Identification and Mutation of Primary and Secondary Proteolytic Cleavage Sites in Murine Stem Cell Factor cDNA Yields Biologically Active, Cell-associated Protein*

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Phenotypic abnormalities of melanocytes, germ cells, and hematopoietic cells of Steel mice demonstrate the critical role of the stem cell factor (SCF) in development. Production of SCF in the hematopoietic microenvironment as either a membrane-associated or soluble factor leads to pleiotropic effects on hematopoietic stem and progenitor cells and significant effects on the production of erythroid cells. Although the production of these two forms of SCF is highly regulated, the physiologic role(s) of membrane-associated and soluble SCF remain unclear. We have demonstrated that the generation of soluble murine SCF by murine stromal cells derived from the fetal hematopoietic microenvironment is dependent on two distinct proteolytic cleavage sites. The primary site in exon 6 is preferentially utilized in these cells. The secondary site located in exon 7 is utilized only in the absence of the primary site. Proteolytic processing at this secondary site appears to be species-specific, since the human protein sequence diverges at this site. The murine SCF cDNA encoding both proteolytic cleavage sites leads to the generation of membrane-associated and biologically active SCF on murine stromal cells. These results suggest that the regulation of processing of the secondary proteolytic cleavage site could play a critical role in the function of membrane-associated SCF protein.

Growth factors and their receptors play important roles in normal development as mediators of intercellular communication by diffusible molecules and direct cell-cell interactions. Many growth factors and some receptors occur in both membrane-bound and secreted forms (1). Several growth factors, such as colony-stimulating factor-1 (2), transforming growth factor-α (TGF-α) (3), and tumor necrosis factor (4) are produced as transmembrane proteins that can be released by specific proteolytic cleavage to generate soluble factors. Such growth factors can influence events at a localized site by adhesive interactions or direct contact with receptors, termed juxtagastric stimulation (5). Following proteolytic cleavage, the secreted products of membrane-bound growth factors can also affect other cells in a regional or systemic manner. Alteration in the balance between the diffusible and membrane-bound forms of growth factors may lead to phenotypic abnormalities as seen in Alzheimer's disease (5) and cutaneous mastocytosis (6).

The hematopoietic microenvironment (HM) represents a heterogeneous group of cells and matrix molecules located in the bone marrow medullary cavity which play a major role in the proliferation and differentiation of hematopoietic cells by a variety of cell-cell interactions (7). The HM has been proposed to regulate hematopoiesis in several ways, including direct cell-cell contact (8), stabilization of growth factors via binding to extracellular matrix proteins (9, 10), production of both positive and negative growth regulatory proteins (11-14), and co-localization of hematopoietic stem and progenitor cells with locally high concentrations of multiple growth factors present on the surface of cells in a local area network (15).

Defects in the HM associated with the Steel (Sl) mutation in mice have recently been demonstrated to be due to abnormalities in the production or presentation of the protein product of the Steel gene, termed stem cell factor or steel factor (also called kit ligand (KL) or mast cell growth factor (MGF)), which exists as a locally secreted or membrane-bound protein (16-19). Stem cell factor (SCF) is present in the HM as a result of both pre- and post-translational processing events of primary SCF mRNA transcripts. The nucleotide sequence of the cloned SCF cDNA predicts a transmembrane protein with a leader sequence, extracellular domain, membrane spanning region, and short cytoplasmic tail (18, 20, 21). A major secreted protein, a 164-amino-acid biologically active growth factor, results from proteolytic cleavage at a site encoded by the primary mRNA (SCF<sup>248</sup>) transcript within exon 6. A membrane-bound 220-amino-acid protein is produced from an alternatively spliced second mRNA transcript (SCF<sup>250</sup>), which lacks exon 6 sequences (22, 23). In some studies, the murine SCF<sup>250</sup> cDNA also encodes a protein released from the cell (24, 25).

The physiologic roles of the various SCF proteins remain unclear. Animals deficient in SCF as a result of deletion of Steel coding sequences (Sl<sup>−/−</sup> homozygotes) died in utero or perinatally with severe deficiencies of germ cells, hematopoietic cells, and melanocytes (26). The presence of pleiotropic abnormalities in these animals is likely due to the absence of SCF in the local area network.
in viable mice with the Steel-Dickie (Stf) mutation, which en-
compases a genomic deletion of the membrane-spanning and cytoplasmic tail of the Steel sequence, has been interpreted as
evidence of the importance of the membrane-bound protein in
adhesive/migratory or cell proliferative responses in these sys-
tems, since presumably only secreted protein is produced in
these mice (22, 24, 27). Differential effects of several forms of
SCF have been demonstrated by our laboratory and other in-
vestigators using in vitro culture systems (23, 28–31).

We have previously reported that stromal cells derived from
the fetal HM of SI/SI mice produce biologically active human
SCF when transfected with the human (h)SCF (24) or hSCF (25)
cDNAs (23). Expression of the cDNA lacking exon 6 (hSCF67)
was associated with SCF which remained almost entirely mem-
brane-bound, while expression of the hSCF (25) cDNA led to high
levels of secreted protein. In contrast, Huang et al. (24) have
reported that expression of both murine (m)SCF (24) and
mSCF (25) cDNAs in COS-7 cells leads to secreted protein, im-
plying a second proteolytic cleavage site exists outside exon 6
which is utilized in these non-HM-derived cells.

To further characterize the processing of SCF in a
HM-derived cell line and to further study the role of various
presentations of SCF protein, we have transfected mSCF (24),
mSCF (25) and plasmids containing mutations at various sites
in exon 6 and exon 7 of mSCF (24) cDNA into SCF-deficient
SU3 (16) stromal cells. We have compared expression of mu-
rine and human protein in these cells. Our results demonstrate
differences in the processing of the human and murine SCF (25)
cDNA-encoded protein and the preferential use of the exon 6
cleavage site of SCF (24) cDNA-encoded protein in murine HM-
derived cells, implying cell or species differences in the protease
responsible for the production of secreted protein. Using in
vitro mutagenesis, we have mapped the location of the primary
proteolytic cleavage site in exon 6 and of a secondary cleavage
site in exon 7 close to the membrane-spanning region and have
generated cell lines containing multiple mutations of mSCF (24)
in which SCF remains membrane bound and biologically ac-
tive.

EXPERIMENTAL PROCEDURES

Cell Lines: SCF Factor-dependent Cells—WB6F, SI/SI (Jackson
Laboratory, Bar Harbor) mouse bone marrow was plated as a cell
suspension and placed in liquid culture in RPMI 1640 (Life Technolo-
gies, Inc./BRL) supplemented with 10% fetal bovine serum (Life Tech-
nologies, Inc./BRL) and 750 ng/ml of recombinant rat stem cell factor
Laboratory, Bar Harbor) mouse bone marrow was prepared as a cell
suspension and placed in liquid culture in RPMI 1640 (Life Technolo-
gies, Inc./BRL). Single-stranded template DNA was purified (33) and oligonucleotide-directed mutagenesis was carried out us-
ing the in vitro mutagenesis system (Amersham Corp.). Mutant clones
were identified by sequencing (34) and were ligated into V19.8 expres-
sion vector (21) and the final plasmid construct was confirmed again
by sequencing.

Generation of Stable Transfectants of SI/SI Stromal Cells with
mSCF cDNAs—Transfection of plasmid DNA into SI/SI cells was per-
formed using DOTAP (Boehringer Mannheim) according to the manu-
ufacturer’s recommendations. SI/SI cells were grown to subconfluence
in DMEM with 10% CS. Plasmid mixtures containing cDNAs of mSCF and
hygromycin-resistance gene in p48 (35) (10:1 ratio) were incubated with
DNA in N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)–
room temperature for 10 min, then added to the cell cultures.
Medium was changed to fresh SI/SI cells and replaced with the
transfection mixture, and the cells were allowed to grow overnight.
The following day, fresh medium with hygromycin (Calbiochem) (300 units/
ml) was added to the cells. Well isolated hygromycin-resistant colonies
arising in 10–14 days were transferred to 24-well plates and expanded.

For mSCF (25) plasmid DNA transfection, reporter RNA was isolated from each of the recombinant plasmids and guinea pig recombinant reagent (Molecular Research Center, Cincinnati, OH) and cloned were screened for mSCF expression by reverse transcriptase-polymerase
chain reaction (Perkin-Elmer).

Metabolic Labeling and Immunoprecipitation—For protein analysis,
subconfluent cells were labeled overnight in methionine-free DMEM,
10% dialyzed CS with 0.5 ml of [35S]Met-Cys (ICN). Conditioned
medium was collected from each cell line 12 h later, filtered through a
0.45-μm filter, and stored at –80°C until used. Labeled cells were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer
(0.5% sodium-deoxycholate, 0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8.0),
and 1 mM phenylmethylsulfonyl fluoride). Immunoprecip-
itation was performed using a rabbit polyclonal antibody made against rat-SCF (32) (kindly supplied by Dr. Larry Bennett, Amgen). 2
ml of polyclonal sera was conjugated to 1 ml of protein A-agarose using
the Affimmia purification kit (Schleicher and Schuell), according to the
manufacturer’s recommendations. The antibody-conjugated protein A-
agarose was washed with PBS and resuspended in 1 ml of PBS with 0.05% sodium azide and stored at 4°C. For immunoprecipitation labeled samples were thawed overnight at 4°C and passed over DEAE-Sepharose fast flow (Pharma-
ice LKB Biotechnology Inc.) equilibrated with PBS at 4°C. The flow-
through was collected (except for cell lysates of mSCF (25), see below),
concentrated by a Centrerpip-10 (Amicon) device according to the
manufacturer’s instructions and transferred to 1.5-ml microfuge tubes.
For mSCF (25) cell lysate, bound protein was eluted with PBS with 0.1 NaCl. 20 μl of the conjugated antibody was added to each sample and
allowed to incubate overnight at 4°C. The samples were centrifuged for
3 min at 4°C, supernatant discarded, and the immunoprecipitates were
washed once with 20 μl of PBS (pH 7.5) and once with 20 μl Tria-Cl (pH
7.5) and 1% Triton) and once with 20 μl Tria-Cl (pH 7.5). The protein
was released from the beads by boiling for 5 min. For glycosidase treat-
ment, half of the protein samples were digested with neuraminidase
(0.8 units/ml), 0-glycansin (0.12 units/ml), and N-glycanase (20 units/
ml) (all Genzyme) in 10 μl Tria-Cl (pH 7.5), 0.1% SDS and 0.1 mM
methanol, overnight at 37°C. An equal volume was added to 20 μl loading buffer (100 μl Tria-Cl (pH 6.8), 200 mM dithiothreitol, 4% SDS,
0.02% bromophenol blue, and 20% glycerol) was added to sample, boiled for 5 min, and the proteins analyzed on 12 or 15% SDS-PAGE
(36).

Biological Activity of Transfected Cell Lines—SCF biological activity
produced by each stably transfected of the murine SCF cDNAs were assayed using the SCF-dependent murine bone marrow-derived SCF+-
cell line and a thymidine incorporation procedure. To analyze biological
activity present as soluble protein, conditioned media was collected from
subconfluent stromal cell transfactants after incubation overnight
in RPMI with 10% fetal bovine serum. Each conditioned medium was
passed through a 0.45-μm filter and concentrated using a Centrerpip-
ten device. The concentrated conditioned medium was filtered again, ali-
quoted, and frozen at –80°C until further use. For analysis of SCF activity, 5 × 105 SCF--
cells were cultured overnight in 100 μl of conditioned concentrated medium in 96-well plates. 1.0 μg of [3H]thy-
mine was added to each well 24 h prior to harvesting and incubated at 37°C for an additional 4 h. The cells were then harvested using an automated cell harvester (96-well Harvester, Brandel, Gaithersburg, MD) and thymidine incor-
poration was determined in a scintillation counter.

The presence of SCF-like activity on the surface of stably trans-
fectants was analyzed using a cell culture assay. On the day prior to assay,
stromal cells were treated with 5 μg/ml mitomycin C (to inhibit further
cell proliferation of the stromal cells), then washed three times in PBS,
trypsinized, counted, and seeded at 3 x 104 cells/well in 0.1% gelatin-
coated 96-well plates. These cultures were incubated in DMEM, 10% CS
at 37°C for 24 h. 5 μl of conditioned medium was added to the stromal cells
for an additional 24 h. Subsequently, 1.0 μCi of [3H]thy-
mine was added to each well for 4 h at 37°C. Cells were then har-
vested and thymidine incorporation was determined as above.
RESULTS

Expression of Murine SCF<sup>248</sup> and SCF<sup>244</sup> cDNAs in SI/SI<sup>4</sup> Cells: Comparison with Expression of Human SCF<sup>248</sup> cDNA—Murine SCF<sup>248</sup> and SCF<sup>244</sup> cDNAs encoding the 248 aa and 220 aa proteins (Fig. 1) were cotransfected into the SI/SI<sup>4</sup> cell line with the hygromycin expression plasmid, p48. Hygromycin-resistant colonies were selected and analyzed for expression of the introduced cDNAs using reverse transcriptase-polymerase chain reaction. Multiple clones expressing mSCF<sup>248</sup> and mSCF<sup>240</sup> were generated. To determine the biosynthesis of mSCF<sup>244</sup> and mSCF<sup>220</sup> proteins, SI/SI<sup>4</sup> stable transfectants expressing each cDNA at similar levels by Northern blot analysis were labeled with <sup>35S</sup>Met-Cys overnight, and conditioned medium and cells were harvested for immunoprecipitation using rabbit polyclonal antibody against rat recombinant SCF. The immunoprecipitated proteins were analyzed on SDS-PAGE gels. As shown in Fig. 2A, expression and processing of both murine SCF<sup>244</sup> and SCF<sup>240</sup> cDNAs leads to secretion of significant amount of SCF protein into the conditioned medium of stable transfectants. After glycosidase digestion (lanes marked +), major protein species of apparent molecular masses of 21 kDa and 18–20 kDa are secreted from the mSCF<sup>244</sup> and mSCF<sup>240</sup> cDNA transfectants, respectively (arrowheads). This difference in apparent molecular mass is consistent with the lack of exon 6 sequences in the secreted protein generated from the mSCF<sup>240</sup> cDNA. In comparison to the amount of protein immunoprecipitated from the conditioned medium (lanes +1), 3–5-fold more cell-associated SCF protein (**) at ~40 and 33 kDa (mSCF<sup>244</sup>) and 27–33 kDa (mSCF<sup>240</sup>) is immunoprecipitated from these cell lines (after glycosidase treatment, arrowheads, Fig. 2B). In sharp contrast, the amount of protein secreted by SI/SI<sup>4</sup> stromal cells transfected with the hSCF<sup>220</sup> cDNA (**) (Fig. 2C) is minimal with an apparent molecular mass of 23 kDa after glycosidase digestion. The difference in the processing of murine and human SCF<sup>240</sup> is even more evident when the relative amount of cell-associated (**) and secreted (+) protein is compared for each cDNA (5:1 versus ~20:1, murine versus human, Fig. 2). Pulse-chase experiments demonstrate the accumulation of soluble protein expressed from the mSCF<sup>240</sup> cDNA in stromal cells is delayed with respect to the primary secreted product derived from the mSCF<sup>244</sup> cDNA (data not shown). These data are consistent with reports by other investigators of a secreted SCF protein derived from the murine cDNA lacking exon 6 (24) and reports from our laboratory demonstrating largely membrane-bound protein derived from the human SCF<sup>220</sup> cDNA in these stromal cells (23).

Purification of SCF from conditioned media of buffalo rat liver cells and amino acid sequence determination has previously demonstrated the carboxyl-terminal amino acid of the secreted rat SCF protein to be either Ala<sup>164</sup> or Ala<sup>166</sup> (37). The region of exon 6 surrounding positions 164–165 contains small nonpolar amino acids which are similar to protease cleavage sites located in the colony-stimulating factor-1 (38) and TGF-α proteins (25). In an effort to better analyze this putative cleavage site, site-directed mutagenesis was utilized to substitute or delete nucleotide sequences corresponding to amino acids surrounding positions 164–165 (Fig. 3). The changes introduced were chosen to facilitate screening and to prevent creation of new protein cleavage sites. The mSCF<sup>240</sup> cDNA represents Ala<sup>164</sup> → Leu and Ala<sup>165</sup> → Glu substitutions with deletion of amino acids at position 163 and 166 of mSCF<sup>248</sup> cDNA and introduction of the XhoI recognition sequence (CTCGAG) in the cDNA. The mSCF<sup>241</sup> cDNA represents deletion of nucleotide sequences corresponding to amino acids 163–166 in mSCF<sup>248</sup> cDNA.

These mutated cDNAs were co-transfected into SI/SI<sup>4</sup>. <sup>35S</sup>Met-Cys-labeled proteins from clones expressing the mutated cDNAs were immunoprecipitated and analyzed on SDS-PAGE (Fig. 4). Surprisingly, SI/SI<sup>4</sup> cells transfected with mSCF<sup>240</sup> and mSCF<sup>241</sup> secrete large amounts of proteins of apparent molecular mass of ~30 kDa after glycosidase treatment. Results from pulse-chase experiments show that accumulation of secreted protein derived from the mSCF<sup>240</sup> and mSCF<sup>241</sup> cDNAs is delayed in comparison to both mSCF<sup>248</sup> and mSCF<sup>220</sup> cDNAs (data not shown). These data are consistent with the utilization of an alternative proteolytic cleavage site in the absence of the exon 6 primary cleavage site. The size of this mutated and secreted protein suggests that the alternative cleavage site must reside in amino acids carboxyl-terminal to the primary site in exon 6 (i.e. closer to the membrane-spanning region).

Examination of the amino acid sequence in this region of the SCF protein revealed a tetrapeptide of Lys-Ala-Ala-Lys at amino acid positions 178–181 in the murine sequence which is similar to the proteolytic cleavage site in exon 6 (Fig. 3). In an effort to further map the alternative proteolytic cleavage site utilized after mutation of the primary site in exon 6, additional mutations were performed. The mSCF<sup>240X323</sup> and mSCF<sup>241T323</sup> cDNAs represent deletions of 12 nucleotides around amino acid 180 in mSCF<sup>248</sup> and mSCF<sup>241</sup>, respectively. These mutated cDNAs were co-transfected with p48 into SI/SI<sup>4</sup>. <sup>35S</sup>Met-Cys-labeled proteins from hygromycin-resistant clones expressing the mutated cDNAs were immunoprecipitated and analyzed on SDS-PAGE (Fig. 5). In contrast to mSCF<sup>248</sup> and mSCF<sup>220</sup> cDNA, a protein of apparent molecular mass of 36–43 kDa after glycosidase treatment which remains almost entirely cell-associated. Similar results were obtained with mSCF<sup>241T323</sup> cDNA (data not shown). These data are consistent with the utilization of a secondary proteolytic cleavage site at or near the tetrapeptide Lys-Ala-Ala-Lys located in exon 7 of mSCF<sup>7</sup> when the primary proteolytic cleavage site in exon 6 is not available. Since the human nucleotide sequence in this area predicts the tetrapeptide Lys-Ala-LysAsn, the data suggest that the difference in processing of the human and murine SCF<sup>220</sup> proteins could be related to sequence differences in this area.

Biological Activity of Murine SCF Proteins Expressed in HM-derived Stromal Cells—To assess the biological activity of proteins expressed from wild-type and mutated mSCF cDNAs, we examined both conditioned medium and cell-associated proteins for the ability to stimulate proliferation of the SCF<sup>240</sup> cell line. Medium conditioned overnight from representative stable transfectants of mSCF cDNAs and SI/SI<sup>4</sup> were concentrated and used in 3H-thymidine incorporation studies with the SCF<sup>240</sup> cell line. As seen in Fig. 6A, conditioned medium from both mSCF<sup>248</sup> and mSCF<sup>220</sup> stimulated thymidine incorporation in the SCF<sup>240</sup> cell line at levels significantly above SI/SI<sup>4</sup>. No stimulation of thymidine incorporation could be detected with mSCF<sup>240</sup> in spite of the presence of immunologically reactive protein in the conditioned medium. Similar results were obtained with mSCF<sup>241</sup> (data not shown). The apparent antagonistic effect of mSCF<sup>240</sup>-conditioned medium was not seen con-
**Stem Cell Factor Proteolytic Cleavage Sites**

**Fig. 2. Expression of murine and human SCF cDNAs in murine S1/S1' stromal cells.** Parental S1/S1' and stable transfectants were labeled overnight with [35S]methionine, conditioned media collected, cells lysed, and immunoprecipitated as described under "Experimental Procedures." A, supernatant of cultured cell lines S1/S1', S1/S1'-mSCF248, and S1/S1'-mSCF220 treated without (+) and with (+) glycosidases. B, cell lysates from the cell lines described in A. C, conditioned medium and cell lysate from S1/S1'-hSCF220 cell line expressing human SCF protein. Arrows show major forms of SCF proteins, * secreted and ** cell-associated forms of SCF.

**Fig. 3. Partial DNA sequence and primary protein structure of murine (M) and human (H) SCF around exon 6 and 7.** Various mutations introduced using site-directed mutagenesis are shown along the left with corresponding abbreviations. Sequence differences in human nucleotides are shown below, amino acid differences shown above the murine sequence. Putative cleavage site in murine sequence are enclosed in boxes.

**Fig. 4. Expression of murine SCF cDNAs mutated in exon 6 in S1/S1' stromal cell lines.** Abbreviations used for the mutated cDNAs are as in Fig. 3. Stable transfectants expressing mutated cDNAs were labeled with [35S]methionine overnight, conditioned media collected, and immunoprecipitated as described under "Experimental Procedures." The results from two mutations are shown without (+) and with (+) glycosidase treatment.

**Fig. 5. Expression of murine SCF cDNAs mutated both in exon 6 and exon 7 in S1/S1' stromal cells.** Abbreviations used for the mutated cDNAs are as in Fig. 3. Stable transfectants expressing mutated cDNAs were labeled with [35S]methionine overnight, conditioned media collected, cells lysed, and immunoprecipitated as in Experimental Procedures. The results of one mutation is shown without (+) or with (+) glycosidase treatment.

As expected, no soluble SCF growth stimulatory activity was present in medium conditioned with stable transfectants of the mSCF248/D3 cDNA. These data are in agreement with the observed lack of secreted protein in the S1/S1'-mSCF248/D3 cell line. The bioactivity demonstrated in the conditioned medium of mSCF248 and mSCF220 was specifically inhibited by neutralizing antibody to murine SCF protein (data not shown).

Analysis of cell-associated SCF-like activity was accomplished using thymidine incorporation assays carried out while SCF<sub>dep</sub> cells were co-cultivated with each stromal transfectant cell line. Although the stromal cell lines were treated with
mitomycin C prior to cultivation to inhibit thymidine incorporation by these cells, some increase in background incorporation was noted on SI/SI4 cells. However, Fig. 6B demonstrates that mSCF248 and mSCF220 proteins stimulates significantly more thymidine incorporation by SCFdep cells than SI/SI4 in cocultivation. In spite of the lack of active protein in conditioned medium, the expression of mSCFXgm3 cDNA in SI/SI4 cells is associated with the presence of biologically active protein on the cell surface. No biologically active protein was detected by co-culture using the mSCFXgm3 transfectant cell line. These results demonstrate that the mSCFXgm3 protein is present on the cell surface of stromal cells and is biologically active. In experiments utilizing primary, adherent cell-depleted murine bone marrow cells, increased numbers of progenitor-derived colonies are seen in cultures utilizing the concentrated conditioned medium from mSCF248 and mSCF220 cell lines. In addition, short co-culture (48 h) experiments demonstrate stimulation of myeloid and mixed primary progenitor colony formation on mSCF248, mSCF220, mSCFXgm3, and mSCFXgm9 cells (data not shown). These data demonstrate that the biological activity assayed in the SCFdep cell line assays is also active on primary bone marrow cultures.

**DISCUSSION**

Phenotypic abnormalities of SI/SI mutant mice demonstrate the critical role of SCF in the normal microenvironments of germ cells, melanocytes, and hematopoietic cells (26). The HM consists of a heterogeneous group of cells, including stromal cells, which have been shown to produce SCF in a regulated fashion (24). The production of SCF in the HM as either a soluble or membrane-associated protein leads to pleiotropic effects on a variety of hematopoietic stem and progenitor cell types as well as significant effects on the production of erythroid-lineage cells both in vitro and in vivo (39). The physiologic roles of membrane-associated and soluble SCF remain unclear. The presence of major phenotypic abnormalities in SI/SI mice, which express a mutated form of SCF lacking the membrane-spanning domain and cytoplasmic domains (22, 24, 27), suggests that the membrane-bound protein is important for many aspects of hematopoietic stem cell biology. Several investigators have demonstrated differential effects of membrane and secreted proteins on germ cell and hematopoietic cell adhesion, survival, and proliferation using in vitro culture systems (23, 28, 29, 40). A major goal of current research is to examine this issue in vivo.

It has become increasingly clear that membrane-associated proteins may have significant effects mediated via soluble forms (2, 23, 24). Stem cell factor is somewhat unique among these proteins in that the secreted form of this growth factor involves both a separate biosynthetic pathway (differential splicing of pre-mRNA to include or exclude exon 6) and post-translational release of the extracellular domain via specific hydrolytic cleavage (19, 21, 24, 25). The possible role of membrane-associated SCF, like TGF-α and tumor necrosis factor, may relate to intercellular communication restricted to adjacent cells, termed juxtacrine stimulation (3).

Intercellular communication by direct cell-cell contact has been previously proposed to be a major mechanism of HM regulation of hematopoiesis (8). In addition to the effects on c-kit-expressing hematopoietic cells, it is possible that binding of SCF to its receptor also induces signaling in the stromal cells presenting SCF. Therefore, advantages of proteolytic release of membrane-associated growth factor could include the rapidity with which a local affect can be changed to a regional stimulation and removal of stimulatory effects of ligandreceptor interactions via the cytoplasmic tail in the presenting stromal cell. The function of the cytoplasmic tail of SCF is currently unknown, although some experimental data support a role in protein processing (41).

Enzymatic cleavage of the proteolytic site in exon 6 of mSCF248 appears to be a highly regulated event with similarities to cleavage of TGF-α (25), including cleavage at a Ala-Val dipeptide and activation via protein kinase C pathways with calcium-dependent mechanisms (24, 42, 43). These characteristics suggest an elastase-like protease (44) may be involved in cleavage of the primary proteolytic site in exon 6. Previous studies (24, 25) have suggested that additional proteolysis in COS-7 cells must take place outside exon 6 to generate a secreted protein from the alternatively spliced mSCF220 mRNA, which lacks exon 6. Protease inhibitor profiles and sequence analysis suggests a distinct protease may be involved in the generation of this secreted form of mSCF220 (25). Our results from stromal cell expression of mSCF220 also indicate a second proteolytic cleavage site outside exon 6. Utilization of this site in stromal cells results in delayed appearance of soluble SCF. Similar results have previously been observed in COS-7 cells (24).

Data presented here also map this putative secondary proteolytic cleavage site within exon 7 and demonstrate that in stromal cells derived from the HM this site is clearly used only when the primary site is not present. In addition, the differential processing of human versus murine SCF220 protein in murine stromal cells, and the sequence divergence in this region, support the suggestion that a distinct protease is responsible for the proteolytic cleavage of the exon 7 site (25).

Secrected forms of mSCF248 and mSCF220 were demonstrated to be biologically active. In the studies presented here mSCF expressed from a cDNA containing mutations in both exon 6 and exon 7 (mSCFXgm3) proteolytic cleavage sites remains membrane-associated. No biologically active SCF-like protein activity was demonstrated in concentrated conditioned me-
from stromal cells expressing this cDNA, although cell-associated protein functioned when SCF-dependent cells were in direct cell-cell contact with producing stromal cells. These results are similar to data previously reported from our laboratory utilizing the hSCF protein, which in the context of murine stromal cells remains membrane-associated and is biologically active. No biological activity could be demonstrated with mSCF protein-containing mutations of the primary proteolytic cleavage site in exon 6. Analysis of SCF secondary structure (45) has shown that exon 6 encodes a variable spacer chain which may be important in the physiologic regulation of dimer formation. Mutation of exon 6 sequences could interfere with dimer formation of this protein resulting in the continued presence in conditioned medium of immunologically reactive SCF protein with diminished or absent biological activity. These data would suggest that inhibition of dimer formation plays a more critical role in the function of soluble versus membrane-associated SCF, since the same mutation when accompanied by additional mutations remains active when presented on the stromal cell surface.

These results provide valuable information about the regulation of murine SCF, including confirmation of the primary cleavage site in exon 6 and the mapping of a secondary proteolytic cleavage site in exon 6. Mutation of both these sites generates a functional but entirely diminished or absent biological activity.

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