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Interspecies Molecular Chimeras of Kit Help Define the Binding Site of the Stem Cell Factor

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The extracellular portion of the *kit*-encoded receptor for the stem cell factor (SCF) comprises five immunoglobulin (Ig)-like domains. To localize the ligand recognition site, we exploited the lack of binding of human SCF to the murine receptor by using human-mouse hybrids of Kit and species-specific monoclonal antibodies (MAbs) that inhibit ligand binding. Replacement of the three N-terminal Ig-like domains of the murine Kit with the corresponding portion of the human receptor conferred upon the chimeric receptor high-affinity binding of the human ligand as well as of human-specific ligand-inhibitory MAbs. By constructing five chimeric murine Kit proteins which individually contain each of these three human Ig-like units or pairs of them, we found that the second human domain confers upon the mouse Kit high-affinity binding of the human ligand and also binding of species-specific SCF-competitive MAbs. Nevertheless, the flanking Ig-like domains also affect high-affinity recognition of SCF. Moreover, it appears that the determinants that define ligand specificity of the murine and the human receptors do not structurally coincide. This observation allowed us to identify a chimeric receptor that displayed a dual specificity; namely, it bound with high affinity either the human or the murine SCF molecules and reacted with mouse- as well as human-specific ligand-inhibitory MAbs. Conversely, another chimera, which included all of the five Ig-like domains, bound neither ligand. In conclusion, interdomain packing involving the second Ig-like domain of human Kit and noncontiguous structural motifs of the receptor are involved in SCF recognition.

Polypeptide growth factors control cell proliferation and differentiation by binding to specific cell surface receptors. The stem cell factor (SCF) or mast cell growth factor (also called Kit ligand and *Steel* factor) is such a ligand that has been recently identified and molecularly cloned (1, 7, 8, 13, 22, 34, 39). SCF is encoded by the well-studied *Steel* locus of the mouse (7, 13, 38), and mutations within this gene cause a defective microenvironment for the development of melanocytes, germ cells, and certain hematopoietic progenitor cells (2, 27, 30). Besides its functions in vivo in hematopoiesis, melanogenesis, and gametogenesis, SCF synergizes in vitro with other lymphokines to increase the number and the size of colonies of hematopoietic progenitors (3).

The cell membrane receptor for SCF is a 145-kDa glycoprotein that spans the plasma membrane and carries a cytoplasm-facing tyrosine kinase activity (26, 35). This receptor tyrosine kinase is encoded by the proto-oncogene c-*kit*, the cellular counterpart of the oncogene v-*kit* of the Hardy-Zuckerman 4 feline sarcoma virus (4). c-*kit* is allelic with the dominant *White spotting* (*W*) locus on mouse chromosome 5 (6, 9). The phenotype of *W* mutant mice is almost identical to that of *Steel* mutants, but various mutations vary in their effects on different cell lineages. The Kit protein shares structural architecture and sequence homology with the receptors for the platelet-derived growth factors (PDGFs) and the receptor for colony-stimulating factor 1 (36). The similarity includes a common structure of the extracellular ligand binding domains of this group of receptors, which include five immunoglobulin (Ig)-like domains. The compact structure of the Ig-like structural unit is expected to fold independently of adjacent regions, as is the case with other members of the Ig superfamily of proteins (14, 33). Apparently this structural organization of the ectodomain of Kit enables it to carry out two functions that are crucial for signal transduction by SCF. First, it contains the ligand binding site, and second, it enables ligand-induced dimerization of the receptor. By constructing a soluble ectodomain of Kit, we recently found that both activities are confined to the extracellular receptor portion (20). Moreover, our studies with heterodimers of the mouse and the human Kit proteins led us to conclude that receptor dimerization is independent of the bivalency of the dimeric structure of SCF, and it may involve a receptor domain distinct from the binding cleft (21).

In this study, we addressed the structural determinants of SCF binding to the Kit receptor by taking advantage of the lack of binding of the human SCF to the murine receptor. Recombinant human-mouse chimeric Kit proteins enabled us to confine the major binding determinant of the human SCF to the second Ig-like domain of the receptor. Unexpectedly, we found that the binding specificity sites of the human and the murine receptors are apparently not coincidental; the third rather than the second mouse domain was found to be essential for binding of the rodent SCF. However, interdomain packing appears also to be involved in ligand recognition, and a model that combines mapping of immunological epitopes with ligand binding analyses is proposed.

MATERIALS AND METHODS

Materials. Polyclonal antibodies to Kit were raised in rabbits that were injected with synthetic peptides corresponding to the C terminus (antibody [Ab] 212) or a portion

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of the interkinase domain (Ab 213) of the receptor. Anti-Kit monoclonal antibodies (MAbs) were obtained by injecting mice with a recombinant ectodomain of human Kit (20). Hybridomas producing anti-Kit antibodies were selected as described previously (5). Rabbit antibodies to phosphotyrosine were raised as described elsewhere (25). Recombinant bacterially made SCF of human or rat origin was kindly provided by Amgen (Thousand Oaks, Calif.). Protein A coupled to agarose was from Pharmacia (Uppsala, Sweden). Molecular weight standards for electrophoresis were from Sigma (St. Louis, Mo.), and radioactive materials were from Amersham (Amersham, Buckinghamshire, United Kingdom). Calf serum was purchased from HyClone (Logan, Utah). Unless otherwise indicated, all chemicals were from Sigma.

Constructions of human-mouse hybrid cDNA molecules. To construct MH-123 and HM-123 chimeric cDNAs, we introduced a new *Kpn*I site in the intervening sequence between Ig-like domains 3 and 4 at homologous positions of both the human and the mouse c-*kit* cDNAs. The sequences of the synthetic oligonucleotides that were used in this site-directed mutagenesis were GACATTTGCGGTACCAAAAG TAT (human) and ATACTTTTGGTACCGCAAATGTCAC (mouse). The underlined bases represent mutations that changed serine (residue 298 in human Kit [35]) to threonine in both receptors and thus enabled the introduction of the novel *Kpn*I site. The chimeric cDNAs were constructed first in pBluescript (Stratagene) by digestion with *Kpn*I and swapping of the corresponding fragments. After the orientation and reading frame of the fusion point were verified by nucleotide sequencing, the chimeric cDNAs were subcloned into the pLSV mammalian expression vector either in an *Xba*I site (MH-123) or in *Xho*I and *Xba*I sites (HM-123).

To construct hybrid cDNA molecules that encode MH-23, MH-12, MH-1, MH-2, and MH-3, we made use of the mutated human and mouse cDNAs that contained the novel *Kpn*I site. In addition, we took advantage of a *Hin*dIII site that lies between the first and second Ig-like domains of human Kit, and also an *Eco*RI site that is localized in the region between domains 2 and 3. These endonuclease restriction sites were introduced into the homologous portions of the murine cDNA by using site-directed mutagenesis and three synthetic oligonucleotides that were annealed simultaneously with the cDNA. The sequences of the oligonucleotides were as follows: CCAGGAAAAGCTTGGCAGGA TC (*Hin*dIII site), CACCACCGTGAATTCGTCCCCTTTC (*Eco*RI site), and GGGGCCCGGTTCCCAGCTTTG. The latter oligonucleotide was designed to destroy an existing *Kpn*I site in the polylinker of the pBluescript plasmid that contained the mouse c-*kit* cDNA. The underlined bases indicate mutations that were introduced into the cDNA. Nucleotide sequencing and endonuclease restriction analyses confirmed the proper introduction of the novel sites in the mouse c-*kit* cDNA. These changes were silent in terms of the translated sequence except for threonine 228 of mouse Kit, which was changed to glutamic acid by introducing the *Eco*RI site. Human Kit contains a glutamic acid at the corresponding position. To construct the chimeric cDNAs, we used the human c-*kit* cDNA (with a novel *Kpn*I site) and the mutated mouse cDNA (with novel *Eco*RI and *Hin*dIII sites), both cloned into pBluescript. The MH-1 hybrid was obtained by swapping an *Xho*I-*Hin*dIII cDNA fragment of mouse c-*kit* with the corresponding region of human c-*kit*. Similarly, a *Hin*dIII-*Eco*RI fragment of mouse c-*kit* was replaced by the same fragment of human c-*kit* to generate MH-2. To construct MH-3, an *Eco*RI-*Kpn*I fragment of human c-*kit* was used, whereas an *Xho*I-*Eco*RI fragment of mouse c-*kit* was replaced to construct MH-12. Lastly, a *Hin*dIII-*Kpn*I fragment of mouse c-*kit* was replaced with the corresponding fragment of human c-*kit* to obtain the MH-23 hybrid cDNA. All of the chimeric cDNAs were checked by endonuclease restriction analysis and by oligonucleotide sequencing of the junction regions. Each hybrid cDNA molecule was then cloned into the pLSV mammalian expression vector following digestion with *Xho*I and *Xba*I.

Establishment of stably expressing cell lines. The HSR cell line was established by cotransfection of pLSV/human c-*kit* and pSV2neo into NIH 3T3 cells by using the calcium phosphate precipitation method as described previously (32). Gentamicin (G418; 1 mg/ml; GIBCO-BRL, Bethesda, Md.) was used to select colonies of cells that were resistant to neomycin. Immunoprecipitation analysis of Kit was used to select an overexpressing clone. To establish cell lines that express the chimeric receptors, we used NIH 3T3 fibroblasts and cotransfection of the chimeric cDNA of interest (in the pLSV expression vector) together with either pSV2neo or pSV2hph. The latter confers resistance to hygromycin. Transfectants were selected for growth in medium that contained gentamicin (1 mg/ml) or hygromycin (50 μg/ml). The MK-37 cell line that overexpresses mouse Kit was established as described previously (21). All cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% bovine serum (HyClone).

Immunoprecipitation and Western immunoblot analyses. Confluent cells grown in 10-cm-diameter dishes were washed once with phosphate-buffered saline (PBS), scraped into 1 ml of lysis buffer [20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml], and incubated on ice for 5 min. After clearing of the cell lysates by centrifugation (12,000 × *g*, 15 min at 4°C), the Kit receptor was immunoprecipitated by using protein A-agarose beads that were precoupled to specific antibodies as indicated. Following 90 min of shaking at 4°C, the immunobeads were washed three times with HNTG (20 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100), boiled in gel sample buffer, and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). In some experiments, the gel-embedded proteins were electrophoretically transferred onto nitrocellulose. The blot was then blocked with Tris-buffered saline (10 mM Tris [pH 7.4], 150 mM NaCl) that contained 10% low-fat milk and 1% ovalbumin. Antisera or purified MAbs were then added in the same solution, and incubation was carried out for 1 h at 22°C. For detection, the filters were washed three times (5 min each wash) with Tris-buffered saline–0.05% Tween 20 and reacted for 45 min at room temperature with horseradish peroxidase-conjugated protein A. The enzyme was removed by washing as described above, and the filters were reacted for 1 min with a chemiluminescence reagent (ECL; Amersham) and exposed to autoradiography film for 1 to 15 min. Analysis of Kit tyrosine phosphorylation following stimulation with SCF was performed exactly as described previously (19).

Radiolabeling of SCF and ligand binding assays. Human or rat SCF was labeled with ^{125}I (Amersham) by using the chloramine T method (15). Ligand displacement analyses were carried out as follows. Cells were grown to confluence in 24-well dishes (Nunc). The monolayers were washed with

PBS and incubated in binding buffer (Dulbecco's modified Eagle medium containing 20 mM HEPES [pH 7.5] and 0.1% bovine serum albumin) for 90 min at 22°C with ^{125}I-SCF (2 ng of human SCF per ml or 5 ng of rat SCF per ml) and increasing concentrations of unlabeled SCF as indicated. The monolayers were then washed three times with ice-cold PBS; the cells were solubilized in 1 ml of solubilization buffer (0.1 N NaOH, 0.1% SDS) per well, and radioactivity was determined by counting γ radiation. For direct ligand binding analyses, the cells were incubated for 90 min at 4°C with increasing concentrations of ^{125}I-labeled human SCF (0.2 to 500 ng/ml), and cell-associated radioactivity was determined as described above. Nonspecific SCF binding was determined by performing parallel experiments with confluent monolayers of parental untransfected NIH 3T3 cells. Each data point was obtained in duplicates, and every binding experiment was repeated at least three times.

Covalent cross-linking of ^{125}I-labeled SCF. Confluent monolayers of Kit-expressing cells were grown in 65-mm-diameter plates. The cells were first washed with PBS and then incubated with radiolabeled SCF (20 ng of human SCF per ml or 60 ng of rat SCF per ml). After 90 min at 4°C, the plates were transferred to 22°C and bis(sulphosuccinimidyl) suberate (BS^3; Pierce Biochemical Co.) was added to a final concentration of 0.5 mM. The cross-linking reaction was terminated by extensive washing with PBS followed by 5 min of incubation with PBS containing 150 mM glycine-HCl buffered at pH 7.5.

RESULTS

We have previously reported that the human SCF binds with high affinity to the human Kit receptor ($K_d \cong 0.5$ nM) but it does not detectably recognize murine Kit (21). By contrast, the affinity of the rat SCF is significantly higher to murine Kit ($K_d \cong 3$ nM) than to the human protein ($K_d \cong 50$ nM [21]). On the basis of this species-specific recognition of SCF, we envisioned that it would be possible to confine the binding site of the human SCF by replacing specific portions of human Kit with the corresponding portions of the murine receptor. In doing so, we were aided by the use of a battery of MAbs to human Kit (5) and ACK-2 MAb to the mouse receptor (24). All of the MAbs that we used are species specific. Moreover, MAbs K44 and K57 to human Kit, and the mouse-specific ACK-2 antibody, inhibit SCF binding and therefore allowed us to perform immunological mapping of the ligand binding site in parallel with direct analysis of SCF recognition.

The three N-terminal Ig-like domains of Kit contain the ligand binding site. To allow swapping of the N-terminal three Ig-like domains between the human and mouse Kit proteins, we introduced, through site-directed mutagenesis, a unique *Kpn*I site between the third and fourth Ig-like domains of both the murine and human c-*kit* cDNAs (see Materials and Methods). This resulted in a conservative replacement of a serine residue, in both receptors, with a threonine. Control experiments (not shown) demonstrated that the specific serine-for-threonine replacement in the wild-type mouse Kit (serine 301 [26]) or the human receptor (serine 298 [35]) had relatively small effects on the affinity to the rat or human SCF, respectively, and did not affect the tyrosine kinase activity of either receptor. The chimeric proteins are MH-123, a murine Kit protein in which the three N-terminal Ig-like domains were replaced by the corresponding domains of human Kit, and the reciprocal chimera HM-123 (Fig. 1A). The cDNA hybrids were inserted into the pLSV mammalian expression vector (20) and were transfected into NIH 3T3 mouse fibroblasts. Individual clones of cells that overexpressed the chimeric proteins were then selected and expanded. We first analyzed the interaction of the expressed receptors with species-specific MAbs by performing immunoprecipitation with these antibodies and Western blotting of the complexes by using a polyclonal antibody to the cytoplasmic domain of Kit. As shown in Fig. 2A, HM-123 was recognized by the mouse-specific and ligand-inhibitory MAb ACK-2 (24) and also by two human-specific MAbs that do not inhibit SCF binding (K94 [19] and K27 [5]). However, a human-specific MAb that inhibits ligand binding, K44, did not interact with the HM-123 hybrid receptor. By contrast, MH-123, which contains the first three Ig-like domains of the human receptor, was recognized by several human-specific antibodies, including the ligand-competitive MAbs K44 and K57 (Table 1). On the basis of these lines of immunological evidence, we concluded that the SCF binding sites of both the murine and human Kit proteins reside in the N-terminal three Ig-like domains.

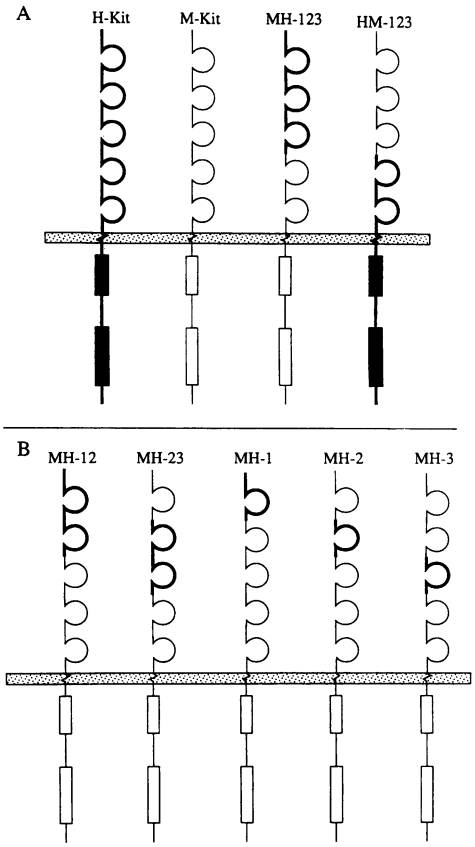

FIG. 1. Schematic presentation of the wild-type human and mouse Kit proteins and the chimeric receptors. The horizontal shaded bar represents the plasma membrane. The five Ig-like units at the extracellular domains are shown as loops, and the split tyrosine kinase sequences are indicated by boxes. The first letters in the names of chimeric receptors indicate the parental receptor (H, human; M, mouse), whereas the second letters and the numbers represent the source of the swapped Ig-like loops and their positions relative to the N terminus. Bold lines and filled boxes indicate sequences that were derived from human Kit.

Covalent cross-linking of SCF to the chimeric receptors confirmed the immunological results. Because recombinant

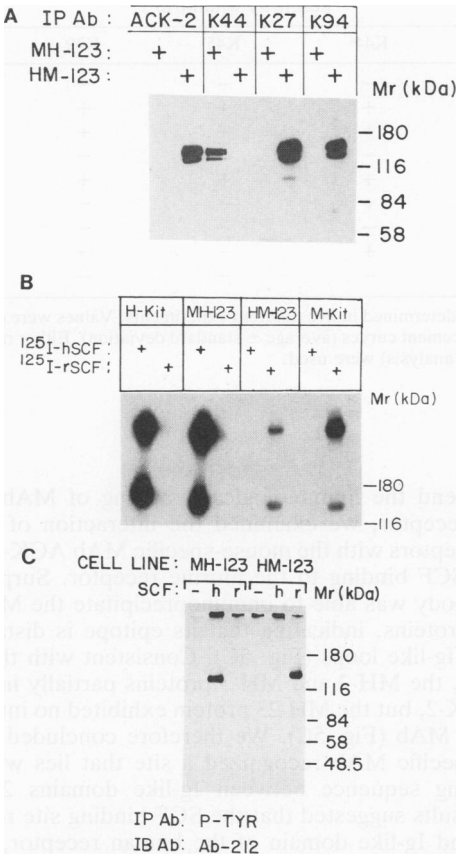

FIG. 2. Biochemical analyses of the chimeric receptors MH-123 and HM-123. (A) Immunoprecipitation with MAbs. Whole lysates were prepared from cell lines that overexpress either MH-123 or HM-123. The chimeric proteins were then immunoprecipitated (IP) by using the indicated MAbs (+), and the immune complexes were separated on a 7.5% acrylamide gel. After electrophoretic transfer to a nitrocellulose filter, Kit proteins were detected by blotting with Ab 212, a rabbit antibody to a synthetic peptide that corresponds to the C terminus of Kit. Detection was performed with horseradish peroxidase-labeled protein A and enhanced chemiluminescence (ECL; Amersham). An autoradiogram (3-min exposure) is shown along with the locations of molecular weight marker proteins. (B) Cross-linking of ^{125}I-SCF. Monolayers of cells that overexpress wild-type human Kit (H-Kit) or mouse Kit (M-Kit) or the indicated chimeras were incubated at 4°C with radiolabeled human SCF (hSCF; 20 ng/ml) or rat SCF (rSCF; 60 ng/ml). After 90 min of incubation, the BS^3 cross-linking reagent was added and incubation was continued for 30 min at 22°C. Cell lysates were then prepared, and the receptor-ligand complexes were immunoprecipitated with a rabbit antibody that recognized both the human and mouse receptors (Ab 212). The covalent complexes were resolved by SDS-PAGE (5.5% acrylamide) and detected by autoradiography (5 h). Note that both receptor monomers and dimers were detectable. The level of expression of HM-123 was lower than that of the other Kit proteins. (C) Ligand-induced autophosphorylation. Monolayers of cell lines overexpressing HM-123 or MH-123 were incubated for 10 min at 37°C with either 150 ng rat SCF per ml (r) or 50 ng of human SCF per ml (h) or were left untreated (−). Cell lysates were prepared and subjected to immunoprecipitation with a rabbit antibody to phosphotyrosine. Following SDS-PAGE of the washed immunocomplexes, the resolved proteins were transferred to a nitrocellulose filter that was immunoblotted with a rabbit antiserum to Kit (Ab 212) and horseradish peroxidase-labeled protein A.

mouse SCF was not available in a radiolabeled form, we used the rat ligand. Radiolabeled SCF of either human or rat origin was incubated at 4°C with cells that overexpress wild-type or mutant Kit proteins; this procedure was followed by covalent cross-linking with the BS^3 reagent and gel electrophoresis of the ligand-receptor complexes after immunoprecipitation (Fig. 2B). As we reported previously, this procedure results in intensive labeling of receptor dimers and some labeling of monomeric SCF-Kit complexes (21). Evidently radiolabeled human SCF underwent cross-linking to the human receptor and also to the MH-123 fusion protein but not to the wild-type murine Kit or to the HM-123 chimera. In comparison, radiolabeled rat SCF efficiently labeled the murine receptor but displayed faint or no cross-linking to the human receptor or to MH-123 (Fig. 2B). To test the validity of the ligand cross-linking assays and also extend them to signal transduction, we analyzed the ability of the human and rat SCF to induce tyrosine phosphorylation of the chimeric proteins. Cells that overexpress each hybrid receptor were incubated for 10 min at 37°C with either human SCF (50 ng/ml) or rat SCF (150 ng/ml). Following cell lysis, the receptors were immunoprecipitated with antibodies to phosphotyrosine, and the immune complexes were electrophoresed and blotted with a rabbit antiserum to the cytoplasmic portion of Kit (Fig. 2C). Whereas the tyrosine kinase of the HM-123 protein was stimulated by rat SCF, human SCF could not stimulate this kinase. However, the latter ligand efficiently activated the MH-123 receptor, indicating that this receptor has the same ligand specificity as does the wild-type human Kit. Taken together, results of the ligand cross-linking analysis (Fig. 2B) and the tyrosine kinase assay (Fig. 2C) implied that the N-terminal three Ig-like domains of Kit confer to the receptor species-specific ligand binding and consequently also kinase activation by the respective ligand.

The qualitative functional equivalence of MH-123 and HM-123 proteins to the human and mouse receptors, respectively, was quantitatively confirmed by analysis of ligand displacement (Fig. 3). In these assays, radiolabeled SCF was incubated, in the presence of increasing concentrations of unlabeled ligand, with cells that overexpress the various Kit proteins. Similar apparent affinities (K_d ~3 nM) were displayed by the wild-type mouse Kit and the HM-123 chimera when rat SCF was tested (Fig. 3B). The same ligand displayed an approximately 30-fold-lower affinity to either the human receptor or the MH-123 protein when its capacity to displace the radiolabeled human SCF was examined (Fig. 3C). Similarly, the apparent affinities of the wild-type human Kit and the MH-123 protein to human SCF were practically identical (Fig. 3A and Table 1). We therefore concluded that the three N-terminal Ig-like domains of the mouse and human Kit proteins contain all of the structural determinants needed for the species-specific binding of SCF.

Binding of SCF-competitive MAbs is conferred by pairs of Ig-like domains that include the second domain. To further confine the ligand binding site within the three N-terminal Ig-like domains, we focused on the binding of the human SCF and constructed two chimeric proteins that separately included consecutive pairs of these structural units (Fig. 1B). Both proteins were derived from the murine Kit, but they contained either the first and second human Ig-like domains (MH-12 protein) or the second and third human domains (MH-23 protein). A series of four MAbs to human Kit was then used to immunoprecipitate the fusion proteins from cells that were transfected with the respective cDNA molecules. As shown in Fig. 4, both ligand-competitive MAbs

TABLE 1. Summary of ligand affinities and MAb reactivities of different Kit proteins

Protein	Ligand affinity (K_d [nM])[a]		Reactivity with MAb[b]:				
	Human SCF	Rat SCF	ACK-2[c]	K44[c]	K45	K49	K57[c]
Mouse Kit		4.3 ± 1.8	+	−	−	−	−
Human Kit	0.55, 0.65 ± 0.28	Low	−	+	+	+	+
MH-123	0.55, 0.6 ± 0.2	Low	−	+	+	+	+
HM-123		3.6 ± 0.6	+	−	−	−	−
MH-12	1.2, 2.9 ± 0.8	5.9 ± 2	+	+	+	+	+
MH-23	0.3, 0.4 ± 0.1	Low	−	+	−	−	+
MH-1		3.5 ± 0.5	+	−	−	−	−
MH-2	0.5, 0.9 ± 0.3	4.5 ± 0.5	+/−	+	−	−	+
MH-3		Low	+/−	−	−	−	−

[a] Dissociation constants of human and rat SCF for the various chimeric proteins of Kit were determined in five different experiments. Values were determined either by Scatchard analysis (28) (first of each pair of values for human SCF) or by ligand displacement curves (average ± standard deviation). Either radiolabeled human SCF (Scatchard analysis) or pairs of radiolabeled and unlabeled ligands (displacement analysis) were used.
[b] Determined by an immunoprecipitation assay.
[c] Ligand-competitive antibody.

(K44 and K57) as well as antibodies that do not inhibit SCF binding (K45 and K49) reacted with the MH-12 protein. However, only the ligand-competitive MAbs recognized the MH-23 chimeric receptor (Fig. 4). It was therefore concluded that the epitopes of MAbs K45 and K49, which do not inhibit ligand binding, are localized to the most N-terminal Ig-like domain or to the region between domains 1 and 2. More importantly, the fact that the ligand-competitive MAbs K44 and K57 recognized MH-12 as well as MH-23 suggested that the SCF binding site is most likely confined to the second Ig-like domain.

The binding sites of human SCF and the murine ligand map immunologically to distinct domains. Although the results obtained with the various MAbs suggested that the determinants that confer species-specific binding of human SCF reside in the second Ig-like domain, interdomain packing and intramolecular interactions could contribute to precise definition of the binding cleft. To address these possibilities, we separately replaced each of the three N-terminal Ig-like domains of murine Kit with the corresponding portions of the human Kit/SCF receptor by using site-directed mutagenesis, thus introducing unique endonuclease restriction sites. Figure 1B illustrates the structures of the chimeric receptors MH-1, MH-2, and MH-3, which respectively contain the first, second, and third Ig-like domains of human Kit in the backbone of the mouse receptor. The results of immunoprecipitation of these recombinant proteins, as well as MH-12 and MH-23, from lysates of transfected cells are presented in Fig. 5A. Comparable expression levels were obtained with all cell lines that express the recombinant receptors, and they all displayed a characteristic doublet protein band corresponding to the precursor and mature Kit proteins. We next determined the interaction between the chimeric receptors and MAbs that compete with binding of the human SCF or the rodent factor (Table 1). As expected from the analysis of MH-12 and MH-23 (Fig. 4), only the ligand-inhibitory antibodies K44 and K57 reacted with MH-2 in an immunoprecipitation assay (Fig. 5B). These antibodies, like MAbs K45 and K49, did not recognize the MH-1 chimera. Similarly, none of our human-specific MAbs reacted with the MH-3 protein (Table 1), indicating that domain 2 and the region between domains 1 and 2 carry the epitopes of MAbs K44, K45, K49, and K57 and therefore may be particularly immunogenic. Together, these results confirmed that the primary site that confers ligand specificity to the human receptor resides in the second Ig-like domain.

To extend the immunological mapping of MAbs to the murine receptor, we examined the interaction of the chimeric receptors with the mouse-specific MAb ACK-2, which inhibits SCF binding to the murine receptor. Surprisingly, this antibody was able to immunoprecipitate the MH-1 and MH-12 proteins, indicating that its epitope is distal to the first two Ig-like loops (Fig. 5C). Consistent with this interpretation, the MH-2 and MH-3 proteins partially interacted with ACK-2, but the MH-23 protein exhibited no interaction with this MAb (Fig. 5C). We therefore concluded that the mouse-specific MAb recognized a site that lies within the intervening sequence between Ig-like domains 2 and 3. These results suggested that the SCF binding site resides in the second Ig-like domain of the human receptor, but the corresponding site of the rodent Kit may flank this domain at its carboxy-terminal side. However, considering the relative size of the ligand-competitory MAb and the SCF binding site, it is possible that the inhibitory effect of ACK-2 is due to steric hindrance.

SCF binding analysis reveals complex interdomain interactions. Direct binding of radiolabeled SCF to the various chimeric proteins was assayed by using ligand displacement and Scatchard analyses; the results are summarized in Table 1. Competition between unlabeled human SCF and the radiolabeled ligand showed that MH-23 and the wild-type human Kit displayed practically indistinguishable displacement curves (Fig. 6). An approximately twofold reduction in affinity was observed with the MH-2 protein, and the MH-12 chimera exhibited somewhat lower affinity to human SCF (Fig. 6). As predicted, both MH-1 and MH-3 recombinant receptors exhibited no detectable specific binding of the human SCF (data not shown). Saturation curves and Scatchard analysis of ligand binding (Fig. 7) almost precisely confirmed the binding constants and established the relative order of affinities of the recombinant receptors toward SCF. Furthermore, this analysis indicated the existence of apparent homogeneous populations of binding sites with all of the proteins that were examined (Fig. 7). In summary, all of the chimeric mouse proteins that contained the second human Ig-like domain displayed a remarkable increase (400- to 800-fold) in affinity to human SCF (Table 1). Although our results indicated that domain 2 provides the core of the SCF specificity determinants, the observed differences in ligand affinities appear to involve other domains in ligand recognition. Thus, whereas the third Ig-like unit increased the strength of ligand-receptor interactions, the most N-terminal

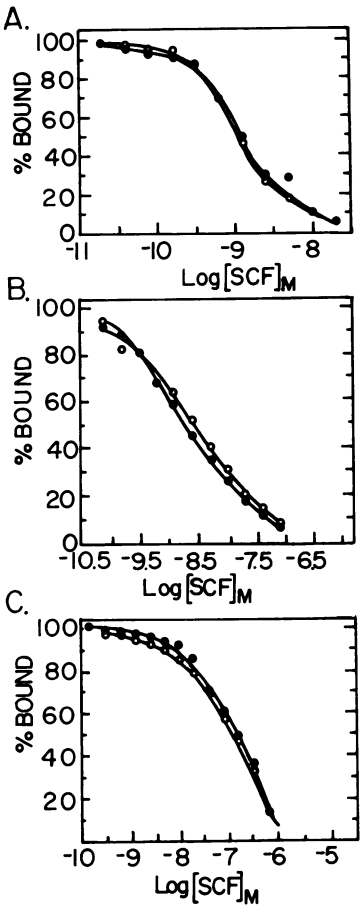

FIG. 3. Ligand displacement analyses of wild-type and chimeric Kit receptors. Monolayers (4×10^5 cells) were prepared from cell lines that overexpress wild-type or mutant proteins. Radiolabeled SCF (2 ng of human SCF per ml or 5 ng of rat SCF per ml) was incubated with the cells at 22°C in the presence of increasing concentrations of unlabeled SCF; 90 min later, the cell-associated radioactivity was determined in a gamma counter and expressed as the percentage of ligand binding in the absence of unlabeled competitor. (A) Binding of radiolabeled human SCF to wild-type human Kit (open circles) or to MH-123 (closed circles) in the presence of unlabeled human SCF; (B) binding of radiolabeled rat SCF to wild-type mouse Kit (open circles) or to HM-123 (closed circles) in the presence of unlabeled rat SCF; (C) binding of radiolabeled human SCF to wild-type human Kit (open circles) or to MH-123 (closed circles) in the presence of unlabeled rat SCF. The experiments were repeated three times with essentially the same results. Each data point represents the average of a duplicate determination (variance, <10%).

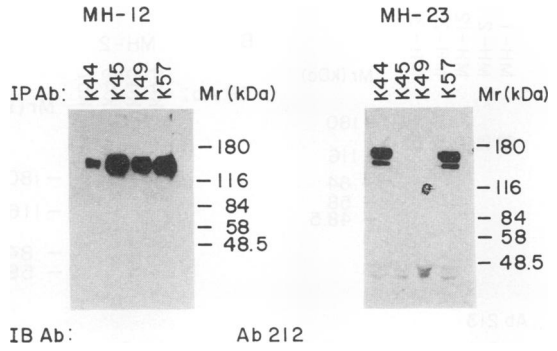

FIG. 4. Recognition of chimeric proteins MH-12 and MH-23 by MAbs to human Kit. Whole cell lysates were prepared from cells that overexpress the chimeric protein MH-12 or MH-23. Kit proteins were immunoprecipitated (IP) from the lysates by using the indicated four MAbs to human Kit. All of the MAbs are human specific, and they were used at 20 μg/ml. The immune complexes were resolved by gel electrophoresis and Western blotting. The immunoblotting antibody was a rabbit antiserum (Ab 212) that recognizes both the human and mouse Kit proteins. The enhanced chemiluminescence signals that were obtained with horseradish peroxidase-labeled protein A are shown (2-min exposure) along with the locations of molecular weight marker proteins.

Ig-like domain apparently imposed a small negative effect on binding of the human SCF.

A chimeric mouse Kit that contains the second human Ig-like domain is a dual-specificity receptor. The observation that the chimeric receptor MH-12 was recognized by the mouse-specific and ligand-competitive MAb ACK-2 (Fig. 5C), as well as by the human-specific antagonistic MAbs K44 and K57 (Fig. 4), led to the prediction that the specificity-determining sites of the human and the mouse Kit molecules are not structurally coincidental. By extension, this could confer to a single chimeric protein dual ligand specificity. To directly test this possibility, we examined the ability of native rat SCF to displace the radiolabeled rat ligand from the various chimeric proteins, including MH-12 and MH-2, that we suspected to function as dual-specificity receptors. The results of this analysis are summarized in Table 1. The second human Ig-like domain, when introduced into the mouse receptor, appeared to confer high-affinity binding of the human SCF (Fig. 6 and 7) without reducing the original affinity of the murine receptor to the rat SCF (Table 1). By contrast, mouse chimeric proteins MH-3 and MH-23, which contained the third human Ig-like domain, practically lost their high affinity to rat SCF (Table 1). We therefore concluded that the specificity for human SCF is associated with the second Ig-like domain, whereas the third murine domain is apparently essential for recognition of the rodent ligand. This conclusion is best exemplified by the fact that two chimeric receptors, MH-2 and MH-12, are dual-specificity receptors; that is, they recognized both the human and rat ligands with affinities that were comparable with those of the parental wild-type Kit proteins (Table 1). Although this interpretation is consistent with the localization of the epitopes of the ligand-competitive MAbs (Fig. 5), according to an alternative interpretation, the third human domain exerts a negative effect on binding of the rat SCF to mouse domain 2.

We next examined the extent of structural overlap between the binding sites of the mouse and human ligands by testing the ability of one of the dual-specificity receptors, MH-12, to simultaneously bind a radiolabeled ligand and a MAb that is inhibitory for binding of the other ligand. Covalent cross-linking of radiolabeled human SCF to MH-12 was found to abolish the interaction between this receptor and the mouse-specific MAb ACK-2 (Fig. 8). Likewise, MH-12, which became covalently cross-linked to rat SCF, was not recognized by the human-specific MAb K44 (Fig. 8). We therefore concluded that despite the dual ligand specificity of MH-12, the two binding sites are probably too close to one another to allow independent accessibility of a ligand and an antagonistic MAb that are directed to distinct sites. This possibility was supported by competition analyses; antibody K44 not only completely inhibited binding of the

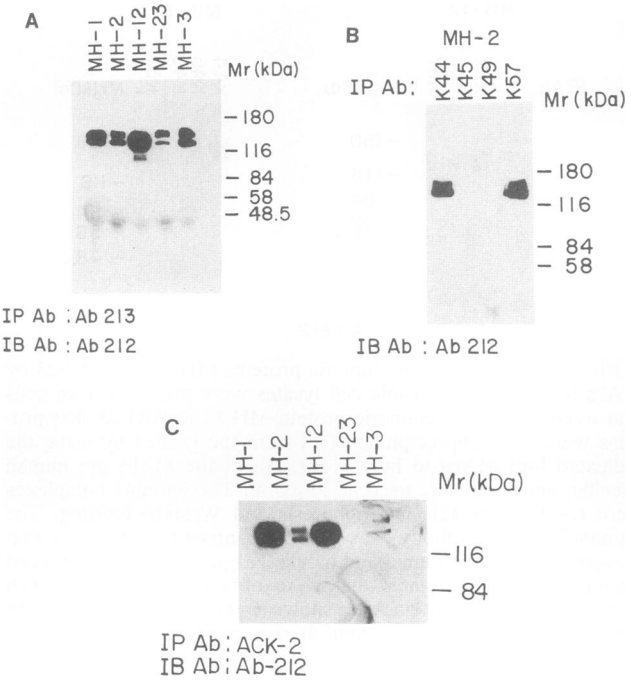

FIG. 5. Antibody reactivities of mouse Kit proteins that contain individual human domains. (A) Expression of chimeric proteins. Cell lines that express the indicated chimeric proteins were established and selected for receptor overexpression. Whole cell lysates were prepared from 5×10^6 cells of each cell line and subjected to immunoprecipitation (IP) with a rabbit antiserum to a synthetic peptide from the cytoplasmic domain of Kit (Ab 213). The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting (IB) with a second rabbit antiserum that is specific to the most C-terminal peptide of Kit (Ab 212). An autoradiogram of enhanced chemiluminescence signals is shown along with the locations of marker proteins. (B) Reactivities of the MH-2 chimera with MAbs. Whole cell lysates were prepared from 10^6 cells that overexpress MH-2. The lysates were subjected to immunoprecipitation (IP) with the indicated human-specific mAbs. The immunocomplexes were resolved by gel electrophoresis and analyzed by immunoblotting (IB) with a rabbit antiserum to Kit (Ab 212) as described in the legend to Fig. 2A. (C) Reactivities of the chimeric receptors with a mouse-specific mAb. Whole lysates from 10^6 cells that overexpress the indicated chimeras were subjected to immunoprecipitation (IP) with the mouse-specific MAb ACK-2, which competes with SCF for binding to Kit. The immunocomplexes were analyzed by immunoblotting (IB) with a polyclonal antibody (Ab 212) to Kit exactly as for panel B.

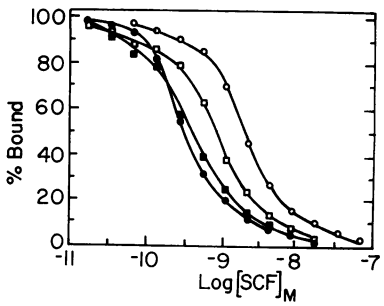

FIG. 6. Ligand displacement analyses of chimeric receptors. Monolayers of cells that overexpress wild-type Kit or various chimeras were incubated at 4°C with 2 ng of ^{125}I-labeled human SCF per ml in the presence of increasing concentrations of the unlabeled human ligand. Cell-associated radioactivity was determined 90 min later as detailed in Materials and Methods and expressed as percentage of the amount of radioactivity that was bound in the absence of unlabeled competitor. The following receptors were analyzed: wild-type human Kit (closed squares), MH-2 (open squares), MH-12 (open circles), and MH-23 (closed circles). Each point is the average of a duplicate determination, and the difference between determinations did not exceed 10%. The results given are representative of three independent experiments.

radiolabeled human SCF to the MH-12 molecules but also inhibited 95% of the binding of the rat SCF to the same receptor. Likewise, the mouse-specific antibody ACK-2 had no effect on binding of the human SCF to the human receptor, but it inhibited 93 and 91%, respectively, of the binding of the rat SCF and the human ligand to MH-12 (data not shown).

DISCUSSION

The aim of this study was to molecularly confine the ligand binding site of the Kit/SCF receptor. To this end, we used the differences in cross-species ligand binding of the human and rodent SCF molecules. Using a panel of human-mouse hybrids of Kit and ligand-competitive MAbs, we concluded that the three N-terminal Ig-like units of Kit define the binding specificity. We further determined that the second domain of the human receptor is the primary determinant of specificity to the human ligand but the adjacent Ig-like units are also involved in ligand recognition. It appears, however, that the third, rather than the second, Ig-like domain of the mouse receptor provides the major contribution to ligand specificity. These two features of ligand binding to Kit, namely, the involvement of noncontiguous segments of the receptor and the apparent lack of structural coincidence of the specificity-determining sites of the human and rodent ligands, are schematically illustrated in a model shown in Fig. 9 and further discussed below. Perhaps the most unexpected observation of this study is the identification of two chimeric proteins, MH-12 and MH-2, as dual-specificity receptors and, on the other hand, the fact that neither the human nor the rodent ligand binds with high affinity to another receptor, MH-3 (Table 1). In analogy with the receptors for PDGFs, which appear to share ligand binding features with Kit, the α receptor, like MH-12, is a dual-specificity receptor for both PDGF-BB and PDGF-AA, whereas the β type of receptor is similar to MH-23 or wild-type Kit in that it binds only one ligand, PDGF-BB (12).

Noncontiguous regions of the extracellular domain define ligand specificity. The three N-terminal Ig-like domains of Kit appear to determine ligand specificity, as revealed by domain swapping experiments (Fig. 2 and 3). Moreover, this portion of Kit is sufficient to confer high-affinity ligand binding to a soluble receptor that lacks the fourth and fifth domains (5). However, within the ligand binding portion, each Ig-like domain appears to play a distinct role in SCF recognition. Thus, the second human domain is the major specificity determinant, as its presence in the mouse receptor is sufficient to confer high-affinity binding of the human SCF to the MH-2 chimeric receptor (Fig. 6 and Table 1). In addition, human-specific MAbs that inhibit SCF binding were found to be directed against this domain (Fig. 5B). On the other hand, the third human Ig-like domain appears to exert an additive effect on the recognition of the human ligand. This conclusion was inferred from the higher ligand affinity of MH-23 than of MH-2 (Table 1). We calculated that

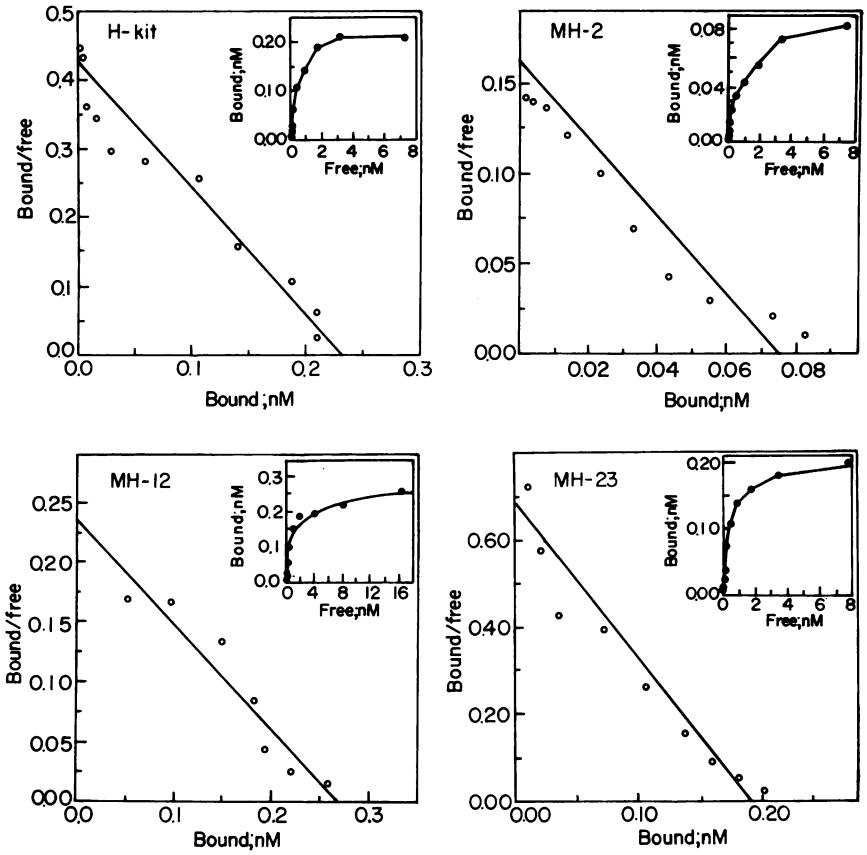

FIG. 7. Scatchard analysis of the binding of human SCF to wild-type and chimeric Kit proteins. Increasing concentrations of radiolabeled human SCF were incubated at 4°C with monolayers of 4×10^5 cells that overexpress wild-type human Kit (H-Kit) or the indicated chimeric receptors. Cell-bound radioactivity was determined following 90 min of incubation and extensive washing on ice. Nonspecific binding was determined in parallel binding experiments that were performed with parental untransfected NIH 3T3 cells and was subtracted from the total amount of cell-bound radioactivity. The binding data were analyzed according to Scatchard (28). The insets show the data in the form of saturation curves. Each point is the average of a duplicate determination, and the experiment was repeated three times with essentially the same results.

the contribution of this domain is 0.30 kcal (1 kcal = 4.184 kJ/mol), a small effect in comparison with the free energy of binding of human SCF to its wild-type receptor, which is 12.27 kcal/mol. Contrary to the positive effect of domain 3, grafting the most N-terminal human domain into the mouse receptor introduced a negative effect on SCF binding (compare the affinities to human SCF of MH-2 and MH-12; Table 1). Energetically, domain 1 reduced the stability of ligand-receptor complexes by 0.5 to 1.2 kcal/mol. Consistent with these relatively small effects on the free energy of ligand binding, the introduction of neither human domain 1 nor domain 3 individually was sufficient to confer measurable binding of human SCF to the chimeric proteins MH-1 and MH-3 (Table 1). Obviously, our biochemical approach cannot determine whether or not the effects of domains 1 and 3 are mediated by direct contact with the ligand or whether they reflect the existence of molecular constraints that indirectly determine the conformation of the ligand binding cleft. However, the identification of domain 2 of Kit as the primary determinant of binding of human SCF is consistent with its being highly immunogenic (Table 1 and Fig. 9). This finding is also reminiscent of the apparent localization of the binding site of PDGF-AA, by means of a deletion mutant, to the second domain of the α-PDGF receptor (11). The corresponding Ig-like unit of Kit belongs to the C2 type of

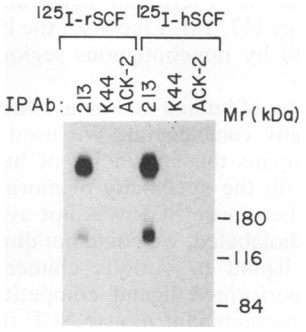

FIG. 8. Antibody recognition of covalent complexes of MH-12 and ^{125}I-SCF. Radiolabeled human or rat SCF was cross-linked to cells that overexpress the MH-12 protein essentially as described in the legend to Fig. 2B. Cell lysates were then prepared, and the radioactive ligand-receptor complexes were subjected to immunoprecipitation (IP) with either a polyclonal rabbit antibody that is directed against the kinase insert of Kit (Ab 213) or two different MAbs that compete with SCF for binding to the receptor (human-specific MAb K44 and the mouse-specific MAb ACK-2). The extensively washed immunocomplexes were resolved by gel electrophoresis (5.5% acrylamide). An autoradiogram (12-h exposure) is shown along with the locations of molecular weight marker proteins.

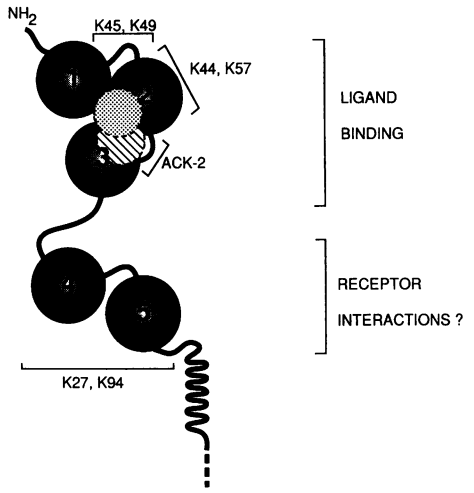

FIG. 9. Functional model of the extracellular domain of Kit/SCF receptor. The Ig-like domains of Kit are represented by circles and labeled 1 through 5 starting at the amino terminus (NH_2). The transmembrane domain is shown by a zig-zag line. The model schematically presents the quantitative data summarized in Table 1. It assumes that the ligand binding site is confined to the three N-terminal Ig-like domains. An epitope map of the ligand-inhibitory MAbs K44 and K57 (for human Kit) and ACK-2 (for mouse Kit) is also shown. The relative contribution of each domain to the species specificity of SCF recognition is shown by a stippled circle (human SCF) and a hatched circle (rat SCF). Note that these domains are not structurally coincidental.

Ig-related sequences (33), but it displays unique structural features that mark it as a nonclassical Ig-like loop. These features include the presence of two additional cysteine residues within the loop and the absence of several canonical residues of the Ig fold.

Our finding that noncontiguous receptor regions stabilize the SCF-Kit complexes are analogous to observations that were made with other growth factor receptors. For example, the binding of epidermal growth factor to its receptor involves mostly a sequence that connects the two cysteine-rich domains, but the N-terminal region also contributes to the binding energy (17, 18). Likewise, the binding pocket of insulin is defined by noncontiguous regions of the insulin receptor (10, 29).

The binding sites of human and mouse Kit overlap but may not be structurally coincidental. We used the rat SCF to structurally compare the specificity of human Kit to the human ligand with the specificity of murine Kit to the rat SCF. Because the mouse SCF was not available in a form that could be radiolabeled, we could not directly test binding of the murine ligand to various chimeric Kit proteins. However, we performed ligand competition experiments with unlabeled recombinant mouse SCF (from Genezyme, Cambridge, Mass.) that displaced radiolabeled rat SCF. In these experiments, the murine SCF displayed two- to five-fold-higher affinity than did rat SCF to the wild-type murine Kit, and the affinities of the mouse SCF were not significantly lower to the chimeric proteins MH-12 and to MH-2 than to the wild-type mouse Kit. It was therefore assumed that the data obtained with radiolabeled rat SCF reliably represent also the binding properties of the murine ligand. Although the N-terminal half of each receptor contains the sequences that discriminate between the two ligands (Fig. 2B and Table 1), the relative contribution of each Ig-like domain to SCF recognition differed between the two receptors. In contrast with the limited effect of human domain 3 on the binding of the human SCF, the corresponding segment of murine Kit was indispensable for high-affinity binding of rat SCF, as indicated by the lower affinity of MH-3 than of MH-12 to rat SCF (Table 1). Consistent with the primary determinant of rat SCF recognition being mouse domain 3, the epitope of a MAb that can displace SCF from the mouse receptor was mapped, at least in part, to this Ig-like domain (Fig. 5C). Moreover, the MH-2 and MH-12 chimeric receptors that apparently contain the major sites for both the human ligand (domain 2) and the murine SCF (domain 3) indeed displayed high affinity to both ligands (Table 1). On the other hand, MH3, a chimeric receptor that includes neither human domain 2 nor mouse domain 3, bound neither ligand. Nevertheless, our data cannot exclude an alternative model in which domain 3 exerts an inhibitory effect on binding of the rat SCF. According to this possibility, both the human and the rat SCF bind to the second Ig-like domain but contact also domain 3. Reciprocal chimeric proteins, such as HM-12 and HM-2, may enable determination of whether human domain 3 is neutral or negative with respect to binding of rat SCF. Independent of the involvement of domain 3 in binding of the rat SCF, the second domain appears to participate also in recognition of the rodent ligand. This conclusion is based on the ability of rat SCF to displace a radiolabeled human SCF from all of the chimeric proteins, albeit with different potencies (data not shown). In addition, despite the fact that MH-12 can bind either the human or rat SCF and also human- and mouse-specific antagonistic MAbs, this chimeric receptor was not simultaneously recognized by a ligand from one species (e.g., human) and an antagonist MAb that is specific to the other species (e.g., mouse) (Fig. 8). We therefore concluded that the binding sites of the rodent and human SCF overlap to some extent (Fig. 9). This feature of the Kit/SCF receptor is analogous to other receptor tyrosine kinases. For instance, the Bek/FGFR2 receptor for fibroblast growth factors (FGFs) binds acidic as well as basic FGF apparently through different portions of its ectodomain, which contains three Ig-like units (23, 31, 37). Similarly, distinct portions of the closely related receptors for insulin and insulin-like growth factor 1 mediate interaction with the corresponding ligands (16, 29). Lastly, the binding sites of PDGF-AA and PDGF-BB on the α-PDGF receptor are not structurally coincidental (11). The studies that we described represent the first attempt to systematically address the structure-function relationships within the five-lobe structure of the ectodomain of Kit. These studies leave many open questions that involve the exact packing of the subdomains and their interactions. In addition, our results underscore the yet unknown function of the fourth and fifth Ig-like domains of Kit. These questions, once solved, may be broadly relevant to the many other growth factor receptors of the Ig superfamily.

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