Structure-Function Relationships of Stem Cell Factor: An Analysis Based on a Series of Human-Murine Stem Cell Factor Chimera and the Mapping of a Neutralizing Monoclonal Antibody

By Jeffrey V. Matous, Keith Langley, and Kenneth Kaushansky

Although much is now known about the biological properties of the c-kit receptor and its ligand, stem cell factor (SCF), little is known of the structural basis for the binding and function of this hematopoietic cytokine. By analyzing the activities of chimeric interspecies and homologue muteins and epitope mapping of a monoclonal antibody (MoAb) to the human protein, we have found that three distinct regions of SCF are essential for full biological function. Homologue and interspecies swapping of polypeptide sequences between the amino terminus and G35, between L79 and N97, and between R121 and D128 reduced or eliminated the ability of the chimera to act in synergy with murine granulocyte-macrophage colony-stimulating factor (GM-CSF) to promote hematopoietic colony formation. Moreover, a nonconformation-dependent MoAb that neutralizes human, but not murine SCF, was found to bind to residues within the L79-N97 segment of the human homologue. As these three regions localize to the putative first, third, and fourth helices of the protein, findings remarkably similar to previous studies of cytokines as diverse as growth hormone, GM-CSF, and interleukin (IL)-4, our results suggest that cytokines of multiple classes share a common functional organization.

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THE TYROSINE KINASE receptor, c-kit, is critically involved in the proliferation and differentiation of early hematopoietic progenitor cells. The cloning of this receptor \(^{1,2}\) and its ligand (here termed stem cell factor [SCF], but also known as c-kit ligand, mast cell growth factor and steel factor \(^{3,4}\)); culminated decades of work begun by developmental biologists who first noted the pleiotropic effects (macrocystic anemia, mast cell deficiency, sterility, and pigmentation defects) of the Steel (S) and White Spotting (W) mutations of mice. \(^{1,2}\) The gene products encoded by the W and S1 loci were subsequently found to be c-kit and SCF, respectively. SCF exists in both soluble and transmembrane forms. Alternative splicing of exon 6 is responsible for the generation of the critical proteolytic signal and cleavage sites responsible for the release of the soluble form of the protein from the cell surface. \(^{1,2}\) The full-length transcript includes a 25-amino acid secretory leader, a 189-amino acid extracellular domain, a 23-amino acid transmembrane region, and a 36-amino acid cytoplasmic tail. The first 164 or 165 residues of the extracellular domain comprise soluble SCF, which exists in solution as a noncovalently linked homodimer. \(^{1,2}\)

The severe hematopoietic defects of the S1 and W mutants, and the dramatic in vitro and in vivo effects of SCF support its role in hematopoiesis. \(^{5,6,10,12,15-19}\) Alone, SCF is a relatively weak proliferative stimulus of marrow hematopoietic colony-forming cells. In contrast, together with erythropoietin (Epo), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, interleukin (IL)-1, IL-3, IL-6, IL-7, IL-11, or thrombopoietin, the cytokine exerts a profound synergistic response in both the number and size of the resultant hematopoietic colonies. \(^{15,17,20-22}\) As a result of these activities, studies have begun to investigate the potential clinical role of SCF both alone and in combination with other growth factors to augment hematopoiesis in states of marrow failure. \(^{23-28}\)

Despite a growing body of data characterizing the biological effects of SCF, very little is known of the structural features of the protein responsible for receptor binding and signal transduction. Several strategies can and have been employed to elucidate the structure-function relationships of the hematopoietins. The methods and results of these studies have been summarized in recent reports. \(^{29,30}\) Briefly, a hematopoietic growth factor can be thought of as a structural scaffold upon which sit critical side chains of proper charge density, hydrogen bonding potential, hydrophobicity, and shape to allow docking with its cell surface receptor. A thorough understanding of the mechanisms involved in receptor-mediated signal transduction is dependent on the detailed study of those regions of ligand required for docking to and triggering of the receptor. In addition, an understanding of the structure-function relationships of any growth factor is imperative when considering the rational design of therapeutic derivatives. Towards this end, we have begun to map regions of SCF critical for receptor engagement and biological activity. Using interspecies chimeric analysis and mapping of a neutralizing monoclonal antibody (MoAb), techniques that have been extremely informative in our and other laboratories, \(^{22,24-28}\) we have identified two nonlinear regions of SCF necessary for full species-specific activity. This strategy was particularly informative for SCF, as the murine growth factor stimulates both human and murine c-kit receptor-bearing cells, but human SCF fails to engage the murine receptor. Therefore, should an interspecies chimera lose murine SCF activity, its tertiary folding and quaternary associations can be assessed by measuring human receptor binding capacity. In addition to the information gained from interspecies chimeras, site directed mutagenesis studies of residues predicted to be in the fourth helix of the folded polypeptide point to an important role for this region in receptor engagement. Based on these findings, we propose that SCF demonstrates a marked conservation of receptor binding topology.
with cytokines seemingly as diverse as growth hormone and GM-CSF.

**MATERIALS AND METHODS**

*Generation of chimeric cDNA expression vectors and proteins.* Full-length cDNA clones for the soluble form of human SCF and murine SCF were subjected to site-directed mutagenesis using a polymerase chain reaction (PCR)-based technique to introduce useful restriction sites for subcloning and to generate interspecies chimeric cDNA. The resultant cDNA were subcloned into the mammalian expression vector pDS50 and the nucleotide sequences confirmed by the method of Sanger.46 Cell lines expressing high levels of recombinant SCF and muteins were established by cotransflecting BHK cells with the various expression vectors and one encoding dihydrofolate reductase (DHFR), as previously described.47,48 Cell lines expressing high levels of SCF-specific mRNA by slot blot analysis and/or expressing high levels of soluble SCF protein by immunoblotting (see below) were identified for further analysis. Cell lines expressing specific muteins were washed in phosphate-buffered saline and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% dialyzed fetal calf serum (dFCS) and antibiotics. Conditioned culture media containing soluble recombinant SCF protein was collected after 3 to 4 days. Sham-conditioned media was transfecting BHK cells with DHFR alone. Recombinant 35S-SCF proteins were generated by metabolically labeling established cell lines in DMEM devoid of cysteine and methionine (Cy5Met), supplemented with 2% dFCS, antibiotics, and 50 μCi/mL 35S CysMet (Express; New England Nuclear, Boston, MA). The conditioned culture medium was then harvested after 2 to 3 days.

*Western blot analysis.* The presence and relative level of specific recombinant SCF proteins were determined by Western blotting, as described previously.49 Aliquots of conditioned medium from appropriate cell lines were denatured, size-fractionated by NaDodSO4/10% polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with a 1:100 dilution of a polyclonal rabbit antiserum raised against a synthetic peptide representing residues 3-31 of human SCF. This antiserum recognizes human SCF and murine SCF equally well, making quantitation of SCF chimeras reliable. An 125I goat antirabbit IgG (Amersham, Arlington Heights, IL) was used to detect immune complexes on the blot. After autoradiography, phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA) was used to quantitate the 125I detecting reagent present, and thereby the relative amounts of specific SCF protein produced by each cell line.

*Radioimmunoprecipitation of metabolically-labeled SCF proteins.* 35S Met/Cys labeled conditioned culture media (2.0 to 2.5 mL) from each of the native and mutein SCF-producing cell lines was incubated with 10 μg of an affinity purified murine antihuman SCF MoAb, 7H6, for 16 to 24 hours at 4°C. Twenty-five micrograms of antibody, 10 μg of murine antihuman SCF, were kindly provided by Dr Sung-Hou Kim at the University of California, Berkeley, CA. Computer modeling to align the structure of SCF on that of M-CSF was performed by Paul O. Sheppard, ZymoGenetics, Inc, Seattle, WA, using software from Biosym Technologies, Inc, San Diego, CA.

**RESULTS**

*Generation of interspecies chimeric SCF molecules.* The constructs illustrated in Fig 1 were designed to independently test each of the four predicted alpha helices of SCF, based on the SCF/M-CSF sequence alignment of Bazan,40 and were generated by PCR-induced mutagenesis of cDNA clones for human and murine SCF. On sequence verification and subcloning into a mammalian cell expression vector, cell lines were generated expressing each construct by methotrexate selection of BHK cells cotransfected with an SCF expression vector and one encoding DHFR. Isolated, methotrexate-resistant colonies were picked, expanded, and culture supernatants then subjected to protein blotting to determine the relative amounts of SCF proteins (Fig 2). As the Western blots used a polyvalent antiserum raised against an amino-terminal peptide of SCF identical in the murine and human proteins, the intensity of antibody staining of the size-fractionated spent tissue culture medium was proportional to the levels of specific protein present. We have successfully used this strategy to quantitate cytokines while studying the structure-function relationships of both GM-CSF and IL-3.43,44

*Biological activity of SCF muteins.* To determine if the chimeric proteins were properly folded and could dimerize, we tested whether the muteins could support the growth of human erythroid progenitor cells. As both human and murine SCF support the growth of human progenitor cells equally well,1 each of the muteins, unless improperly folded, should be active on human cells. This was the case. In the presence of suboptimal levels of IL-3 and optimal levels of Epo, all
of the chimeric SCF proteins acted in synergy to enhance the growth of BFU-E, thereby assuring the tertiary and quaternary structural integrity of the muteins. These results were confirmed by assessing the capacity of the muteins to compete for binding to the human SCF receptor, c-kit, on a human leukemic cell line. As shown in Table 1, all of the interspecies chimera competed for binding to M07e cells in the presence of \(^{125}\)I human SCF.

Next, based on the findings that rodent SCF supports the growth of murine hematopoietic progenitor cells, but the
the murine specific residues beyond position L59 also contribute to murine SCF receptor-specific activity. Comparison of the partial activity of hm2 and the inactivity of hm3 suggest that these additional interactive residues lie between positions L79 and N97. This conclusion was supported by analysis of the biological activities of constructs hm5 and mh5. The partial activity of hm5, a construct in which murine SCF-specific residues from L79 to N97 were substituted into a human SCF background, indicates the importance of this region for murine SCF function. Using a similar strategy, substitution of human specific residues from L79 to N97 into a murine SCF background lead to substantial reduction in the biological function of murine SCF (construct mh5).

Analysis of a neutralizing MoAb of human SCF. The binding epitope of the murine antihuman SCF MoAb 7H6 was next mapped using the chimeric constructs illustrated in Fig 1, in an attempt to glean further information from our analysis. Previous work has shown that both intact antibody and an Fab fragment of 7H6 neutralize the activity of human SCF, but fail to bind the murine homologue (data not shown). Moreover, the antibody is highly reactive in a Western blotting format, even when the target protein is denatured and carboxymethylated on the blot. Such characteristics strongly suggest that the antibody binds to a simple, linear epitope on human SCF. Thus, identifying its binding epitope might shed light on at least one of the sites necessary for receptor binding. Using metabolically-labeled human SCF and SCF muteins, 7H6 was found to immunoprecipitate construct mh3, but not hm1 or hm2, and to bind to mh1, mh2, and mh5, but not mh3 (Fig 4). From this data it can be deduced that 7H6 binds to residues between L79 and N97, precisely the same residues found to be responsible for some of the synergistic activity in the colony-forming assays.

Analysis of putative D-helix residues. The results of chimeric analysis are highly dependent on the presence of critical surface residue differences between two homologues of a protein. Often, the sites critical for binding of a cytokine to its receptor are made up of both species-specific and species-

### Table 1. Human c-kit Receptor Competition Assay

<table>
<thead>
<tr>
<th>Protein</th>
<th>% Specific Competition</th>
</tr>
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<tbody>
<tr>
<td>Human SCF</td>
<td>100</td>
</tr>
<tr>
<td>Murine SCF</td>
<td>100</td>
</tr>
<tr>
<td>hm1</td>
<td>76</td>
</tr>
<tr>
<td>hm2</td>
<td>80</td>
</tr>
<tr>
<td>hm3</td>
<td>90</td>
</tr>
<tr>
<td>hm5</td>
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<tr>
<td>mh1</td>
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<tr>
<td>mh2</td>
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<tr>
<td>mh3</td>
<td>90</td>
</tr>
<tr>
<td>mh5</td>
<td>100</td>
</tr>
<tr>
<td>~D-helix</td>
<td>20</td>
</tr>
<tr>
<td>Sham</td>
<td>6</td>
</tr>
<tr>
<td>gIL-3</td>
<td>0</td>
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</tbody>
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Conditioned media from BHK-derived cell lines expressing soluble SCF chimeric proteins was tested for the ability to compete with 125I-labeled human SCF in an M07e c-kit receptor competition assay. Equivalent amounts of protein, as determined by quantitative Western blotting (Fig 2), were used. The maximal percent competition was defined as 100% specific competition. The data represent the mean of triplicate determinations.

* A fivefold excess of this construct was used in the assay.

Moreover, the antibody is highly reactive in a Western blotting format, even when the target protein is denatured and carboxymethylated on the blot. Such characteristics strongly suggest that the antibody binds to a simple, linear epitope on human SCF. Thus, identifying its binding epitope might shed light on at least one of the sites necessary for receptor binding. Using metabolically-labeled human SCF and SCF muteins, 7H6 was found to immunoprecipitate construct mh3, but not hm1 or hm2, and to bind to mh1, mh2, and mh5, but not mh3 (Fig 4). From this data it can be deduced that 7H6 binds to residues between L79 and N97, precisely the same residues found to be responsible for some of the synergistic activity in the colony-forming assays.

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![KL Chimeric Protein](image-url)

*Fig 3. Murine bone marrow colony forming assay. The data represent the mean number of CFU-GM-derived colonies ± SEM produced in the presence of equivalent amounts of each of the chimeric SCF proteins and a suboptimal amount of murine GM-CSF, from at least three separate experiments. The relative amount of protein assayed was determined so that CFU-GM colony growth for the native murine SCF would be submaximal, thereby ensuring accurate representation of the relative activities of the various SCF proteins.*

human homologue does so only very poorly (800-fold reduction in specific activity; Martin et al9), we tested the proteins for their ability to synergize with suboptimal levels of murine GM-CSF to enhance the growth of murine granulocyte and macrophage colonies. Synergistic assays of SCF activity were used in this study, as by itself, SCF is a poor stimulus of any type of hematopoietic cell.13 As shown in Fig 3, only mutein mh3 was as active in the murine GM-CSF synergy assay. Thus, the human SCF-specific regions downstream of P95 were not required for species-specific activity. This result was independently confirmed by analysis of construct mh3. It showed absolutely no capacity to support the growth of murine CFU-GM derived colonies, indicating that alone, sequences downstream of N97 are insufficient for either murine SCF receptor engagement or signal transduction.

The results illustrated in Fig 3 also indicate that constructs hm1 and hm2 were equally, but only partially, active in this assay of synergistic activity. The partial activity of hm1, compared with murine SCF, indicates that the region between the amino terminus and G35 of SCF is necessary for full activity. However, as construct mh1 failed to fully stimulate GM colony growth, this region is not sufficient for full biological responsiveness.

Additional inferences can be drawn from the data in Fig 3. The equal activity of hm1 and hm2 indicates that the region between W44 and L59 does not contribute to the synergistic activity of murine SCF. Otherwise, construct hm2 should have been less active than hm1. This conclusion is also supported by comparing constructs mh1 and mh2. The addition of murine specific sequence between W44 and L59 (mh2 compared with mh1) failed to favorably affect murine-specific biological function of these partially active proteins. Moreover, as hm2 is partially active, despite having human specific sequence in the amino terminal region, it appears...
conserved residues. As human and murine SCF share 79% sequence identity, this event is likely indeed. Structure-function analysis of a number of cytokines have shown that the fourth alpha helix provides a critical site of receptor interaction. However, based on our computer modeling, human and murine SCF are almost identical in the putative fourth helix. Thus, chimeric analysis of this region (as exemplified by mh3 murine SCF) may not reliably identify critical fourth helix residues.

There are striking similarities between the c-kit/SCF receptor-ligand pair and that of another tyrosine kinase receptor, c-fms, and its ligand, M-CSF. Both are involved in developmental processes, occur in soluble and membrane-bound forms, assemble into homodimers, and share significant sequence and probable structural homology. To further explore the putative fourth helical region of SCF, we constructed a chimeric SCF/M-CSF mutant, based on their structural similarities. Although only four highly polar residues of murine SCF were substituted by the homologous murine M-CSF residues (Fig 1), all shown to be on the surface of M-CSF and predicted to be surface-oriented on murine SCF, the pD-helix construct was devoid of biological activity and bound very poorly to the c-kit receptor (Table 1). Thus, using homologue chimeric analysis, the putative fourth helix of SCF appears to be essential for receptor engagement.

**DISCUSSION**

SCF is an early-acting hematopoietic cytokine produced by the marrow stroma. Its physiologic importance is certain, owing to its role in a congenital macrocytic anemia in mice. In addition, potential therapeutic roles are under active investigation. Little is known, however, of the structural basis through which SCF interacts with its receptor, c-kit, to produce biological effects. To date, only two reports detailing truncation mutagenesis have been reported, demonstrating that at least 141 of the 165 residues of soluble SCF are required for activity. In the present studies, we have explored the structure-function relationships of the soluble form of SCF by assaying the species-specific activity of a series of human-murine chimeric polypeptides. We report that at least two nonlinear regions of the glycoprotein are critical for species-specific activity. In addition, based on the activity of a site-directed mutant of a region close to the carboxyl terminus of the protein, an eight amino acid region was also identified as critical for the biological activity of SCF. Finally, we mapped the binding epitope of a neutralizing anti-human MoAb to one of these regions, corroborating some of the findings derived from biological activity assays.

The ability of chimeric structure-function analysis to identify important ligand-receptor interactive regions and residues is well documented for hematopoietic cytokines, including GM-CSF, IL-4, IL-5, and leukemia inhibitory factor (LIF), and for other important proteins. In contrast to the results from simple deletional mutagenesis, in which regions critical for both structural integrity and receptor binding will result in ablation of functional activity, and hence be indistinguishable, the risk that loss-of-function interspecies chimeric mutants are due to gross structural abnormalities are substantially reduced, because proteins that share as little as 25% primary sequence identity display conserved tertiary folding patterns. As human and murine SCF share 79% sequence identity, they are almost certain to retain highly similar tertiary structures. Therefore, substituting the structural backbone regions of one homologue (eg, human SCF) for those of another (eg, murine SCF) should not affect biological activity through gross changes in structural folding. Consistent with this assumption is our finding that all of the chimeric SCF mutants bind to and activated the human c-kit receptor.

In contrast to human c-kit bearing cells, in which both human and murine SCF are equally active, only the murine homologue can synergize with GM-CSF and stimulate hematopoietic colony formation from murine marrow cells. Thus, substitution of human SCF-specific sequences involved in receptor interaction, for the homologous regions of murine SCF, would be expected to alter the capacity of the resultant interspecies chimeric protein to activate murine cells. Based on this strategy, two regions of the protein, between residues 1-35 and residues between positions 79-97, were identified.
as contributory to the synergistic biological activity of murine SCF. At least part of these sequences reside in the predicted first and third helices of murine SCF.

In related studies reported herein, we mapped the binding epitope of a neutralizing MoAb of human SCF to many of these same residues, located between L79 and N97. As this MoAb blocks the biological activity of human SCF, both as an intact Ig and as a Fab fragment, it is unlikely that neutralization is accomplished by steric hindrance. Also, as 7H6 recognizes denatured human SCF in a Western blotting format, even when the target protein is denatured and acetylated under harsh conditions on the blot, it is unlikely that the MoAb binds to a complex epitope so that neutralization of SCF activity is accomplished by MoAb binding to a site distant from where our chimeric protein analysis mapped it. Determining the binding epitopes of more neutralizing and nonneutralizing antibodies would yield additional important information about the structure-function relationships of SCF. However, it is of considerable interest that the binding epitope of MoAb 7H6 was assigned to residues between positions 79-97, further emphasizing the critical nature of this region.

Chimeric analysis is not without its deficiencies. The biological activity of a growth factor may not be due entirely to species-specific activity. Although not required for species-specific activity, other surface residues that are conserved between species may also be essential for receptor engagement or for triggering signal transduction. Such residues are not identified by chimeric analysis. One striking example of the failure of chimeric analysis to identify critical receptor interactive residues is E21 of hGM-CSF. Despite the fact that our previous chimeric analysis of GM-CSF suggested that residues E14 through E21 were necessary for full activity, the importance of position 21 was not appreciated as both the murine and human homologues contain glutamic acid at this site. It was not until this residue was specifically mutated to Arg that its critical role was identified. Thus, it remained possible that amino acid residues outside of regions 1-35 and 79-97 contribute to the functional activity of SCF.

The D or fourth helix of the hematopoietic cytokine family assumes great importance in receptor-ligand interactions. As the residues of the predicted fourth helix of SCF are nearly identical in the human and murine homologues (only R117 [to S] and V130 [to M] vary in the two SCFs), the usefulness of interspecies chimeras in this region was limited. Instead, we chose to substitute some of the predicted surface residues of murine SCF for the corresponding residues in M-CSF. The justification for this strategy is sound. First, helical wheel analysis in this region of SCF clearly delineates hydrophilic and hydrophobic faces, and the crystal-derived structure of M-CSF establishes the orientation of these sites. Second, we mutated only four of the putative surface residues in SCF to the corresponding M-CSF surface residues, all highly polar amino acids (thus unlikely to represent internal, structure-determining core residues). Third, we easily obtained mammalian cell lines that secreted significant quantities of mutant protein. As it is clear from the literature and our own personal experience that abnormally folded proteins are rarely secreted from mammalian cells, the ease of obtaining high level μD-helix expression argues against its loss of activity based on gross structural alteration. Fourth, this region is not involved in the formation of M-CSF homodimerization, again making it unlikely that the crippling effect of the μD-helix mutation is mediated by mechanisms other than alteration of a critical site of receptor interaction. And fifth, similar types of helix swapping experiments have been reported for IL-5 and GM-CSF, in which a critical yet conserved helical region of the former was substituted into the latter, without any adverse effect on biological activity. Thus, the loss of activity and receptor competition of the μD-helix construct clearly points to the importance of the putative fourth helix of SCF in its biological function.

The results of the present study support a model in which the first, third, and fourth helices of SCF are necessary for engagement of c-kit and signal transduction. This conclusion is remarkably similar to the functional anatomy of growth hormone, GM-CSF, and IL-4, whose receptors are members of the hematopoietic cytokine receptor family, a group of transmembrane proteins distinct from the receptor tyrosine kinases for SCF and M-CSF. However, the data for the model are clear, interspecies chimeras and site-directed mutants of the three distinct regions of SCF clearly affect biological activity. And a neutralizing, conformation independent MoAb binds to one of these regions. Additional experiments using single alanine substitution mutants will be required to further localize the precise binding sites involved in the SCF/c-kit interaction, but the present work allows us to extend the common functional organization of the hematopoietic cytokine receptor family to those of the receptor tyrosine kinase family of ligands.

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