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Role of Dimerization of the Membrane-associated Growth Factor Kit Ligand in Juxtacrine Signaling: The $S\text{I}^{17\text{H}}$ Mutation Affects Dimerization and Stability—Phenotypes in Hematopoiesis

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Summary

The Kit ligand (KL)/Kit receptor pair functions in hematopoiesis, gametogenesis, and melanogenesis. KL is encoded at the murine steel ($S\text{I}$) locus and encodes a membrane growth factor which may be proteolytically processed to produce soluble KL. The membrane-associated form of KL is critical in mediating Kit function in vivo. Evidence for a role of cytoplasmic domain sequences of KL comes from the $S\text{I}^{17\text{H}}$ mutation, a splice site mutation that replaces the cytoplasmic domain with extraneous amino acids. Using deletion mutants and the $S\text{I}^{17\text{H}}$ allele, we have investigated the role of the cytoplasmic domain sequences of KL in biosynthetic processing and cell surface presentation. The normal KL protein products are processed for cell surface expression, where they form dimers. Both $S\text{I}^{17\text{H}}$ and the cytoplasmic deletion mutants of KL were processed to the cell surface; however, the rate of transport and protein stability were affected by the mutations. Deletion of cytoplasmic domain sequences of KL did not affect dimerization of KL. In contrast, dimerization of the $S\text{I}^{17\text{H}}$ protein was reduced substantially. In addition, we have characterized the hematopoietic cell compartment in $S\text{I}^{17\text{H}}$ mutant mice. The $S\text{I}^{17\text{H}}$ mutation has only minor effects on hematopoiesis. Tissue and peritoneal mast cell numbers were reduced in mutant mice as well as in myeloid progenitors. Interestingly, long-term bone marrow cultures from $S\text{I}^{17\text{H}}$ mice did not sustain the long-term production of hematopoietic cells. In addition, homing of normal hematopoietic progenitors to the spleen of irradiated $S\text{I}^{17\text{H}}/S\text{I}^{17\text{H}}$ recipient mice was diminished in transplantation experiments, providing evidence for a role of Kit in homing or lodging. These results demonstrate that the membrane forms of KL exist as homodimers on the cell surface and that dimerization may play an important role in KL/Kit-mediated juxtacrine signaling.

Polypeptides which transmit extracellular signals are most often secreted as soluble factors. An increasing number of growth factors that derive from membrane-anchored precursors and growth factors which function both as soluble and as membrane growth factors have been described in the past several years. They include members of the epidermal growth factor family, e.g., epidermal growth factor, TGF-α, and c-erb/H ER 2 ligand (1), several hematopoietic growth factors, including the c-kit ligand (KL), CSF-1, IL-1, and TNF (2, 3). Transmembrane growth factors have also been identified in the nematode Caenorhabditis elegans (lin-3) (4) and in Drosophila, bride of sevenless (boss) (5, 6). Several of these transmembrane growth factors release soluble growth factors by proteolytic processing or shedding (7–9). The nonprocessed membrane-anchored growth factors themselves possess biophysical activities and promote cell adhesion and juxtacrine stimulation in adjacent cells expressing cognate receptors on their cell surface (3). Furthermore, the recently described ligands of the eph-family receptor tyrosine kinases with roles in axon guidance are obligate membrane growth factors (10).

The protooncogene c-kit encodes a receptor tyrosine kinase which is a member of the platelet-derived growth factor receptor subfamily (11). c-kit and its cognate ligand, KL, also called steel factor, are encoded at the murine white-
spotting (W) (12, 13) and steel (Sl) loci (14–16), respectively. The phenotypes of mice with mutations at the W and Sl loci imply functions of Kit/KL in germ cell development, melanogenesis, and hematopoiesis (17–21). In target cells, KL promotes cell proliferation, survival, cell adhesion/migration, and differentiation. The steel gene encodes two KL protein products, KL-1 and KL-2, produced by alternative splicing (2, 22). Both the KL-1 and KL-2 proteins are synthesized as transmembrane proteins and are expressed on the cell surface (2). Both membrane-bound KL-1 and KL-2 can mediate direct cell-cell contact with c-kit-expressing cells, e.g., bone marrow-derived mast cells (BMMCs) (22, 23) and primordial germ cells (24, 25). Proteolytic processing of both membrane-bound KL-1 and KL-2 releases biologically active soluble KL proteins, although with different efficiencies and through distinct proteolytic cleavage mechanisms (2, 9, 26). Molecular characterization of various Sl alleles has provided important insight into the role of the soluble and membrane-anchored forms of KL (14, 15, 22, 27). Homozygotes for the Steel-didie mutation (SlD) are viable and less severely affected, implying some residual functional activity of KL, but they display all of the pleiotropic effects normally associated with steel mutations (17, 18). Molecular analysis showed that the SlD allele arose as a result of an intragenic deletion including the transmembrane domain and COOH terminus, generating a secreted KL protein product with normal biological activity (2, 22, 27). The biological characteristics of mice carrying the SlD mutation imply that the SlD KL protein sustains some activity but is largely defective in facilitating proliferation and survival of target cells, indicating that the membrane-anchored forms of KL play pivotal roles in c-kit function. The Sl17H allele contains a splice donor site mutation resulting in the substitution of amino acids 239–273 in the cytoplasmic domain of KL, with KL protein sequences of murine and human KL being 79% identical. The strong conservation of the cytoplasmic domain sequences of KL throughout evolution suggests a function for these sequences. The cytoplasmic domain sequences of KL may interact with itself or other molecule(s), and these interactions may play a role in processing to the cell surface, cell surface presentation, and juxtacrine signaling. We have investigated the role of cytoplasmic domain sequences of wild-type KL and the Sl17H protein maturation and cell surface presentation. We have also investigated the characteristics of hematopoietic cells in mice carrying the Sl17H allele. The results obtained in this study are discussed in the context of mechanisms governing juxtacrine signaling.

**Materials and Methods**

Construction of KL Deletion Mutants. Deletion mutations in the cytoplasmic domain of KL were created by the primer-directed PCR mutagenesis method. One 5′ primer covering the initiation codon of KL (5′-GACGGAAAGAATCTTCTCTGTGT-3′) and several 3′ primers containing a termination codon located at the desired position were used in the PCR reaction. A plasmid containing the KL cDNA clone 19.1.1 (2) was used as template. The 3′ primers used to create the following mutants were 5′-CTCTCTAGATTTAACAATCTTATCTC-3′; for KL-L263; 5′-ATTTCCTAGATTTAATCTG-TATATT-3′; for KL-N254; 5′-ATTCTAGAAACTGCCTTGTGACTG-3′; for KL-V248; and 5′-CCCTTCTAGACGTTTACTGTGTCCTCT-3′ for KL-Q241. For generation of the internal deletion mutation, KL-Δ241/254, and SL17H, an M13 phage DNA containing the KL cDNA clone 19.1.1 and the following oligonucleotides were used: for KL-Δ241/254, 5′-ATTATCCTCTTTATCTGTTTCTTTCTCCA-3′, and for KL-S17H, 5′-TCTTTCTGTTGCAACATACCTTTCAGTAAGGCTCC-3′. All mutations were confirmed by sequence analysis. The mutant KL cDNAs were subsequently cloned into pcDNA 8 for expression in COS-1 cells.

Transient Expression of KL cDNAs in COS-1 Cells. COS-1 cells were transfected with the DEAE-dextran method described previously (2, 30) with minor modifications. Briefly, COS-1 cells were grown to subconfluence 1 d before use, trypanosed, and seeded on 150-mm petri dishes at a density of 6 × 10^5 cells per dish. After 24 h, the cells had reached ~70% confluence and were transfected with 5 μg of plasmid DNA in the presence of DEAE-dextran (Sigma Chemical Co., St. Louis, MO) for 6–12 h. The medium containing the plasmid DNA was removed, and the cells were shocked with 10% DMSO/PBS– for 1 min. Residual DMSO was removed by washing the cells with PBS– twice. Transfected COS-1 cells were grown in DME containing 10% FCS, 100 μg/ml L-glutamine, and antibiotics.

Pulse chase and immunoprecipitation analyses of KL Proteins. At 72 h after transfection, COS-1 cells expressing KL proteins were used for pulse chase experiments. Cells were incubated with methionine-free DME containing 10% dialyzed FCS for 30 min and labeled with 35S-methionine (DuPont-NEN, Boston, MA) at 0.5 μCi/ml. At the end of the labeling period, the labeling medium was replaced with regular medium containing an excess amount of methionine. Cell lysates and supernatants were collected at the indicated times. Cell lysates were prepared as described previously (31) in 1% Triton X-100, 20 mM Tris (pH 7.5), 150 mM NaCl, 20 mM EDTA, 10% glycerol, and protease inhibitors phenylmethyl sulfonyl fluoride (1 mM) and leupeptin (20 μg/ml). For immunoprecipitation analysis of KL protein products, equal volumes of cell lysates or supernatants were immunoprecipitated with excess anti-KL antiserum. KL antiserum was obtained by immunization of rabbits with soluble native and recombinant murine KL, and both antisera were used with indistinguishable results. The anti-KL serum was conjugated to protein A–Sepharose (Pharmacia Biotech, Inc., Piscataway, NJ) and washed three 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 supernatants and cell lysates at 4°C for at least 2 h. The immunoprecipitates were washed three times with wash A.
were then washed once 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, 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 min, and analyzed by SDS-PAGE (12%) and autoradiography. For endoglycosidase digestion, endo H treatment, digestion with endo H (Boehringer Mannheim Biochemicals, Indianapolis, IN) was performed by incubating immunoprecipitates for 16 h at 37°C with 5 mM of endo H in 50 mM of 0.1 M sodium phosphate buffer, pH 6.1, containing 0.1% Triton X-100, 0.03% SDS, and 20 mM EDTA.

Immunofluorescence Microscopy. At 40 h after transfection, COS-1 cells were trypanized and reseeded on 12-mm round coverslips. After 32 h, the cells were fixed for 15 min at 25°C with 2% (vol/vol) formaldehyde in PBS and rinsed twice with PBS. Cells were then either used directly for immunofluorescence staining or permeabilized by incubating for 15 min at 25°C with 0.1% (wt/vol) saponin in PBS. For staining, cells were incubated at 25°C for 30 min consecutively with 100 μl of normal goat serum, anti-KL serum (1:100 dilution) (32), or an ER mAb (2 μg per 10^7 cells) (Genzyme Corp., Cambridge, MA) for 1 h at 37°C. The reaction was terminated with 100 μl Tris-HCl (pH 7.5) for 10 min. Cell lysates were prepared by incubating equivalent amounts of cell lysate with anti-mouse stem cell factor rat Fab (2 μg per 10^7 cells) (Genzyme Corp., Cambridge, MA) for 3 h at 4°C. Protein G-Sepharose beads (Pharmacia Biotech, Inc.) were used to collect the antigen-antibody complexes. For gel analysis, immunoprecipitates were solubilized in SDS sample buffer by boiling for 5 min, and analyzed by SDS-PAGE (10%). Proteins were electrophoretically transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). After transfer, the membrane was incubated with 4% skim milk/TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 12 h at room temperature, and Western blot analysis using anti-mouse stem cell factor polyclonal antibody (1:500 dilution; Genzyme Corp.) and enhanced chemiluminescence detection (Pierce Chemical Co., Rockford, IL) were used for identification.

Mast Cell Adhesion Assay. Transfected COS cells were grown in 24-well plates (7.5 × 10^4 per well). BM MCs, obtained as described previously (34), were washed three times with RPMI 1640 medium and incubated for 1 h with transfected COS cells (1.5 × 10^6 cells per well). Nonadherent BM MCs were then removed by washing three times with medium, and the BM MCs attached to the COS cells were counted by visual inspection in the microscope. BM MCs were counted as total number in each well or the number attached to individual COS cells. To compete the adhesion of BM MCs to COS cells, the c-kit antagonistic antibody ACK2 (a gift from Dr. Nishikawa, Kyoto University, Kyoto, Japan) was used.

A analysis of Peripheral Blood Parameters and Hematopoietic Progenitor Aays. Blood samples for platelet and white blood cell (WBC) counts were drawn from the retroorbital plexus or the tail vein with a capillary pipette (Unopette; Becton Dickinson Labware, Rutherford, NJ). Platelet and WBC numbers were determined using a hemacytometer and phase-contrast microscopy. The hematocrit was determined using heparinized microhematocrit tubes (Fisher Scientific Co., Pittsburgh, PA).

For in vitro progenitor assays, bone marrow was harvested, and the cellularity was determined as described previously (35). 10^6 bone marrow cells were resuspended in 1 ml of IMDM/0.8% methylcellulose (Fisher Scientific Co., Bethesda, MD) 30% FBS (Hyclone Laboratories, Inc., Logan, UT) 0.2 mM hemin (Sigma Chemical Co.) containing 50 ng/ml mouse IL-3 (BioSource International, Camarillo, CA), 3 U/ml human erythropoietin (Amgen, Thousand Oaks, CA), and 20 ng/ml recombinant mouse KL. Cultures were incubated at 37°C in a 5% CO2 atmosphere. After 7 d of incubation, colonies were scored on the basis of gross morphology as erythroid burst (BFU-E), granulocyte/macrophage (CFU-GM), and mixed colonies (CFU-GEMM).

Splenic CFU (CFU-S)–day 12 were assessed by injection of 10^5 donor bone marrow cells suspended in MEM supplemented with 5% FBS and 10% fetal bovine serum into the lateral tail vein of lethally irradiated (9.5 Gy) recipient mice. Mice were killed 12 d later, their spleens were fixed in Bouin's solution and 10% formalin, and macroscopically visible colonies on the surface of the spleens were enumerated.

For long-term bone marrow cultures, bone marrow cells from one femur were flushed into a T-25 flask in Fisher's medium containing 20% horse serum and dexamethasone. Cultures were incubated at 33°C and semidepopulated at weekly intervals.

Results

Construction of KL-1 Cytoplasmic Domain Variants and Analysis of Their Turnover Characteristics in COS-1 Cells. The cytoplasmic domain sequences of KL are highly conserved in evolution. To study roles for cytoplasmic domain sequences of KL, COOH-terminal deletion mutations of varying lengths were constructed (Fig. 1). The wild-type KL-1 cDNA plasmid was used as a template for mutagenesis. The deletion mutations were named according to the position of the COOH-terminal amino acid, e.g., KL-L263, KL-N254, KL-V248, and KL-Q241. In addition, an internal deletion mutation, KL-Δ241/254, and KL-S17H were constructed. All constructs were subcloned into the eukaryotic expression vector pCDM8 suitable for transient expression in COS-1 cells.

The primary KL translation products are progressively
processed primarily by carbohydrate modification as they are transported through the endoplasmic reticulum (ER) and Golgi compartments to the cell surface (2). Biosynthetic processing of the protein products of the KL cytoplasmic domain variants and KL-SI17H was investigated by performing pulse chase experiments in transiently transfected COS-1 cells by using 35S-methionine. Immunoprecipitation analysis using anti-KL antiserum and SDS-PAGE indicated that the biosynthetic processing pattern of all cytoplasmic domain mutants and KL-SI17H was similar to that of wild-type KL-1 (Fig. 2). Smaller molecular mass (Mr) proteins representing the unglycosylated forms of KL were synthesized: KL-1 (35,000), KL-L263 (33,000), KL-N254 (31,000), KL-V248 (33,000, not shown), KL-D241/254 and KL-SI17H (33,000), and KL-Q241 (30,000). These proteins were progressively modified by glycosylation to form more mature products representing the cell surface forms. The respective Mr for the mature proteins were as follows: KL-1, 45,000 daltons; KL-L263, 43,000 daltons; KL-N254, 41,000 daltons; KL-V248 (42,000 daltons, not shown), KL-D241/254 and KL-SI17H (33,000), and KL-Q241 (30,000). These proteins were progressively modified by glycosylation to form more mature products representing the cell surface forms. The respective Mr for the mature proteins were as follows: KL-1, 45,000 daltons; KL-L263, 43,000 daltons; KL-N254, 41,000 daltons; KL-V248 (42,000 daltons, not shown), KL-D241/254 and KL-SI17H (33,000), and KL-Q241 (30,000). These proteins were progressively modified by glycosylation to form more mature products representing the cell surface forms. The respective Mr for the mature proteins were as follows: KL-1, 45,000 daltons; KL-L263, 43,000 daltons; KL-N254, 41,000 daltons; KL-V248 (42,000 daltons, not shown), KL-D241/254 and KL-SI17H (33,000), and KL-Q241 (30,000). These proteins were progressively modified by glycosylation to form more mature products representing the cell surface forms. The respective Mr for the mature proteins were as follows: KL-1, 45,000 daltons; KL-L263, 43,000 daltons; KL-N254, 41,000 daltons; KL-V248 (42,000 daltons, not shown), KL-D241/254 and KL-SI17H (33,000), and KL-Q241 (30,000). These proteins were progressively modified by glycosylation to form more mature products representing the cell surface forms.
the protein was not properly transiting the Golgi complex. A more thorough time course comparing KL-1 and KL-SI\textsuperscript{17H} showed that at 45 and 60 min of chase, KL-SI\textsuperscript{17H} displayed some persistence of endo H sensitivity, whereas KL-1 had become fully resistant (Fig. 4B). Thus, KL-SI\textsuperscript{17H} is delayed in exiting the ER. Immunofluorescence staining of KL-1 expressing COS-1 cells revealed a pattern characteristic of a membrane protein which outlined the shape of the cytoplasm (Fig. 5). The KL-SI\textsuperscript{17H} staining pattern was indistinguishable from that of KL-1. KL-Q241 staining was observed at the cell surface as well, but appeared less intense than KL-1 staining. Upon permeabilization, staining patterns of all KL-expressing cells included a reticular pattern surrounding the nucleus. Relative to cell surface staining, KL-Q241 immunofluorescence was more pronounced in this juxtanuclear region, and this intracellular staining pattern appears to overlap with that of an E.R. marker antibody. Therefore, these results are consistent with the idea that the delay in maturation of the KL-Q241 protein and to a lesser extent of the SL17H protein is a consequence of a delay in passage through a pre-Golgi compartment.

**Efficient Formation of Homodimers of Cell Membrane–associated KL and Cytoplasmic Domain Deletion Variants, and Diminished Formation of Dimers of the Sl17H Protein.**

Growth factors and cytokines commonly activate their receptors by mediating receptor dimerization. Recent studies (see references 50 and 51) with the Kit receptor suggest that bivalent binding of KL dimers is driving Kit receptor dimerization. Soluble KL is predominantly in a dimeric state. However, studies of KL dimerization kinetics imply that in vivo, 90% of KL circulating in serum exists as monomers. It is not known if the membrane-associated KL protein on the cell surface is monomeric or dimeric. To address this question, we set out to identify KL dimers by using chemical cross-linking methodology. COS-1 cells transiently transfected to express KL-1 and KL-2 were treated with a hydrophilic and a hydrophobic cross-linker (DSS and BS\textsuperscript{3}) cell lysates were then fractionated by SDS-PAGE, and KL protein products were identified by Western blotting (Fig. 6). Both cross-linkers efficiently cross-linked the normal KL-1 and KL-2 transmembrane proteins to produce dimeric molecules of 80–85 kD (Fig. 6). These results suggest that a major fraction of the cell-associated KL-1 and KL-2 molecules are in a dimeric form. It was next of interest to determine whether the cytoplasmic domain sequences of KL and/or the SL\textsuperscript{17H} cytoplasmic domain sequences affect the formation of cell-associated KL dimers. Whereas deletion of cytoplasmic domain sequences did not affect KL dimer formation as determined by cross-linking (Fig. 6), importantly, SL\textsuperscript{17H} dimer formation was diminished significantly (Fig. 7), and removal of the SL\textsuperscript{17H} cytoplasmic domain sequences by deletion restored the ability to form dimers (Fig. 7).

**Cell Adhesion Properties of KL Cytoplasmic Domain Variants.**

In an earlier study, Cheng and Flanagan found a significant reduction in binding of a Kit ectodomain alkaline phosphatase fusion protein to COS cells expressing the KL SI\textsuperscript{17H} protein compared with COS cells expressing the normal KL protein (26). The reduction in KL SI\textsuperscript{17H} dimerization we observed in COS cells may contribute to the reduced binding of Kit ectodomain. KL/Kit interactions may also be important in cell-cell adhesion. Therefore, we explored the functional consequences of the cytoplasmic domain deletion and SL\textsuperscript{17H} mutations on cell-cell adhesion.
Cell adhesion between kit receptor-expressing mast cells and KL-expressing COS-1 cells or fibroblasts had been demonstrated previously (22, 23). In 24-well dishes, BMMCs (1.5 × 10⁵ cells per well) were plated on top of COS-1–expressing KL-1, KL-Q241, and KL-SⅭH, grown on coverslips were fixed with 2% formaldehyde (NP, nonpermeabilized control) and permeabilized with 0.1% saponin (P). The cells were incubated with anti-KL antibody or an ER marker antibody and FITC–conjugated goat anti-rabbit antibody. Photographs were taken with fluorescent microscopy.

Adhesion of BMMCs to COS-1 cells expressing KL-Q241 was reduced dramatically (Fig. 8). Similar numbers of BMMCs attached to COS cells transfected with either KL-Q241, the secretory KL-1S (2), or a control plasmid. Interestingly, the number of BMMCs attached to COS cells expressing KL-SⅭH was only about half of that attached to COS cells expressing KL-1 (Fig. 8).

Effects of the SⅭH mutation on hematopoiesis in vivo and in vitro. Previous analysis of mutant SⅭH/SⅭH mice suggested mild effects in the hematopoietic system, i.e., a mild macrocytic anemia was observed (29). We have reexamined the effects of the SⅭH mutation on the hematopoietic system in vivo as well as in vitro. Careful analysis of the in vivo hematopoietic parameters indicates that hematocrit values, WBC, neutrophil, and platelet numbers, as well as bone marrow cellularity are normal in homozygous mutant mice (Table 1). In contrast, the numbers of skin mast cells were reduced by 45% and peritoneal mast cells by 85%, indicating an effect of the SⅭH mutation on mast cell development and/or number (Table 1). In agreement with a lack of an erythroid deficiency, in vitro analysis of hematopoietic progenitors revealed normal numbers of BFU-E. However, mixed colonies (CFU-GEMM) and CFU-GM were reduced slightly, by ~33%. We also established long term bone marrow cultures and followed output of he-
matopoietic cells at weekly intervals. These experiments showed that cultures from Sl17H/Sl17H bone marrow were not able to sustain long-term production of hematopoietic cells (Fig. 9). This result is reminiscent of the defect observed in cultures derived from bone marrow of Sl/Sld mice, which produce only the soluble form of KL and no membrane-associated KL.

Kit-mediated adhesion of hematopoietic cells is known to be mediated by at least two distinct mechanisms: (a) tethering via the membrane growth factor–receptor interaction, and/or (b) by inside-out activation of integrin-mediated adhesion (very late antigen [VLA]-4 and VLA-5) (22, 23, 39, 40). Furthermore, it has been established that VLA-4–mediated adhesion is critical for homing or lodging of early hematopoietic progenitors to the bone marrow in transplantation experiments (41, 42). Therefore, we determined whether Sl17H/S17H mice were suitable recipients in spleen colony assays using normal donor bone marrow. Interestingly, we observed a reduced number of spleen colonies (65% of normal) in Sl17H/Sl17H recipient mice (Table 2). This may suggest that the Sl17H mutation impairs lodging/homing of transplanted CFU-S progenitors to the spleen of irradiated mice.

**Table 1.** Mast Cells and Hematopoietic Progenitors in S17H/S17H and C3H Control Mice

| Genotype       | Skin mast cells | Peritoneal mast cells | Bone marrow cellularity | CFU-GEM M | CFU-GM       | BFU-E       |
|----------------|-----------------|-----------------------|-------------------------|-----------|--------------|-------------|
| +/+            |                 |                       |                         |           |              |             |
| Exp. 1         | 162.5 ± 4.5     | 49.5 ± 9.9            | 11.54 ± 0.77            | 2,626 ± 335 | 29,003 ± 2,871 | 1,955 ± 430 |
| Exp. 2         |                 |                       |                         | 770 ± 191 | 9,717 ± 1,215 | 2,677 ± 204 |
| Sl17H/Sl17H    |                 |                       |                         |           |              |             |
| Exp. 1         | 91.6 ± 12.8     | 8.0 ± 1.6             | 12.42 ± 1.67            | 1,634 ± 608 | 19,923 ± 3,163 | 1,925 ± 515 |
| Exp. 2         |                 |                       |                         | 583 ± 210 | 10,266 ± 1,811 | 2,391 ± 657 |

**Discussion**

The Sl allele arose as a result of an intragenic deletion including the transmembrane domain and COOH terminus, generating a secreted KL protein product with normal biological activity (2, 22, 27). Analysis of the Sl phenotype has been of great value in understanding the differential biological roles of membrane-associated and soluble forms of KL. The biological characteristics of homozygous Sl/Sl mice and of Sl/Sl mice indicate that the Sl protein supports some level of KL function. However, Sl mice display major defects in facilitating proliferation and survival of tar-
get cells. Therefore, the cell-associated form of KL plays a critical role in c-kit function, and the cytoplasmic domain of KL is potentially important to the processes mediated by juxtacrine signaling. This notion is supported by the mutant phenotypes of the Sl17H allele, a splice site mutation that results in the substitution of amino acids 239–273 in the KL cytoplasmic domain with 27 extraneous amino acids (28). Therefore, the Sl17H allele potentially important to the processes mediated by dimerization. The decreased efficiency of dimer formation of the Sl17H protein suggests that the cytoplasmic domain of KL does not have an active role in dimer formation. The decreased efficiency of dimer formation of the Sl17H protein may not be a rate-limiting event in juxtacrine signaling between KL and Kit. This is in contrast to receptor tyrosine kinases such as Kit which primarily exist as monomers on the cell surface and which form dimers in response to engagement with the cognate ligand (50, 51). Our analysis of various cytoplasmic domain deletion mutations suggests that the cytoplasmic domain of KL does not have an active role in dimer formation. The decreased efficiency of dimer formation of the Sl17H protein may not be a rate-limiting event in juxtacrine signaling between KL and Kit.

Our findings that the KL cytoplasmic domain is required for normal processing to the cell surface are consistent with reports on a variety of secreted or membrane-anchored proteins in which cytoplasmic domain mutations disrupted intracellular trafficking and maturation. For example, mutations removing the four COOH-terminal cytoplasmic residues of α-1 proteinase inhibitor or the COOH-terminal 22 amino acids of thyroxine-binding globulin caused nascent protein to be retained in the ER with resultant lack of secretion (43, 44). Additionally, single point mutations of glycines in the cytoplasmic domain of P-glycoprotein or a single point mutation in the cytoplasmic kinase domain of the Kit receptor caused these proteins which are normally membrane-anchored to be inefficiently glycosylated and retained in the ER (45, 46). Cytoplasmic domain mutant proteins may not interact properly with molecules involved with transport from the ER to the cell surface. Also, incorrect folding as a consequence of cytoplasmic domain mutations may be responsible for the maturation defects (47).

Soluble KL forms noncovalent dimers in solution (15, 48, 49), and it is thought that dimerization of the ligand as well as of the receptor are essential steps for receptor activation. Therefore, it seemed reasonable that juxtacrine signaling by cell-associated forms of KL may involve membrane KL dimers. Our finding of efficient dimer formation of cell-associated KL-1 and KL-2 molecules on transfected COS cells supports this conjecture. Importantly, the apparent large fraction of KL dimers on the cell surface in the absence of cognate receptor implies that dimer formation may not be a rate-limiting event in juxtacrine signaling between KL and Kit.

Table 2. Reciprocal T transplantation of bone marrow from Sl17H/Sl17H and Control C3H Mice into Irradiated Control and Sl17H/Sl17H Mice

| Donor | Recipient | CFU-S/10^5 cells |
|-------|-----------|-----------------|
| +/+   | +/+       | 25.3 ± 2.1 |
| Sl17H/Sl17H | +/+       | 36.9 ± 2.0 P = 0.0008 |
| +/+   | Sl17H/Sl17H | 25.1 ± 1.7 |
| +/+   | Sl17H/Sl17H | 16.5 ± 1.0 P = 0.0024 |

First, reciprocal transplantation of normal bone marrow to Sl17H/Sl17H and control Sl17H mice revealed a reduced number of tissue mast cells was appreciably affected by the Sl17H mutation. Second, the bone marrow of Sl17H/Sl17H mice did not deviate significantly from normal controls, although slightly reduced numbers of mixed colonies (CFU-GEMM) and slightly increased numbers of CFU-S were observed. Evidence that the hematopoietic microenvironment is compromised in Sl17H/Sl17H mice comes from two different experiments. First, reciprocal transplantation of normal bone marrow into Sl17H/Sl17H and normal mice revealed a reduced number of spleen colonies in the Sl17H/Sl17H recipients, suggesting that homing or lodging to the spleen is affected by the mutation. Second, the bone marrow of Sl17H/Sl17H mice.
does not support long-term culture of hematopoietic progenitors. The biochemical defects we observed for the $Sl^{17H}$ protein, including delayed processing and reduced dimer formation, may contribute to these hematopoietic deficiencies. The observation that homing or lodging of progenitors to the spleen of irradiated mice is affected is of great interest. KL had been shown to mediate adhesion processes in hematopoietic cells and mast cells by means of an inside-out activation of the integrins VLA-4 and VLA-5 (39, 40, 52, 53). Also, VLA-4-mediated adhesion has been demonstrated to play a role in homing or lodging of hematopoietic progenitors to the spleen (41). In addition, tethering via the membrane growth factor–receptor interaction is thought to provide a mechanism for KL/Kit-mediated cell–cell adhesion (2, 22). Although our results provide evidence that supports a role for KL/Kit in homing or lodging of hematopoietic progenitors to the spleen possibly by an integrin-mediated mechanism, a role for KL/Kit in homing to the bone marrow as suggested by Papapanopoulou and Craddock (54) remains to be investigated.

The number of germ cells in neonatal gonads is reduced similarly in male and female $Sl^{17H}$/Sl$^{17H}$ mice as a result of an effect on primordial germ cells; however, the $Sl^{17H}$ allele affects male but not female fertility (28, 55). In postnatal spermatogenesis, KL-2 is more abundant than KL-1, whereas in oogenesis, KL-1 and KL-2 are equally abundant (2, 32). Since the KL-2 protein is more resistant to proteolytic processing and therefore is more stable on the cell surface, the preferred expression of KL-2 in testis development implies a more selective role for transmembrane KL in spermatogenesis. Therefore, the defect in dimerization of $Sl^{17H}$ would more severely affect spermatogenesis and may in part account for the sterility of $Sl^{17H}$ males, while females remain fertile. In addition, immunohistochemical studies showed that the transmembrane KL protein accumulates in the basolateral region of the seminal epithelium in Sertoli cells at the time when c-kit–expressing spermatogonia begin to mature (32). KL-expressing Sertoli cells supporting KL/c-kit-dependent spermatogenic proliferation have the morphology of a polarized epithelium. Possibly, the cytoplasmic domain of KL may direct sorting of this protein to the basolateral region of Sertoli cells and/or affect its stability in the basolateral region of these cells. In the adult ovary, follicle cells surrounding the oocyte are less polarized than Sertoli cells. Therefore, although a 50% decrease in $Sl^{17H}$ protein may be tolerated in oogenesis, misdirected expression of KL–$Sl^{17H}$ in polarized Sertoli cells may affect spermatogenic proliferation and survival and result in male sterility.

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