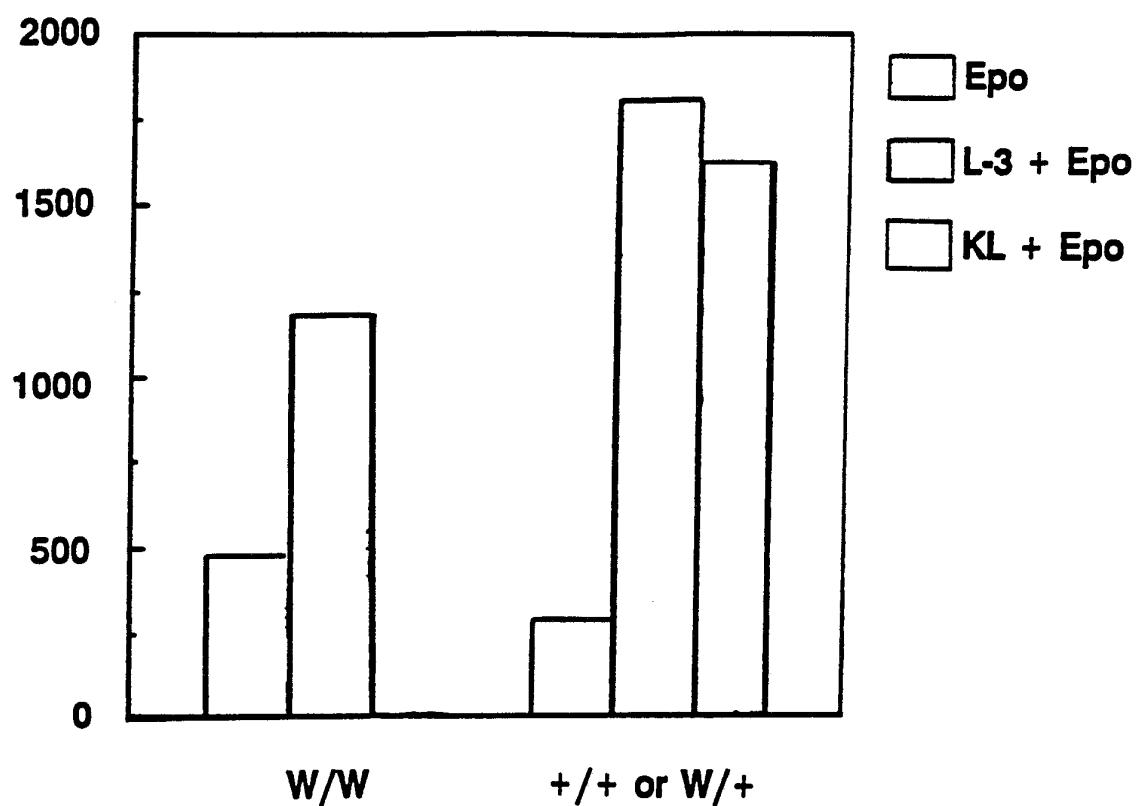
**FIGURE 5 C**

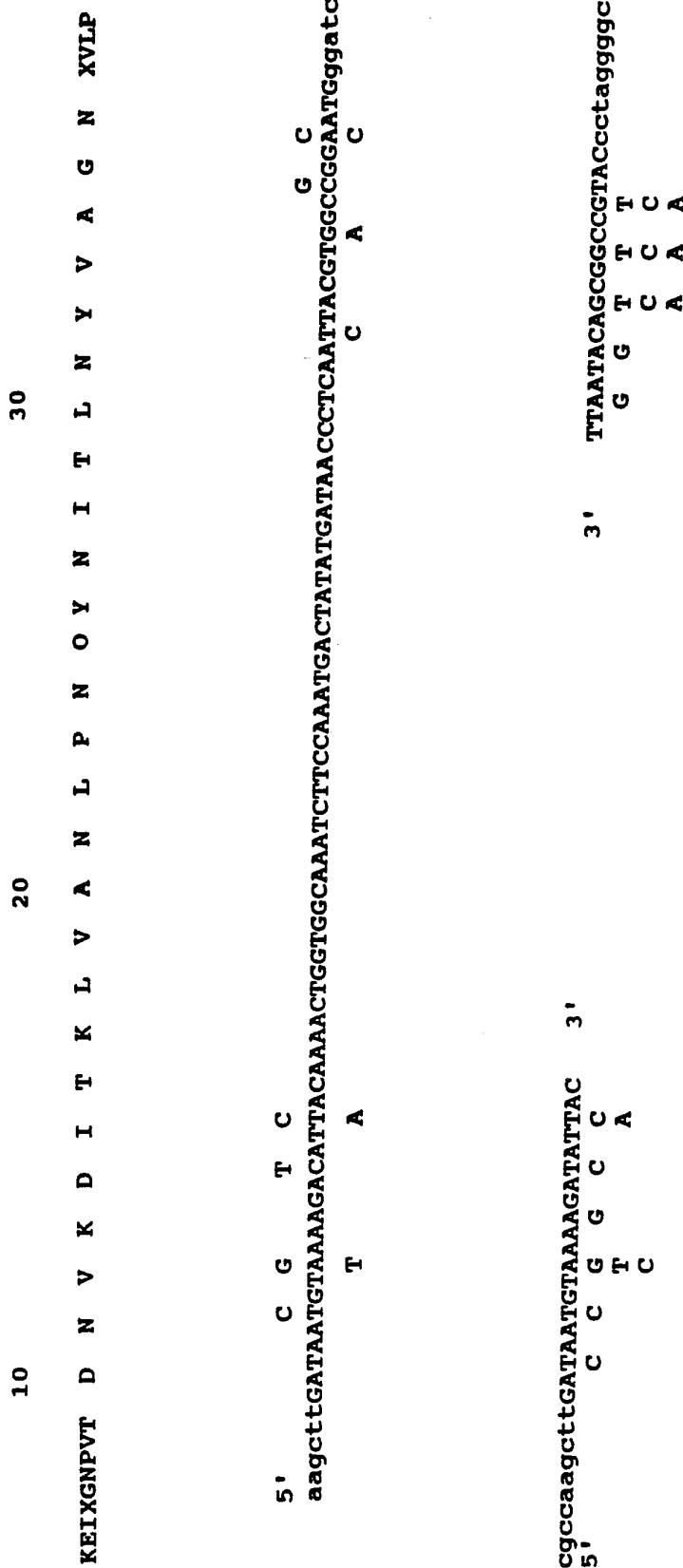
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FIGURE 6



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FIGURE 7**BFU-E/Fetal Liver****SUBSTITUTE SHEET**

**FIGURE 8**

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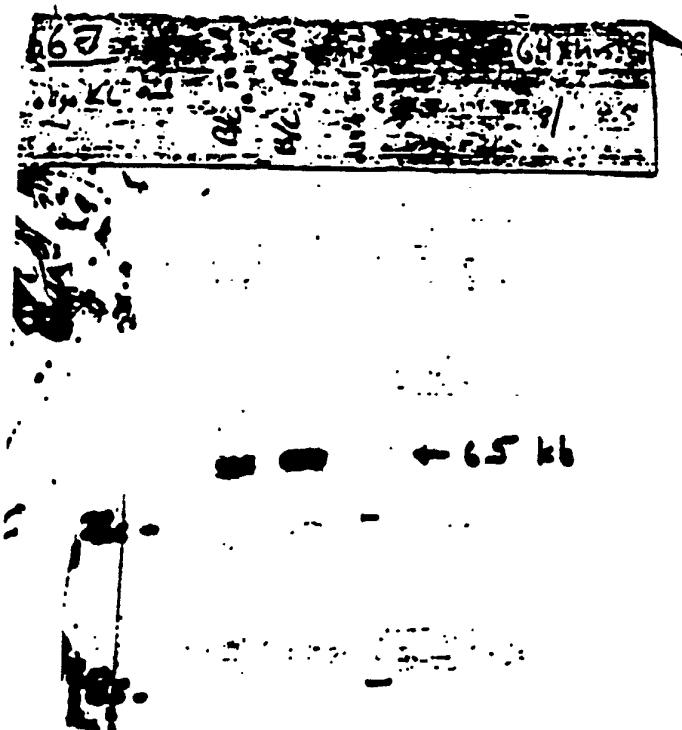


FIGURE 9

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FIGURE 10

SUBSTITUTE SHEET

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| SP

M K K T Q T W I I T C I Y L Q 15
 GCGGTGCCCTTCCTTATGAAGAACACAAACTGGATTATCACTGCATTATCTTCAA

.....| L L L F N P L V K T K E I C G N P V T Q 35
 CTGCTCCTATTTAACCTCTCGTCAAAACCAAGGAGATCTGCGGGAACTCCTGTGACTGAT

M V K Q I T K L V A N L P N D Y N I T L 55
 AATGTAAAAGACATTACAAAACGGTGGCAAATCTTCAAATGACTATATGATAACCCCTC

M Y V A G N D V L P S N C W L R D N V I 75
 AACTATGTCGCCGGATGGATGTTGCCTAGTCATTGGCTACGACATATGGTAATA

Q L S L S L T T L L D K F S N I S E G L 95
 CAATTATCACTCAGCTTGA C T C T G G A C A A G T C T C A A A T A T T C T G A A G G C T T G

S M Y S I I D K L G K I V D Q L V L C M 115
 AGTAATTACTCCATCATAGACAAACTGGAAAATAGTGGATGACCTCGTGTATGCATG

E E N A P K N I K E S P K R P E T R S F 135
 GAAGAAAACGCACCGAAGAATATAAGAATCTCCGAAGAGGCCAGAAACTAGATCCTT

T P E E F F S I F N R S I D A F K D F M 155
 ACTCCTGAAGAATTCTTAGTATTTCAATAGATCCATTGATGCCTTAAGGACTTTATG

V S S D T S D C V L S S T L G P E K D S 175
 GTGGCATCTGACACTAGTGA C T G T G C T C T C A A C A T T A G G T C C C G A G A A G A T T C C

R V S V T K P F M L P P V A A S S L R N 195
 AGAGTCAGTGTACAAAACCATTATGTTACCCCTGTTGCAGCCAGCTCCCTAGGAAT

D S S S S N R K A A K S P E D S G L Q W 215
 GACAGCAGTAGCAGTGATAGGAAAGCCGCAAAGTCCCCTGAAGACTCGGGCCTACAATGG

| TMS
 T A N A L P A L I S L V I G F A F G A L 235
 ACAGCCATGGCATTGCCGGCTCTCATTCGCTTGTAAATTGGCTTTGCTTTGGAGCCTTA

.....| Y W K K K Q S S L T R A V E N I Q I N E 235
 TACTGGAAGAACAGTCAGTCTTACAAGGGCAGTTGAAATACAGATTAATGAA

E C N E I S M L Q Q K E R E F 270
 GAGGATAATGAGATAAGTATGCTGCAACAGAAAGAGAGAGAATTT

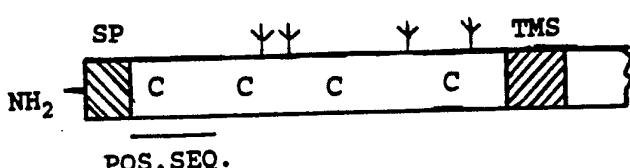
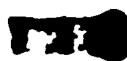


FIGURE 11

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16s - 26s -



160

FIGURE 12

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FIGURE 13

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FIGURE 14

Figure 14 A

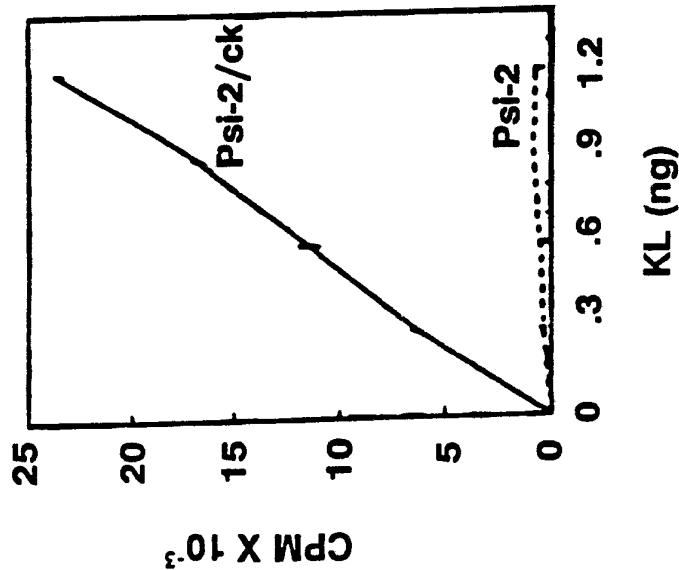
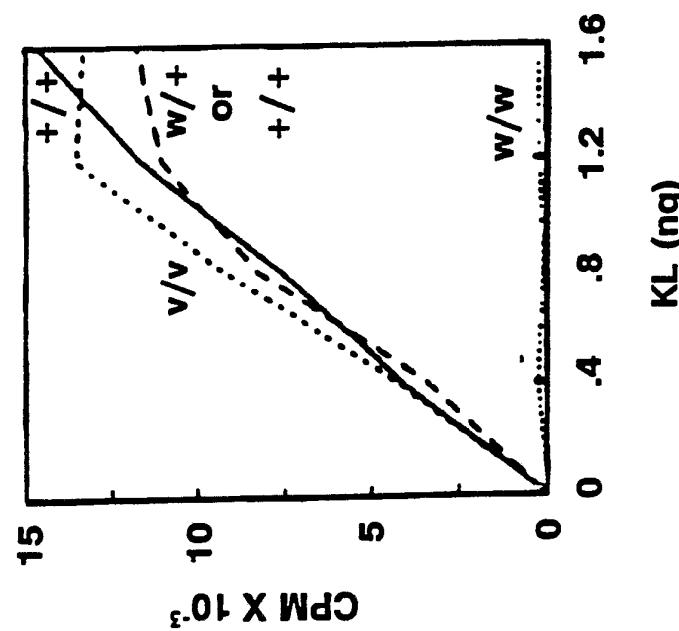


Figure 14 B



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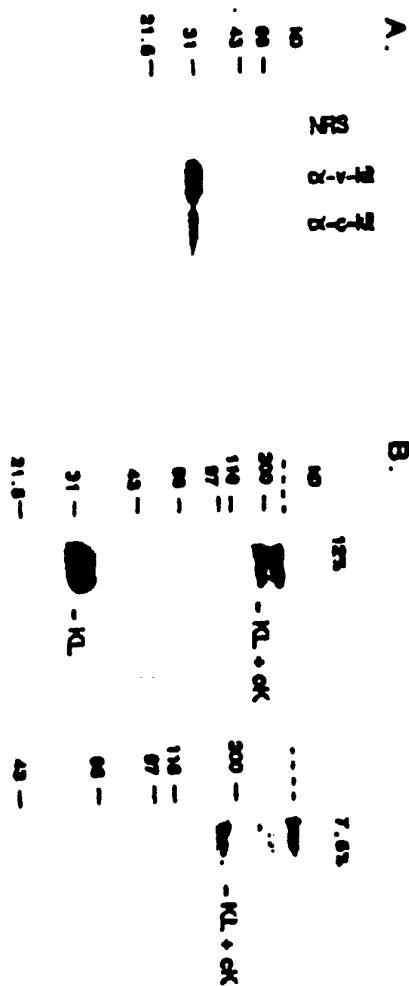


FIGURE 15

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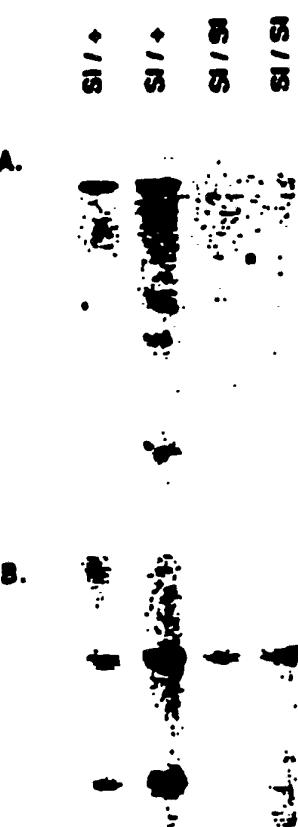


FIGURE 16

SUBSTITUTE SHEET

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60

120

	M	K	K	T	Q	5
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SP

180

ACTTGGATTATCACTGCATTATCTTCAACTGCTCCTATTTAATCCTCTTGTCAAAACC
 T W I I T C I Y L Q L L L F N P L V K T 25
 AAGGAGATCTGGGGAACTCTGTGACTGATAATGTAAGAACATTACAAAAGTGGCA 240
 K E I C G N P V T D N V K D I T K L V A 45
 AATCTTCAAATGACTATGATAACCTCAACTATGTCGCCGGATGGATTTGCCT 300
 N L P N D Y M I T L N Y V A G M D V L P 65
 AGTCATTGTTGGCTACGAGATATGTAATACAATTACTCAGCTTGAECTACTCTG 360
 S H C W L R D M V I Q L S L S L T T L L 85
 GACAAGTCTCAAATATTCTGAAGGCTTGAAGTAACTCCATCATAGACAAACTGGG 420
 D K F S N I S E G L S N Y S I I D K L G 105
 AAAATAGTGGATGACCTCGTGTATGCATGAAAGAACGACCGAAGAATAAAAGAA 480
 K I V D D L V L C M E E N A P K N I K E 125
 TCTCCGAAGAGGCCAGAAACTAGATCCTTACTCCTGAAGAATTCTTAGTATTTCAAT 540
 S P K R P E T R S F T P E E F F S I F N 145
 AGATCCATTGATGCCTTAAGGACTTTATGGTGGCATCTGACACTAGTGAECTGTGCTC 600
 R S I D A F K D F M V A S D T S D C V L 165

▼

TCTTCAACATTAGTCCCAGAAAGATTCCAGAGTCAGTGTACAAAACCATTATGTTA 660
 S S T L G P E K D S R V S V T K P F M L 185

▼

CCCCCTGTTGCAGCCAGCTCCCTAGGAATGACAGCAGTAGCAGTAATAGGAAAGCCGCA 720
 P P V A A S S L R N D S S S S N R K A A 205

▲

GTCTCTCTTGACAA---- s1^d
 AAGGCCCTGAAGACTCGGGCCTACAATTGACAGCCATGGCATTGCCGGCTCTCATTG 780
 K A P E D S G L Q W T A M A L P A L I S 225
 A S L - s1^d

TMS

CTTGTAATTGGCTTGGAGCCTTACTGGAAGAACAGTCAAGTCTTACA 840
 L V I G F A F G A L Y W K K K Q S S L T 245
 AGGCAGTTGAAATATACAGATAATGAAGAGGATAATGAGATAAGTATGTTGCAACAG 900
 R A V E N I Q I N E E D N E I S M L Q Q 265

-----GGTGGAGAAGTCAGTGAATGGAGAAAGGCTGGCTCTATTGACA s1^d
 AAAGAGAGAGAATTCAAGAGGTGAATTGGACGTATCACATTGTTACCTCGCACA 960
 K E R E F Q E V * 273

▼

GA s1^d
 GTGGCTGGTAACAGTTCATGTTGCTTCAAAATGAAGCAGCCTAAACAAATTCCATT 1020

CTGTCTCAAGTGACAGACCTCATCCTTACCTGTTCTGCTACCCGTGACCTTGTGGAT 1080
 GATTCAAGTTGGAGCAGAGTGCTTCGCTGTGAACCCCTGCACTGAATTATCATCTGTAA 1140
 AGAAAAATCTGCACGGAGCAGGACTCTGGAGGTTTGCAAGTGAATGGACAAGAAC 1200
 ATGTGTCCAGTCTACTTGCACCGTTGCATGGCTTGGAAACGTCTGAGTGTGCTGAAAC 1260
 CACCCAGCTTGTCTTCAGTCACAACCTGCAGCCTGCGTTAATTATGGTCTCTGCAAG 1320
 TAGATTTCAGCCTGGATGGTGGGG 1344

FIGURE 17

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3T3 fibroblast
spleen

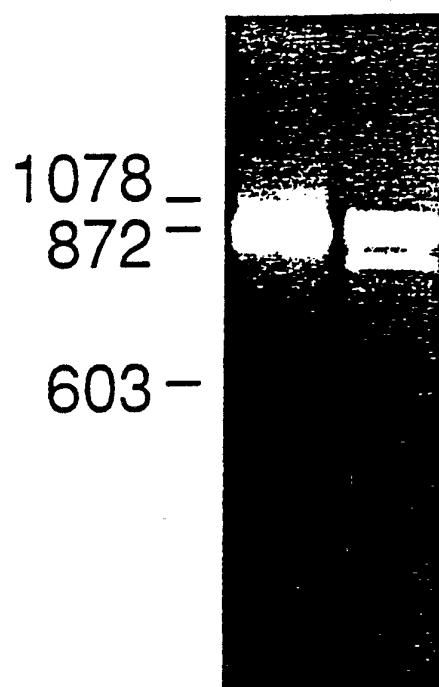


FIGURE 18A

SUBSTITUTE SHEET

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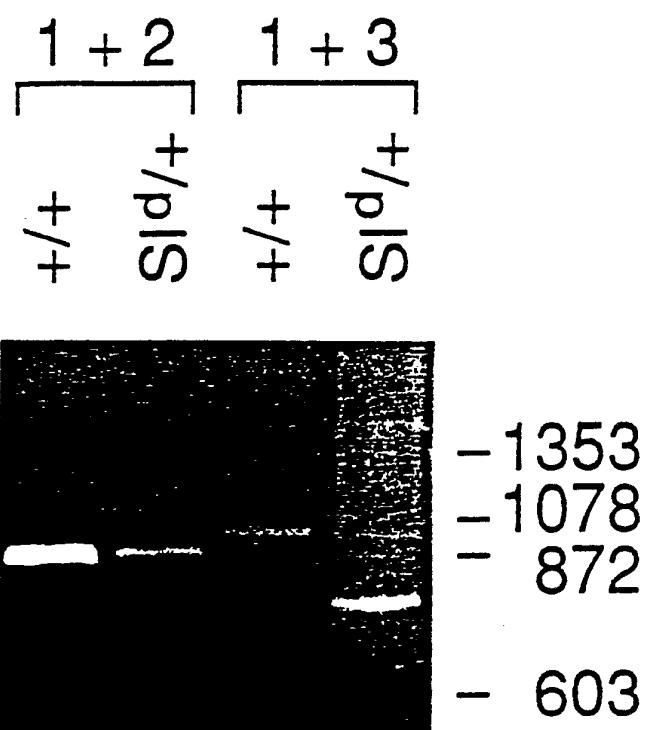


FIGURE 18B

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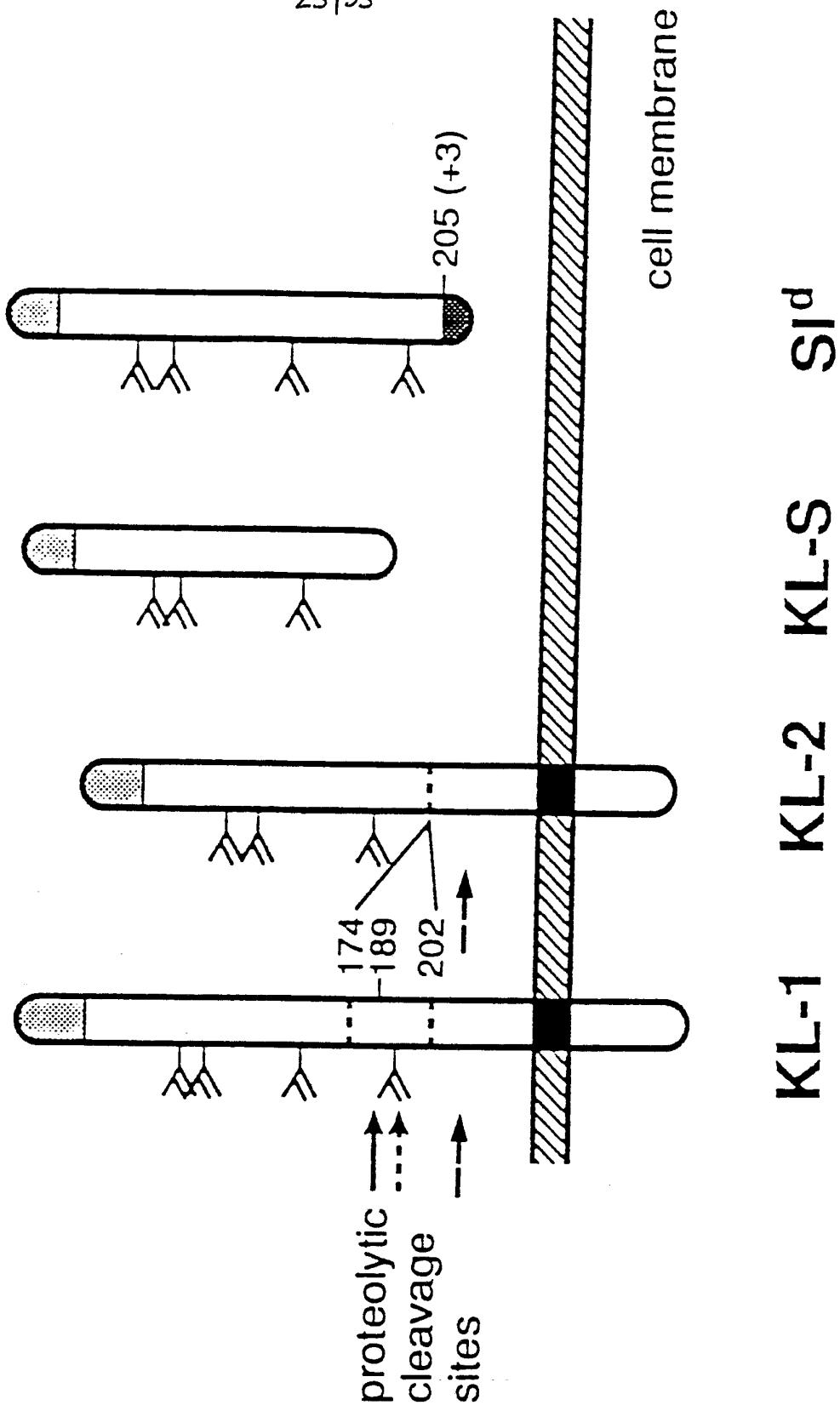


FIGURE 19

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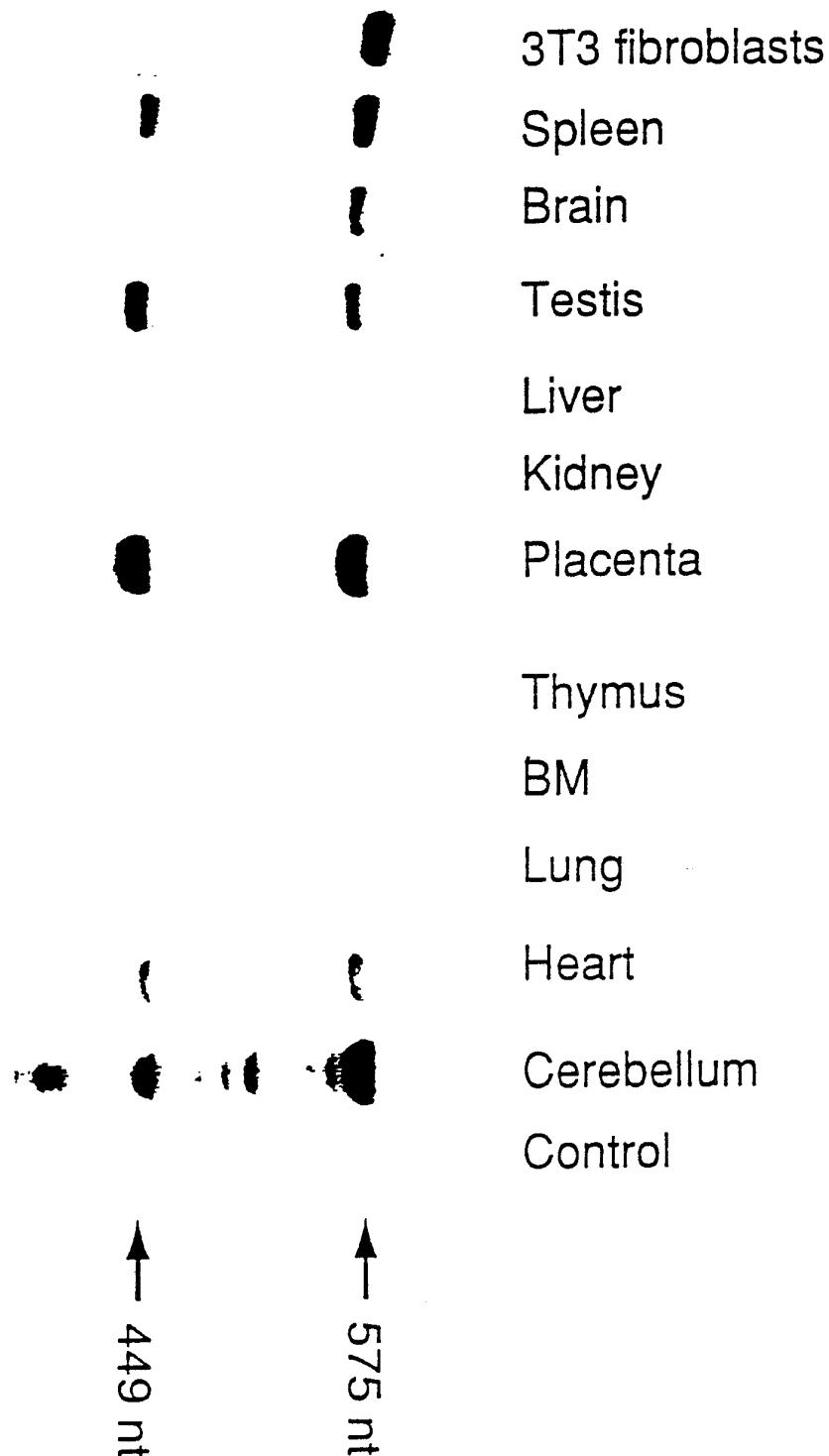


FIGURE 20

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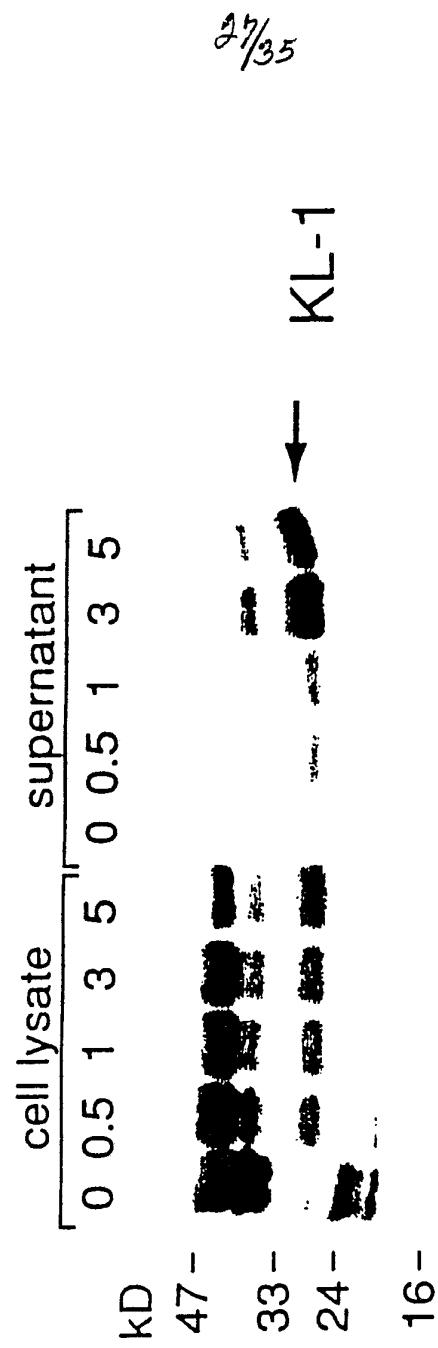


FIGURE 21A

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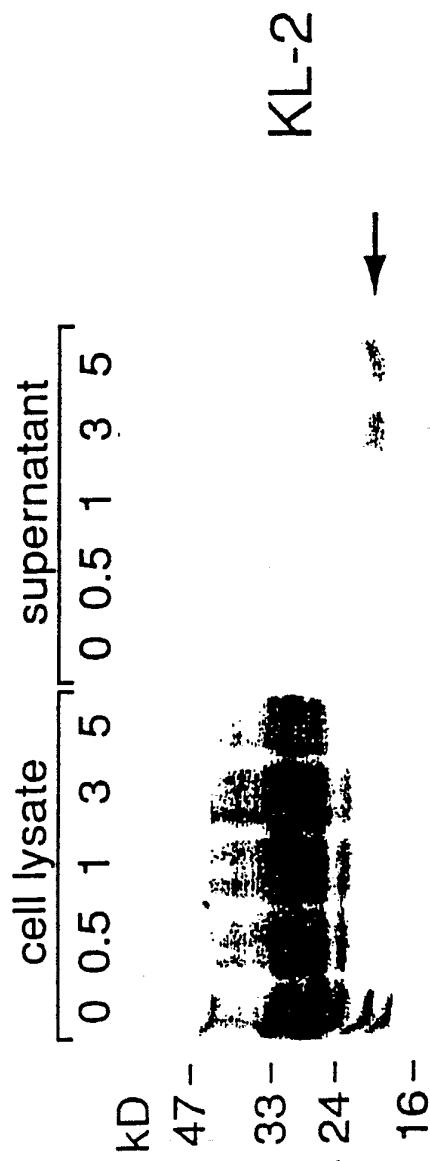


FIGURE 21B

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COS-1

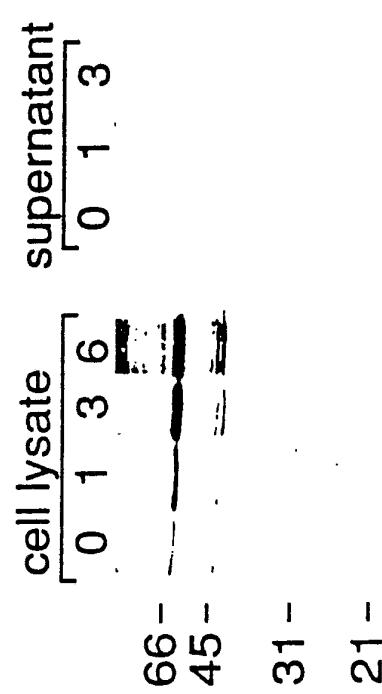


FIGURE 21C



FIGURE 22A

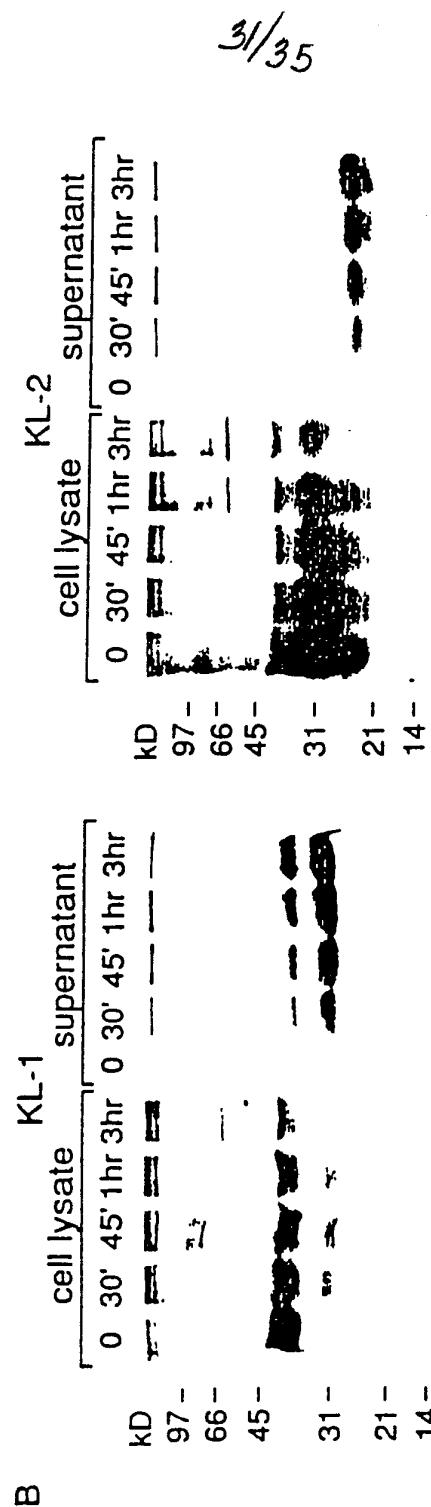


FIGURE 22B

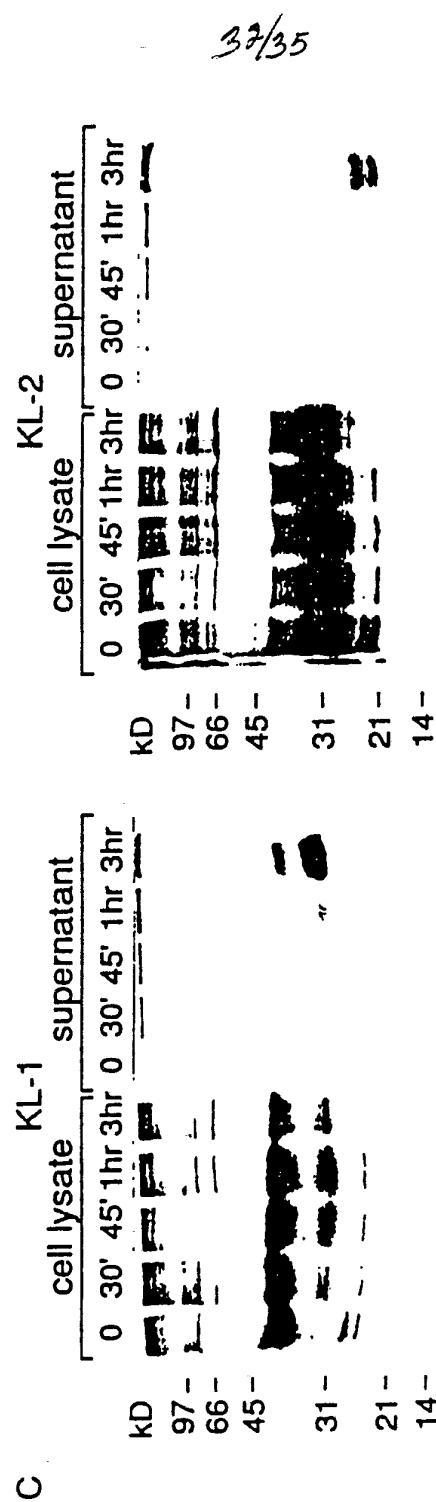


FIGURE 22C

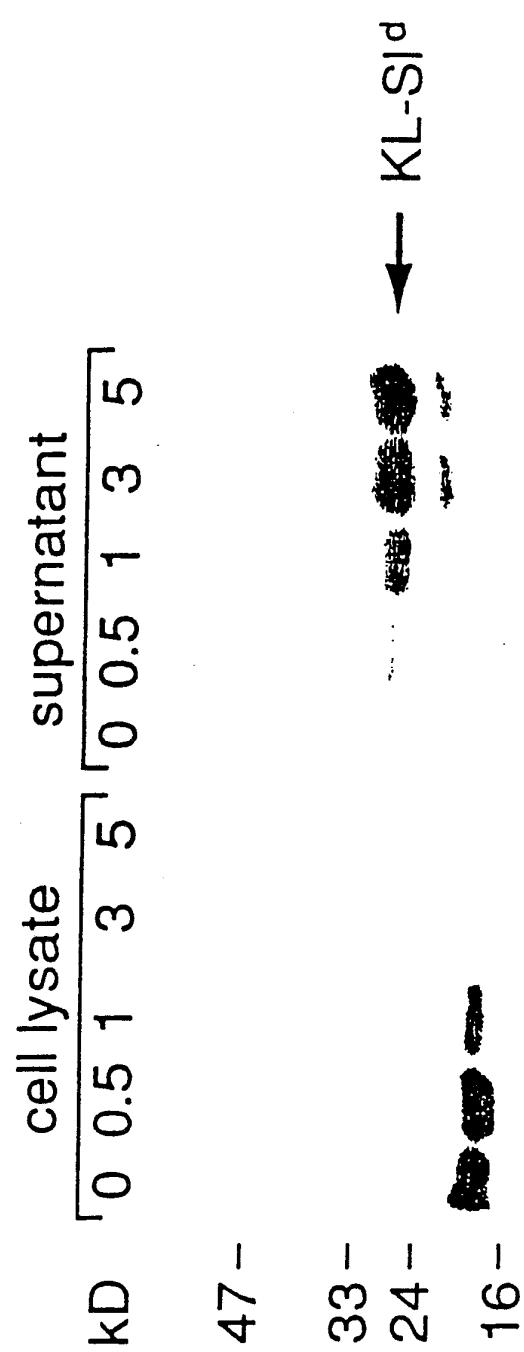


FIGURE 23A

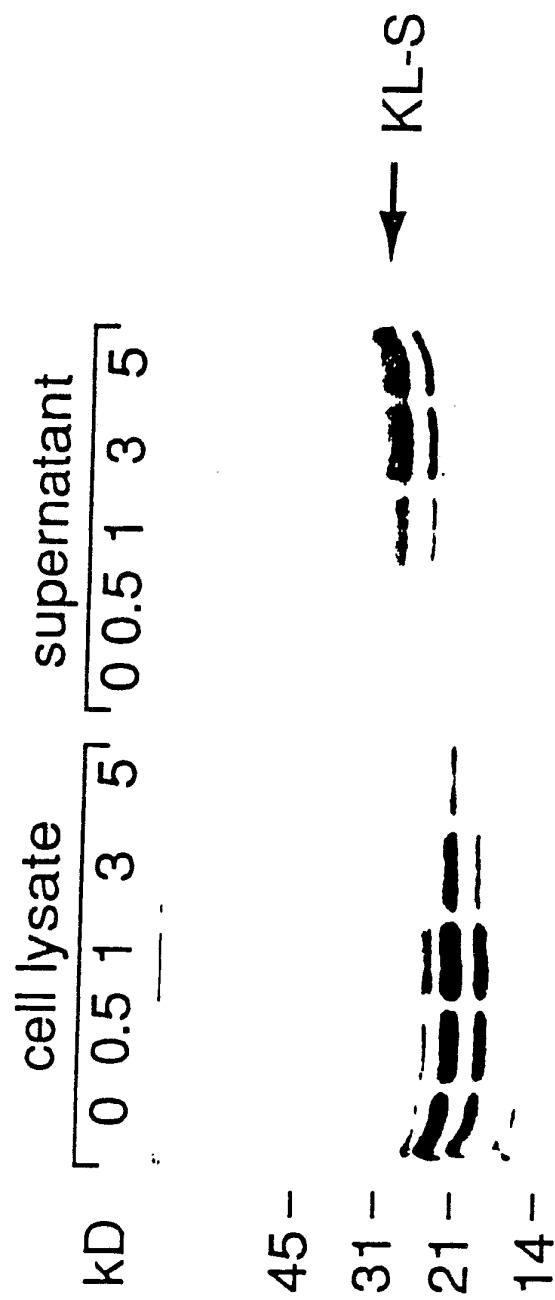


FIGURE 23B

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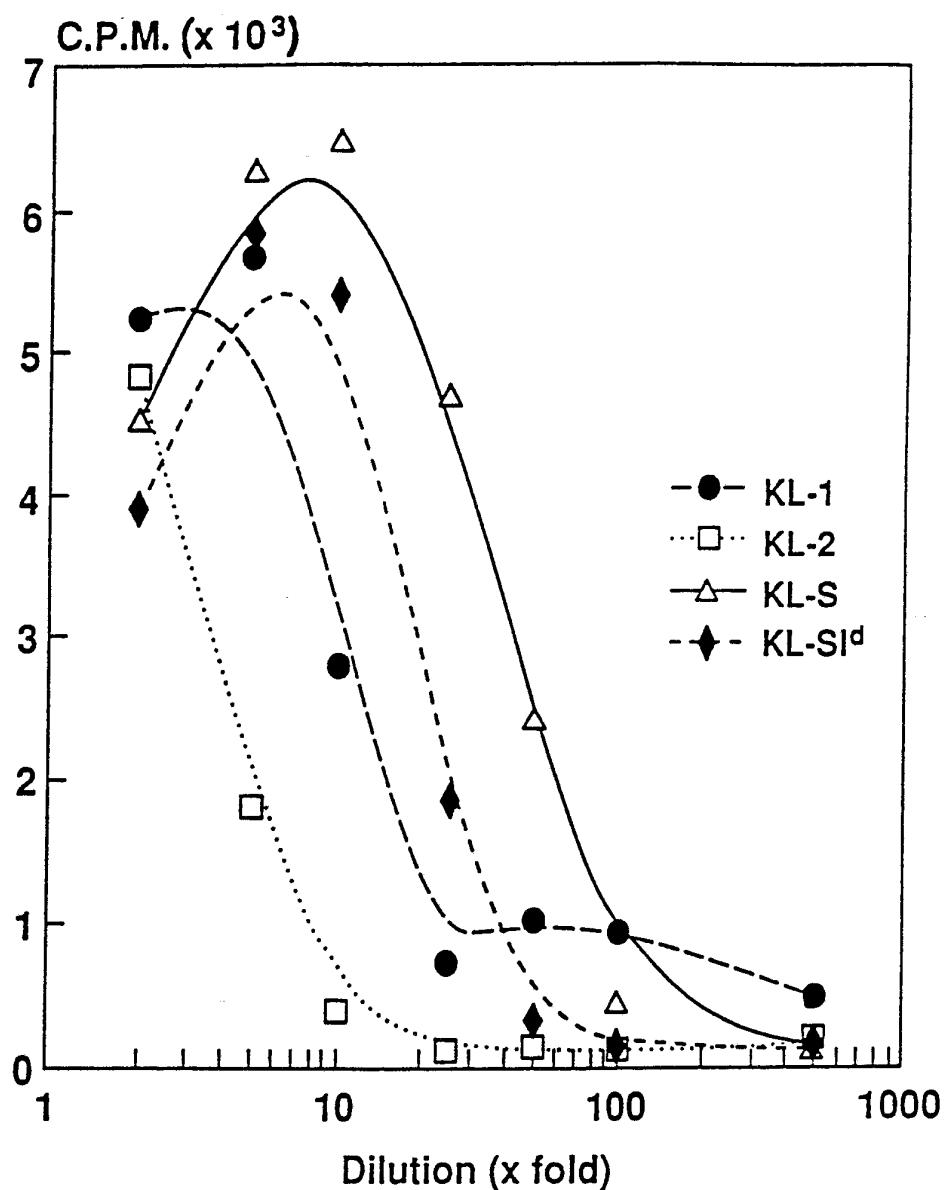


FIGURE 24

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06130

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC
 IPC(5): C07H, 15/12; C07K 13/00; C12N 1/14, 1/20, 5/10, 15/00, 13/00; C12P 21/00
 U.S.: 435/69.1, 240.1, 252.1, 254, 320.1; 530/350; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.	435/69.1, 240.1, 252.1, 254, 320.1; 530/350; 536/27

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

APS, Biosis, Medline

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	Nature Vol. 335, issued 01 SEPTEMBER 1988, B. Chabot et al., "The proto-oncogene <u>c-kit</u> encoding a transmembrane tyrosine kinase receptor <u>maps to the mouse w locus</u> ," pages 83-89. See entire document	1-5, 11-37, 46-47, 49-53
X, P	EMBO JOURNAL, Vol. 9, no. 10, issued OCTOBER 1990, K. Nocka et al., "Candidate ligand for the <u>c-kit</u> transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors," pages 3287-3284, See entire document.	1-5, 11-37 46-47, 49-53
Y	DEVELOPMENT, Vol. 109, issued 1990, A. Orr-Urtreger et al., "Developmental expression of <u>c-kit</u> , a proto-oncogene encoded by the <u>w locus</u> ," pages 911-923. See entire document.	49-53
X, P	CELL, vol. 63, issued 05 OCTOBER 1990, E. Huang et al., "The Hematopoietic Growth Factor KL is Encoded by the <u>sl</u> locus and is the Ligand of the <u>c-kit</u> Receptor, the Gene Product of the <u>w locus</u> ," pages 225-233. See entire document.	1-5, 11-37, 46-47, 49-53

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

21 November 1991

Date of Mailing of this International Search Report

23 DEC 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Dian Cook

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers _____, because they relate to subject matter¹² not required to be searched by this Authority, namely:

2. Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹³, specifically:

3. Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

See attachment

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-5, 11-37, 46-47, 49-53

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

The additional search fees were accompanied by applicant's protest.
 No protest accompanied the payment of additional search fees.