Soluble c-Kit Proteins and Antireceptor Monoclonal Antibodies Confinet the Binding Site of the Stem Cell Factor*

(Received for publication, September 11, 1992)

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The binding of the stem cell factor (SCF) to the c-kit-encoded receptor tyrosine kinase stimulates a variety of biochemical responses that culminate in cellular proliferation, migration, or survival. The extracellular domain of p145<sup>Kit</sup> consists of five immunoglobulin-like domains. To confine the ligand binding site to this portion of the receptor we generated a panel of murine monoclonal antibodies (mAbs) to the Kit protein and identified two mAbs that efficiently displaced receptor-bound SCF and also inhibited proliferation of SCF-dependent human megakaryocytes. To map the epitopes of these mAbs we constructed and expressed soluble portions of the extracellular domain of Kit, which included either the two amino-terminal Ig-like domains (denoted Kit 1-2), three Ig-like domains (Kit 1-2-3), or the entire extracellular portion (Kit-X). All three recombinant proteins were recognized by the ligand inhibitory mAbs, suggesting that the SCF binding site resides in the amino-terminal half of the ectodomain. Consistent with this conclusion, all of the soluble proteins inhibited SCF binding to Kit-expressing cells, and they also underwent specific covalent cross-linking to the radiolabeled ligand. However, whereas Kit 1-2-3 and Kit-X displayed comparable ligand affinities, deletion of the third Ig-like domain, in Kit 1-2, involved significant reduction in SCF binding. Hence, the binding site of SCF probably includes Ig-like domains 1 and 2, but structural determinants distal to this portion may also participate in ligand recognition.

Mice with mutations in the Steel (Sl) locus on chromosome 10 exhibit a complex phenotype that includes defects in melanogenesis (white hair coat), gametogenesis (sterility), melanogenesis (macrocytic anemia) (Bennett, 1956; McCulloh et al., 1964, 1965; Mintz and Russell, 1979; Silvers, 1979; Russell, 1979). SI alleles contain deletions in a gene that encodes a transmembrane protein which functions as a precursor for a peptide growth factor (Zsebo et al., 1990a; Huang et al., 1990; Copeland et al., 1990). The soluble growth factor was identified independently on the basis of its ability to promote formation of colonies from early hematopoietic progenitors (Zsebo et al., 1990a), its differential proliferative effect on normal mast cells, but not mast cells derived from W mutant mice (Nocka et al., 1990), and by its action on MC-6 mast cells (Williams et al., 1990). This cytokine was respectively named stem cell factor (SCF), c-kit ligand, and mast cell growth factor. Besides its essential in vivo role in hematopoiesis, melanogenesis, and gametogenesis, in vitro SCF acts synergistically with other growth factors including erythropoietin, granulocytemacrophage colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukins (ILs) 3 and 6 (reviewed in Bennett, 1991). SCF exists both as a cell surface form and as a soluble form that corresponds to 164 amino-terminal residues of the long protein. In addition, two alternatively spliced SCF mRNAs encode variant transmembrane proteins that differ in the rate of processing into the soluble form (Flanagan et al., 1991).

All of the biological effects of SCF are initiated by its binding to a cell surface receptor that is encoded by the c-kit proto-oncogene (Yarden et al., 1987; Qiu et al., 1988). The mouse kit gene is allelic with the white spotting (W) locus on chromosome 5 (Chabot et al., 1988; Geissler et al., 1988). Early observations indicated that W mutants are phenotypically indistinguishable from Sl mutant mice and therefore predicted that these two loci interact on the same functional pathway (Bennett, 1956; Russell, 1979). This prediction has been confirmed recently through the identification of the Sl and W gene products as a ligand (SCF) and its cognate receptor (Kt), respectively. High affinity binding of noncovalently held dimers of SCF to p145<sup>Kit</sup> protein rapidly induces dimerization of the receptor (Blume-Jensen et al., 1981; Lev et al., 1992a). This stimulates the intrinsic cytoplasm-facing catalytic domain of Kit that functions as a tyrosine-specific protein kinase. The next step in the signaling pathway includes tyrosine phosphorylation and physical association with a distinct set of proteins that generate intracellular second messengers (Lev et al., 1991; Rottapel et al., 1991; Funasaki et al., 1992). The interactions of the ligand-stimulated Kit with cellular substrates is mediated, at least in part, by the hydrophilic kinase insert domain that lies within the catalytic core and strongly couples to proteins that contain src homology domains (Lev et al., 1992b; Rottapel et al., 1991).

The Kit protein belongs, both structurally and functionally, to the stem cell factor (SCF), colony stimulating factor; CSF, colony stimulating factor; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; PDGF, platelet-derived growth factor; mAb, monoclonal antibody; Kit-X, a recombinant extracellular domain of Kit/SCF receptor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EGF, epidermal growth factor.

*The abbreviations used are: SCF, stem cell factor; CSF, colony-stimulating factor; G-CSF, granulocyte CSF; GM-CSF, granulocyte-macrophage CSF; IL, interleukin; PDGF, platelet-derived growth factor; mAb, monoclonal antibody; Kit-X, a recombinant extracellular domain of Kit/SCF receptor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EGF, epidermal growth factor.

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to a subgroup of receptor tyrosine kinases which includes the receptors for the platelet-derived growth factors (PDGFs), colony-stimulating factor 1, and a few receptors for still unknown ligands. These type III receptor tyrosine kinases (Yarden and Ullrich, 1988) are characterized by extracellular ligand binding portions that contain five immunoglobulin (Ig)-like domains (Williams and Barclay, 1988; Hunkapiller and Hood, 1989). In addition, the ectodomains of these receptors share many common short stretches of amino acids that are distributed throughout the whole length of the extracellular portions (Yarden et al., 1987). This raises the question of where among the extracellular structures the differential ligand binding specificities reside. One possibility is that a specific Ig-like domain, or a specific interdomain stretch, functions as a ligand binding site. The Ig homology unit is characterized by a primary sequence of 70–100 residues in length, with an essentially invariant disulfide bridge spanning 40–60 amino acids. Several other relatively conserved residues are involved in establishing a tertiary structure referred to as an antibody fold (Amzel and Poljak, 1979). This structure is shared by all members of the Ig supergene family, which includes molecules with immunological functions, and proteins with no known immune function, such as cell adhesion molecules and components of growth factors (Williams and Barclay, 1988; Hunkapiller and Hood, 1989).

Kit, like other nonimmunological members of the family, contains Ig homology units that correspond to the C2 type or the V type of Ig-like domains.

It is presently unclear how the compact and globular structures of the Ig homology units of receptor tyrosine kinases participate in the establishment of ligand binding. However, soluble recombinant portions of the PDGF receptor (Duan et al., 1991) and Kit/SCF receptor (Lev et al., 1992c) fully retain high affinity ligand binding and also undergo ligand-induced dimerization. These observations indicated that all of the structural determinants of ligand binding are confined to the extracellular portions of these receptors. To confine the ligand binding cleft of Kit further we generated ligand-competitive monomonalic antibodies (mAbs) and recombinant soluble portions of the ectodomain. By using these reagents we obtained results that provide strong evidence that the SCF binding site is confined to the amino-terminal portion of Kit.

**EXPERIMENTAL PROCEDURES**

**Materials**—Radioactive materials were purchased from Amersham Corp. Protein A coupled to Sepharose was from Pharmacia LKB Biotechnology Inc. or prepared in our laboratory. Recombinant human SCF and transforming growth factor-α (TGF-α) were from Biotechnology Inc. and prepared in our laboratory. Recombinant human GM-CSF was a gift from Sandoz (Basel, Switzerland). Molecular weight standards were from Pharmacia-LKB Biotechnology Inc. or prepared in our laboratory. Recombinant human GM-CSF was also purchased from Amgen (Thousand Oaks, CA). Recombinant human IL-3 was a gift from Genetic Institute (Cambridge, MA), and recombinant human GM-CSF was a gift from Sandoz (Basel, Switzerland). Molecular weight standards for gel electrophoresis were obtained from Bio-Rad. Calf serum was from HyClone Laboratories (Logan, UT). Rabbit antibodies to mouse Ig's were from Jackson ImmunoResearch. Unless otherwise indicated, all other chemicals were from Sigma. Polyclonal antibodies to human Kit/SCF receptor were raised in rabbits that were injected with a plasmid (Lev et al., 1992a) fully retain high affinity ligand binding and also undergo ligand-induced dimerization. These observations indicated that all of the structural determinants of ligand binding are confined to the extracellular portions of these receptors. To confine the ligand binding cleft of Kit further we generated ligand-competitive monoclonal antibodies (mAbs) and recombinant soluble portions of the ectodomain. By using these reagents we obtained results that provide strong evidence that the SCF binding site is confined to the amino-terminal portion of Kit.

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Radiolabeling of Proteins—Affinity-purified rabbit antibodies to mouse F(ab)′2 were labeled with 125I by using the chloramine-T method (Hunter and Greenwood, 1962). SCF was labeled by using IODO-GEN (Pierce Chemical Co.) as follows. IODO-GEN-coated tubes (2 pg/50 μl of chloroform) were incubated with SCF (8 ng, and NaCl at 22 °C for 16 h in the same medium supplemented with 10% dialyzed calf serum and 50 μCi of [125I]methionine/ml. The cell supernatants were spun for 10 min at 4 °C in an Eppendorf centrifuge and subjected to immunoprecipitation. Protein A-conjugated Sepharose beads (3 mg/sample) were suspended in HNTG buffer and incubated for 30 min at 22 °C with either a polyclonal rabbit antisera to Kit-X (10 μl) or with rabbit anti-mouse F(ab)′2 (5 μg). The latter was followed, after a brief wash, by incubation with mAbs (10 μg). The antibody-conjugated beads were washed three times with HNTG and incubated with the cell supernatants for 2 h at 4 °C. The immune complexes were washed twice with each of the high, medium, and low salt buffers. Then gel sample buffer was added to the washed immunoprecipitates, and they were heated at 95 °C (5 min) and resolved by SDS-PAGE and autoradiography. In the case of 125I-labeled samples or samples that were not radioactively labeled, the immunoprecipitates were washed three times with HNTG.

Western Blotting—Washed immunoprecipitates were mixed with SDS gel sample buffer and subjected to SDS-PAGE. The gel-separated proteins were transferred electrophoretically onto nitrocellulose filters. Filters were first saturated for 1 h at 22 °C with blocking solution (10% low fat milk in 20 mM Tris-HCl (pH 7.6) and 17 mM NaCl). Antisera were then added in the same solution, and the incubation was carried out for 1 h. For detection the filters were washed three times (5 min each wash) with PBS, 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 above. The filters were reacted for 1 min with a chemiluminescence reagent (ECL, Amersham) and exposed to an autoradiography film for 1–15 min.

Binding Assays of Radiolabeled Ligands—The binding buffer containing Dulbecco’s modified Eagle medium supplemented with 0.1% bovine serum albumin and 20 mM HEPES-buffered at pH 7.5. The assays were carried out in 24-well dishes, and the monolayers were equilibrated in binding buffer 15 min prior to adding the radiolabeled ligand. For the determination of antibody binding the monolayers were incubated at 22 °C for 90 min with hybridoma supernatants or with various concentrations of the polyclonal mAbs. Following the brief wash with PBS, 0.1% bovine serum albumin, [125I]-labeled rabbit antimouse F(ab)′2 (106 cpm/well) was added, and the incubation was continued for an additional 90 min. Cell-bound radioactivity was determined by solubilization and harvest in 0.2 M NaOH and 0.1% SDS. For the determination of 125I-SCF binding cell monolayers were incubated at 4 °C with [125I]-labeled SCF for 2 h in the presence of various concentrations of unlabeled SCF. The amount of nonspecific binding was determined by performing the assay in the presence of a 100-fold excess of unlabeled SCF. At the end of the binding reaction the cells were washed, and their bound radioactivity was determined as described above. Ligand displacement analyses were performed in a single assay except that incubated with cellular monolayers for 4 h at 4 °C in the presence of increasing concentrations of soluble Kit proteins.

Covalent Cross-linking of Radiolabeled SCF—Affinity-purified recombinant proteins (Kit-X, Kit 1-2-3, Kit 1-2, and Neu-X) were incubated in PBS, 0.1% bovine serum albumin with [125I]-labeled SCF (100 ng/ml, 106 cpm/ng) in the presence of various concentrations of unlabeled SCF or transforming growth factor-α. The total reaction volume was 0.06 ml. After 4 h at 22 °C the proteins were subjected to covalent cross-linking by adding diisuccinimidyl suberate to a 0.5 mM final concentration, and the reaction was allowed to continue for 40 min at 22 °C. Gel sample buffer was then added, and the reaction was terminated by boiling and SDS-PAGE. For cross-linking of SCF to c-kit-overexpressing cells, the cells were incubated with 2 μg/ml of scFv antibody for 2 h at 4 °C, washed, and the ligand-receptor complexes were covalently cross-linked by adding EDAC (15 mM) in PBS and further incubation for 40 min at 22 °C. The cells were then lysed in solubilization buffer, and the Kit receptor was immunoprecipitated as described above.

RESULTS

Generation of Ligand-Competitive Monoclonal Antibodies to the Kit/SCF Receptor—In an attempt to determine the contribution of specific regions of the extracellular domain of Kit to the binding of SCF, we generated a panel of site-specific mouse mAbs to Kit by immunizing mice with a recombinant whole ectodomain of human Kit (Kit-X; Lev et al., 1992c). The capacity of selected hybridoma clones to recognize specifically human Kit was tested by immunoprecipitation of unlabeled Kit-X and Western blotting with rabbit polyclonal antibodies (Fig. 1). The specificity of this blotting assay was indicated by the lack of signal in immunoprecipitates of the extracellular portion of p185(Kit) (denoted Neu-X), which was used as a control antigen. To confirm the specificity of the mAbs to p145(Kit), they were assayed for binding to c-kit-transfected CHO cells (cell line T-18). All of the antibodies that immunoprecipitated Kit-X also recognized T-18 cells but did not react with the parental untransfected CHO cells (data not shown). The results of the binding of two mAbs, K44 and K45, to c-kit-overexpressing cells are depicted in Fig. 2A. Evidently both antibodies displayed saturable surface binding.

To test the possibility that some of the mAbs that we generated are directed to the ligand recognition site of Kit, the binding of 125I-SCF to T-18 cells was determined in the presence or absence of each mAb. Fig. 2B depicts the results that were obtained with mAbs K44 and K45. In contrast to the K45 antibody, which did not affect binding of 125I-SCF even at high antibody concentrations, mAb K44 stoichiometrically inhibited ligand binding at an approximately 1:1 ratio of antibody to SCF. Only one other antibody, K57, similarly affected the binding of SCF (data not shown), implying that both mAbs K44 and K57 are directed to the SCF binding site or to a nearby determinant. To support this possibility further we determined the effect of these mAbs on c-kit cross-linking of 125I-SCF to p145(Kit). In the absence of SCF two ligand-receptor complexes could be immunoprecipitated from T-18 cells (Fig. 3A). These complexes correspond to receptor monomers and dimers (Lev et al., 1992a). However, incubation of the cells with 125I-SCF in the presence of K44 or K57 mAb completely abolished both cross-linked receptor forms (Fig. 3A). In contrast, mAbs that did not inhibit SCF binding to T-18 cells (e.g. K45 and K49) exerted only limited effects on cross-linking of 125I-SCF to the receptor. Similarly, mAbs K44 and K57 did not immunoprecipitate preformed covalent complexes of 125I-SCF with p145(Kit) (Fig. 3B), although they did recognize the Kit-X protein (Fig. 1B) and the full-length protein (200 ng) as indicated. After gel electrophoresis and protein transfer to nitrocellulose filter, the blot was probed with a polyclonal rabbit antibody to Kit-X followed by horseradish peroxidase-labeled protein A. Chemiluminescence (ECL kit, Amersham) and autoradiography (5-min exposure) were used to obtain the shown autoradiogram. IP, immunoprecipitated; IB, immunoblotted.

FIG. 1. Selection of hybridomas producing Kit-specific antibodies. Supernatants of individual clones of hybridoma cells were incubated with Kit-X protein (approximately 200 ng) or with Neu-X protein (200 ng) as indicated. After gel electrophoresis and protein transfer to nitrocellulose filter, the blot was probed with a polyclonal rabbit antibody to Kit-X followed by horseradish peroxidase-labeled protein A. Chemiluminescence (ECL kit, Amersham) and autoradiography (5-min exposure) were used to obtain the shown autoradiogram.

[Fig. 1 Image]
receptor (data not shown) when it was not cross-linked to the ligand. On the basis of these experiments we concluded that mAbs K44 and K57 recognize determinants that are close to the SCF binding site of ~145 nm, unlike other antibodies that interact elsewhere on the receptor molecule. In separate experiments we addressed the possibility that K44 and K57 are directed against the same epitope. To this end, we radiolabeled K44 and analyzed its displacement from Kit-expressing cells by native SCF or K57 mAb. Interestingly, SCF efficiently displaced surface-bound 125I-K44, but K57 was ineffective at concentrations that inhibited SCF binding to T-18 cells (data not shown). We therefore concluded that the epitopes of mAbs K44 and K57 are nonoverlapping.

The capacity of mAb K44 to inhibit SCF binding to Kit was examined in vitro by using the M07e human megakaryoblastic cell line. These cells display absolute dependence for survival and proliferation on GM-CSF and IL-3 (Avanzi et al., 1990). More recently it has been reported that M07e cells can proliferate also in the presence of SCF (Hendrie et al., 1991). When tested on M07e cells, mAb K44 completely inhibited SCF-induced incorporation of [3H]thymidine into DNA was determined by a 4-h pulse that was given after 20 h of incubation at 37 °C with antibodies and growth factors. The results given are averages of triplicate determinations in which the variation did not exceed 10%. The experiment was repeated twice.

### Table I

| Treatment          | [3H]Thymidine incorporated | cpm |
|--------------------|----------------------------|-----|
| Deprived cells     | 1,463                      |     |
| SCF (30 ng/ml)     | 57,966                     |     |
| GM-CSF (10 ng/ml)  | 59,632                     |     |
| K44                | 1,069                      |     |
| 10 µg/ml           | 1,196                      |     |
| 5 µg/ml            | 3,536                      |     |
| SCF (30 ng/ml) + K44 | 2,622                    |     |
| 0.5 µg/ml          | 2,977                      |     |
| 5 µg/ml            | 2,773                      |     |
| SCF (30 ng/ml) + K49 | 1,552                    |     |
| 0.5 µg/ml          | 2,367                      |     |
| 5 µg/ml            | 2,977                      |     |
| SCF (30 ng/ml) + K49 | 1,552                    |     |

**FIG. 2.** Antibody binding to Kit-overexpressing cells and competition with SCF. Panel A, confluent monolayers of T-18 cells, a CHO subline that overexpresses the human Kit protein (Lev et al., 1991), were incubated for 90 min at 22 °C with different concentrations of mAb K44 (closed circles) or K45 (open squares). After a brief washing step the monolayers were further incubated for 90 min at 22 °C with radiolabeled rabbit Ig specific to mouse IgG. This was followed by extensive washing of the unbound radioactivity and counting the cell-associated radioactivity in a y-counter. Panel B, confluent monolayers of T-18 cells were incubated with 125I-SCF (2.5 ng/ml, 105 cpm/ng) in the presence of different concentrations of mAb K44 (closed circles) or K45 (open squares). Following 4 h of binding at 4 °C the monolayers were washed extensively with ice-cold PBS, and the cell-associated radioactivity was determined in a y-counter. Nonspecific binding of 125I-SCF was determined by performing the binding reaction in the presence of a 100-fold excess concentration of unlabeled SCF, and it was subtracted from the total amount of bound radioactivity. The results shown are representative of two experiments.

**FIG. 3.** Effect of monoclonal antibodies on cross-linking of 125I-SCF to Kit and immunoprecipitation of the ligand-receptor complexes. Monolayers of T-18 cells (approximately 106 cells) were incubated with 2 nm 125I-SCF in the presence of the indicated mAbs at 2 µg/ml (panel A) or without antibodies (panel B). Following 2 h at 4 °C the cells were washed, and the ligand-receptor complexes were covalently cross-linked by a 40-min incubation at 22 °C with 15 mM EDAC. The complexes were then immunoprecipitated (IP) with a polyclonal antibody to Kit-X (panel A) or with the indicated mAbs (panel B) and analyzed by electrophoresis in 5% acrylamide gels followed by autoradiography (24 h at -70 °C with an intensifier screen). The locations of molecular mass marker proteins are indicated by bars, and the corresponding masses are given in kDa. Note that both dimers and monomers of Kit were resolved.

**TABLE I**

Effect of anti-Kit mAbs on DNA synthesis of M07e megakaryocyte cells

M07e cells (50,000/well) were deprived of growth factors 24 h before starting the experiment. Antibodies were added to the cells 50 min prior to the addition of growth factors. The incorporation of [3H]thymidine into DNA was determined by a 4-h pulse that was given after 20 h of incubation at 37 °C with antibodies and growth factors. The results given are averages of triplicate determinations in which the variation did not exceed 10%. The experiment was repeated twice.
(Yarden et al., 1987) were used to introduce stop codons at these sites, thereby terminating translation at either the amino- or the carboxyl-terminal side of the third Ig domain of Kit (Fig. 4). The modified cDNAs were ligated separately into an eukaryotic expression vector that contained the dhfr gene as a selectable marker. These plasmids were used to transfect dhfr-deficient CHO cells (Urlaub and Chasin, 1980), and drug-resistant colonies were grown individually. Clones that overexpressed the shorter deletion mutant, containing Ig domains 1 and 2 (denoted Kit 1-2), or the longer protein that contained also domain 3 (Kit 1-2-3), were selected by using an immunoprecipitation assay. A representative immunoprecipitation analysis of the selected clones is shown in Fig. 5. Monolayers of the selected cell lines and a control untransfected cell line were incubated for 16 h with $^35$S-methionine. The biosynthetically labeled Kit proteins that were secreted into the growth medium were then immunoprecipitated with a polyclonal rabbit antibody that was raised against the whole extracellular domain. As shown in Fig. 5, a 36-kDa protein was detectable in the medium of cells that were transfected with the kit 1-2 plasmid, whereas a 65-kDa protein was secreted by kit 1-2-3-transfected cells. These molecular masses are in agreement with the sizes of the recombinant glycosylated proteins (Fig. 4 and Qiu et al., 1988). In addition to the indicated p36 and p65 proteins, two other bands of 120 and 180 kDa appeared in the immunoprecipitates. We attribute these molecules to nonspecific interactions with the polyclonal antisera because they were precipitated also from untransfected cells (Fig. 5).

**Ligand Inhibitory mAbs Recognize the Recombinant Kit Proteins**—In the next step we analyzed the ability of Kit-specific mAbs to recognize Kit 1-2-3 and Kit 1-2 proteins by using an immunoprecipitation assay. As shown in Fig. 6, Kit 1-2-3 was recognized by four mAbs, including the ligand-competitive K44 and K57 antibodies. In addition, these two antibodies could immunoprecipitate the shorter mutant (Fig. 6, right panel), implying that their epitopes are confined to Ig domains 1 and 2 and the intervening sequences. Interestingly, mAbs K27 and K69 did not react with Kit 1-2-3, suggesting that their recognition sites are distal to the first three Ig domains of Kit. Both K45 and K49 mAbs, which do not interfere with ligand binding, recognized Kit 1-2-3 and also Kit 1-2 (Fig. 6 and data not shown). In conclusion, the portion of the Kit protein which is included in the short deletion mutant Kit 1-2 contains the binding sites of mAbs K44 and K57, and by extension also the SCF binding site or part of it.

**Inhibition of Binding of Radiolabeled SCF to Wild-type Kit by Soluble Recombinant Proteins**—Because of the soluble nature of the recombinant truncation mutants of Kit, simple ligand binding assays were not feasible. In addition, immobilization by using antibodies could interfere with the binding assay. We therefore tested the ability of soluble Kit proteins...
that were affinity purified over a mAb column (Fig. 7A) to inhibit binding of SCF to Kit-overexpressing cells. This type of analysis has indicated previously that Kit-X and wild-type Kit display comparable affinities to SCF (Lev et al., 1992c). The results presented in Fig. 7B further indicate that Kit 1-2-3 is practically as potent as Kit-X in inhibiting SCF binding. However, Kit 1-2, which lacks domain 3, was much less efficient than Kit 1-2-3 in displacing Kit-bound SCF (Fig. 7B). Since Kit 1-2 did not exceed 50% inhibition of SCF binding in several independent experiments, we could not determine the relative ligand affinity of this mutant protein. However, we estimate that removal of Ig-like domain 3 involves 5–7-fold reduction in apparent ligand affinity.

Covalent Cross-linking of Radiolabeled SCF to Soluble Kit Proteins—To demonstrate directly the specific interaction between SCF and soluble Kit proteins, we employed covalent cross-linking of the radiolabeled ligand. Immunoaffinity-purified Kit-X, Kit 1-2-3, or Kit 1-2 (or Neu-X as a negative control) were incubated with 125I-SCF, and the cross-linking reagent disuccinimidyl suberate was added later to stabilize ligand-receptor complexes covalently. Gel electrophoresis that was performed afterward revealed radioactive bands of sizes that correspond to complexes of 125I-SCF with the respective Kit protein (Fig. 8A). Thus, two bands that were identified previously as monomers and dimers of the extracellular domain of Kit (Lev et al., 1992c) were seen with Kit-X, whereas 85- and 55-kDa radioactive bands were observed with Kit 1-2-3 and Kit 1-2, respectively (Fig. 8A). The specificity of the covalent cross-linking was indicated by the absence of signal with the Neu-X protein (Fig. 8A).

Additional experiments were performed to confirm the specificity of interaction of 125I-SCF with the recombinant Kit proteins (Fig. 8B). These analyses showed that unlabeled SCF (at 0.5 or 2 nM), but not unlabeled transforming growth factor-α (TGF-α, 2.8 µM), or no addition (−) as indicated above each lane. Covalent cross-linking was then induced by the addition of disuccinimidyl suberate and gel electrophoresis as in panel A. Autoradiography was for 24 h. Arrows indicate the locations of the radiolabeled complexes of SCF and the corresponding Kit proteins. Note that a dimer of Kit-X was resolved, but no dimers of Kit 1-2-3 or Kit 1-2 were detectable.

**DISCUSSION**

Receptor tyrosine kinases that contain Ig-like domains in their ligand-binding portions now outnumber receptor tyrosine kinases with other extracellular landmarks (Hanks, 1991), and they include the PDGF receptor group, the receptors for the fibroblast growth factors as well as receptors for neurotrophic factors (reviewed in Ullrich and Schlessinger, 1990). Nevertheless, ligand binding to these receptors is less
understood than in the case of the EGF receptor and the insulin receptors. By using chimeras between chicken and human EGF receptors (Lax et al., 1989) and direct peptide mapping of the binding sites of 125I-EGF (Lax et al., 1988; Wu et al., 1990) and EGF-competitive mAbs (Wu et al., 1989), it was shown that the receptor's portion that lies between the two cysteine-rich sequences specifies ligand binding. However, it appears that the amino-terminal portion of the EGF receptor also participates in the formation of the ligand binding cleft. Similarly, the binding of insulin and insulin-like growth factor-1 to their related receptors appears to involve both the amino-terminal portions of the receptors (Wedekind et al., 1989; Kjeldsen et al., 1991) and their cysteine-rich domains (Gustafson and Rutter, 1990; Yip et al., 1991). The latter may function as a core binding cleft whereas ligand specificity is probably determined by adjacent sequences.

Interestingly, studies of ligand interactions with receptor tyrosine kinases that contain Ig-like motifs also indicate that noncontiguous receptor segments may create the ligand binding sites of such receptors. In addition, determinants of specificity to different ligands may not coincide. For example, the amino-terminal three Ig-like domains of the PDGF receptor confer the ability to bind different PDGF isoforms (Heidaran et al., 1990), but specificity of the α receptor to PDGF-AA resides in the second Ig-like domain and it is distinct from the PDGF-BB specificity determinant (Heidaran et al., 1992). Likewise, the carboxy-terminal half of the third Ig-like domain of variants of the fibroblast growth factor receptors determines ligand specificity to the keratinocyte growth factor or basic fibroblast growth factor, but binding of acidic fibroblast growth factor is determined elsewhere in the receptor (Werner et al., 1992; Miki et al., 1992; Yayon et al., 1992).

By using SCF-competitive mAbs we localized the ligand binding site to the two amino-terminal Ig-like domains of Kit (Fig. 3). However, a recombinant protein that contained only the first two Ig-like domains (Kit 1-2) displayed reduced affinity to SCF, as compared with the whole ectodomain of Kit or a soluble protein encompassing the three amino-terminal Ig-like domains (Fig. 7B). It is therefore conceivable that part of the high affinity binding site resides in the receptor's portion that is defined by the amino-terminal two Ig-like domains, but determinants which are included in the third Ig loop are also involved in the formation of the ligand binding cleft. Covariant cross-linking experiments (Fig. 8) also support our conclusion that the major portion of the SCF binding cleft is confined to the first and second Ig-like domains of Kit. However, this type of experiment cannot resolve differences in ligand binding affinities.

An independent support to the amino-terminal localization of the SCF binding site was obtained recently in our laboratory by utilizing chimeric human-mouse Kit proteins. Since murine Kit does not bind human SCF, it was possible to localize the binding site of human SCF through replacement of individual Ig-like loops of mouse Kit by the corresponding parts of human Kit. This approach identified the second Ig-like domain of human Kit as the core of the binding site but indicated that the adjacent Ig domains are also involved in ligand recognition. Our preliminary data further suggest that the effect of domain 3 is caused by a decrease in the rate of ligand dissociation. Presumably, SCF binding to the second Ig-like domain of Kit is followed by a conformational change that allows the third domain to fold over the binding cleft and thereby inhibit ligand dissociation. Unfortunately, our soluble Kit proteins do not provide a convenient system in which to test this model, and we currently employ chimeric Kit proteins to address the mode of involvement of individual domains.

The possibility that interdomain packing, rather than a contiguous protein sequence, defines the ligand binding site of Kit, is consistent with our observation that the SCF-competitive mAbs (K44 and K57) recognize conformation-dependent epitopes of Kit (data not shown). The amino-terminal location of the binding site is also reminiscent of the finding that domain 2 of the αPDGF receptor confers specificity to PDGF-AA (Heidaran et al., 1992). Interestingly, the first and the second Ig-like domains of another receptor with five such motifs, the intercellular cell adhesion molecule 1, contain the binding site for the integrin LFA-1 and also for a subgroup of human rhinoviruses (Staunton et al., 1990). Presumably, distal localization of the binding sites for soluble ligands (i.e., SCF and PDGF), integrin, or a bulky virus makes these cellular receptors accessible to interactions.

In conclusion, our present study confined the ligand binding site of Kit to its amino-terminal portion and implicated the involvement of noncontiguous protein sequences in SCF recognition. Further dissection of the amino-terminal half of the ectodomain of Kit by internal deletions should make it possible to define more precisely the interaction between SCF and Kit. Alternatively, crystallization of the recombinant Kit proteins, and especially Kit 1-2-3, may provide molecular insights into the binding of SCF to its target cells.

Acknowledgments—We thank Dr. Kris Zeebe (Amgen Center) for recombinant human SCF, Magda David for help in the generation of mAbs, and Miriam Fagan for typing this manuscript.

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