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Chymase cleavage of stem cell factor yields a bioactive, soluble product

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ABSTRACT **Stem cell factor (SCF) is produced by stromal cells as a membrane-bound molecule, which may be proteolytically cleaved at a site close to the membrane to produce a soluble bioactive form. The proteases producing this cleavage are unknown. In this study, we demonstrate that human mast cell chymase, a chymotrypsin-like protease, cleaves SCF at a novel site. Cleavage is at the peptide bond between Phe-158 and Met-159, which are encoded by exon 6 of the SCF gene. This cleavage results in a soluble bioactive product that is 7 amino acids shorter at the C terminus than previously identified soluble SCF. This research shows the identification of a physiologically relevant enzyme that specifically cleaves SCF. Because mast cells express the KIT protein, the receptor for SCF, and respond to SCF by proliferation and degranulation, this observation identifies a possible feedback loop in which chymase released from mast cell secretory granules may solubilize SCF bound to the membrane of surrounding stromal cells. The liberated soluble SCF may in turn stimulate mast cell proliferation and differentiated functions; this loop could contribute to abnormal accumulations of mast cells in the skin and hyperpigmentation at sites of chronic cutaneous inflammation.**

Stem cell factor (SCF) is the ligand for KIT, the protein product of the c-*KIT* protooncogene (1–4). In addition to playing a critical role in hematopoiesis and germ cell development, SCF stimulates the development and differentiated functions of mast cells and melanocytes (5–12). SCF is one of a number of polypeptide growth factors that are produced as transmembrane molecules that can be subsequently cleaved to yield biologically active soluble forms (13). Cleavage of SCF is known to occur most efficiently at a membrane proximate, extracellular site encoded by exon 6 of the SCF gene (1, 14–17). Exon 6 may be alternately spliced, and the isoform of SCF that lacks exon 6 encoded sites remains predominantly bound to the membrane of the cells that produce it, particularly with human SCF which lacks known alternate cleavage sites. Although both membrane and soluble forms of SCF stimulate the membrane-bound KIT receptor tyrosine kinase, soluble and membrane SCF can have somewhat different effects on their target cells (18–20), and alterations in the balance of soluble and cell-bound SCF may play a key role in disease states such as cutaneous mastocytosis (8, 21, 22). Despite the identification of a primary cleavage site encoded within exon 6 of the SCF gene (1, 14–17), little is known about the enzymes that release the soluble form of SCF.

Chymase is a chymotrypsin-like protease expressed by a subset of human mast cells that typically predominate in connective tissues, including the dermis (23). It is stored at high amounts within mast cell secretory granules (4.5 μg/million mast cells compared with 1 μg of elastase/million neutrophils) and is therefore presumed to have an extracellular function (24). Mast cell degranulation is an early event in some cutaneous inflammatory reactions and is best known for its role in the allergic response. However, degranulation may be produced by other physiologically relevant and irrelevant stimuli (substance P, anaphylotoxins, opioids), and may result in the release of granule contents including chymase, without production of the allergic response (25). Analysis of the substrate specificity of human chymase with model peptide–NA substrates (26), protease inhibitors (27), and biological peptides (28, 29) reveals that chymase preferentially cleaves proteins at sites with aromatic residues in the P1 position [P1–P1′ define the site of cleavage (30)] and a Pro residue in the P2 position. The amino acid sequence encoded by exon 6 of SCF contains a Pro–Phe sequence which fits this motif, ≈7 amino acids from the C terminus of the soluble form of SCF that is normally found in cell cultures.

In this study, we investigate the possibility that human SCF is a substrate for human chymase. We found that chymase cleaves SCF to yield a soluble, bioactive form of the molecule that is 7 amino acids shorter than the usual soluble form. These results are the first identification of a specific protease capable of producing bioactive soluble SCF, and suggest the possibility of a novel feedback loop involving chymase and SCF that could affect mast cells and other cells expressing KIT in both neoplastic and inflammatory diseases.

MATERIALS AND METHODS

Sources of SCF. Nonglycosylated recombinant human SCF^{165} ($rHuSCF^{165}$) purified from transfected *Escherichia coli* as previously described (1, 31) was used for protein sequence analysis and bioactivity assays. This material includes a portion of the protein encoded by exon 6, the putative chymase digestion site, and the site previously identified as susceptible to cleavage by unknown enzymes (see Fig. 1). In addition, the transfected bone marrow stromal cell line, Sl^4–Hu^{248}, was used as a source of membrane-bound, glycosylated SCF (20).

Enzymes and Digestions. Human chymase was purified from skin as described (32, 33). Its concentration was estimated from its specific activity for hydrolysis of the substrate Suc-Ala-Ala-Pro-Phe-4-nitroanilide (32–34).

$rHuSCF^{165}$ (100 ng) was digested in 0.05 M Tris·HCl (pH 8.0) and 0.26 M NaCl with 100 nM human chymase. Reactions were stopped at the desired times by addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 3 mM.

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Abbreviations: SCF, stem cell factor; rHuSCF, recombinant human SCF.

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Under these conditions chymase activity is virtually completely inhibited in less than 1 min. After PMSF inhibition, reactions were frozen and kept at −80°C until analysis. In control experiments to demonstrate cleavage specificity, PMSF or 3.5 μM of a recombinant form of α1-antichymotrypsin (rACT–P3–P3′), another chymase inhibitor with more specific properties (34), was added to the reaction prior to addition of substrate. Additional digestions were performed in the presence of heparin (0.1 mg/ml).

SCF Bioactivity Assay. Ligand activation of KIT induces homodimerization and phosphorylation of KIT tyrosine. We determined bioactivity by evaluating the ability of chymase cleaved SCF to induce tyrosine phosphorylation of KIT expressed by human melanocytes *in vitro*. Briefly, melanocytes were grown to confluence and starved of growth factors for 20 h. After addition of test samples for 20 min, cell lysates were prepared by collecting the melanocytes in hot 3× Laemmli's sample buffer and boiling for 10 min. KIT phosphorylation was detected by Western blot analysis using a primary anti-phosphotyrosine antibody.

Western Blot Analysis. Proteins were separated by SDS/PAGE (12% for SCF, 8% for KIT) and transferred to nylon membranes at constant current. Immunoblotting for SCF was performed with a monoclonal anti-human SCF antibody (7H6; Amgen) (35) for phosphotyrosine using a monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY), and for KIT using a monoclonal anti-human anti-KIT antibody (SR-1; Amgen) (36). Bands were detected by chemiluminescence (DuPont/NEN) according to the manufacturer's instructions.

Mass Spectroscopy and Protein Sequencing. Sample aliquots were injected onto an HPLC system [HP1090 liquid chromatograph with a reversed-phase C18 narrow bore column (2.1 mm × 10 cm)] at a flow rate of 250 μl/min. Elution was accomplished with an acetonitrile-trifluoroacetic acid gradient and was monitored by absorbance at 215 and 280 nm as described (16). One-third of the eluate was also subjected to on-line electrospray mass spectrometry analysis (model API100 instrument; Sciex, Thornhill, ON, Canada) and some samples were analyzed by mass spectrometry after elution and collection. Protein samples of chymase digested rHuSCF were also subjected to direct Edman N-terminal sequence analysis using an automated sequencer (model 477; Applied Biosystems), as described (16).

RESULTS

Chymase Cleavage of rHuSCF Produces a Stable, Bioactive Product. To characterize chymase cleavage of SCF, we digested rHuSCF^{165} produced in *E. coli*. rHuSCF is a nonglycosylated, bioactive 18.6-kDa soluble protein corresponding to the protein portion of the secreted form of SCF (Fig. 1). It contains a portion of the exon 6 encoded region of SCF, including two C-terminal alanines at the normal endogenous cleavage site. Gel electrophoresis and Western blot analysis of rHuSCF^{165} gives a single sharp band (Fig. 2). Cleavage by chymase at the predicted site should result in release of 7 amino acids and a decrease in the size of the protein by about 0.7 kDa. As shown in Fig. 2, treatment of 2 μM rHuSCF with 100 nM chymase resulted in the processing of rHuSCF^{165} to a slightly faster migrating species, consistent with cleavage near the C terminus of the protein. Based on band intensities, the reaction appeared half complete by 1 h of incubation and almost fully complete after 4 h. The fully digested material was soluble and capable of inducing KIT phosphorylation in human melanocytes. Assuming that a single site of cleavage was responsible for the conversion of rHuSCF to a slightly lower molecular weight form, and that the concentration of rHuSCF^{165} was well below the K_m for the reaction, the apparent second order rate constant (k_{cat}/K_m) for chymase processing was calculated according to the relationship $k_{cat}/K_m = 0.693 \times (t_{1/2})^{-1} \times [\text{chymase}]^{-1}$. Assuming a $t_{1/2}$ of 60 min, a value of 2,000 $M^{-1}\cdot s^{-1}$ is obtained.

Chymase digestions monitored for 24 h showed no further change in the M_r of the product, indicating that SCF was not highly susceptible to further digestion by chymase. Day-long incubation of chymase in control reactions not containing recombinant SCF, showed between a 30% and 50% loss of enzyme activity, confirming that the stability of the SCF digestion product was not due to a complete loss of enzyme activity over the extended incubation (data not shown). Cleavage also occurred in the presence of 0.1 mg/ml heparin, although at a slightly slower rate (data not shown). Digestion of membrane preparations from *Sl*^{4}–Hu^{248} cells was complicated by instability of membrane based SCF; we were unable

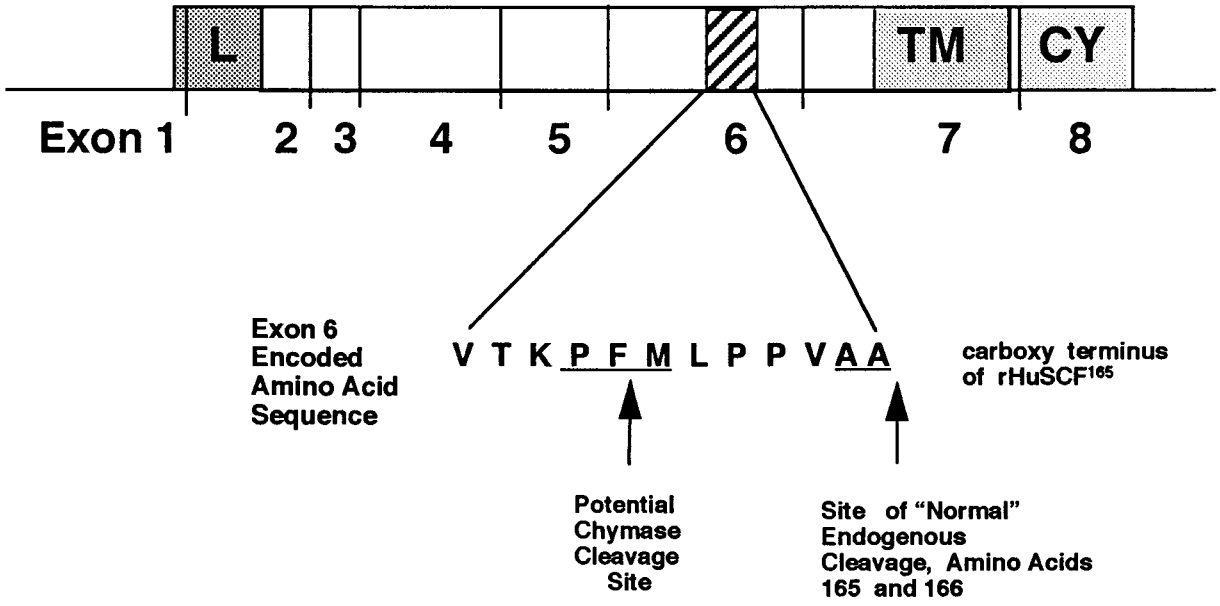

FIG. 1. Schematic representation of SCF protein and cDNA. Cross-hatching in exon 6 encoded area represents the region of known protease sensitive sites, whose amino acid sequences are shown below. L, leader; TM, putative transmembrane region; CY, cytoplasmic domain.

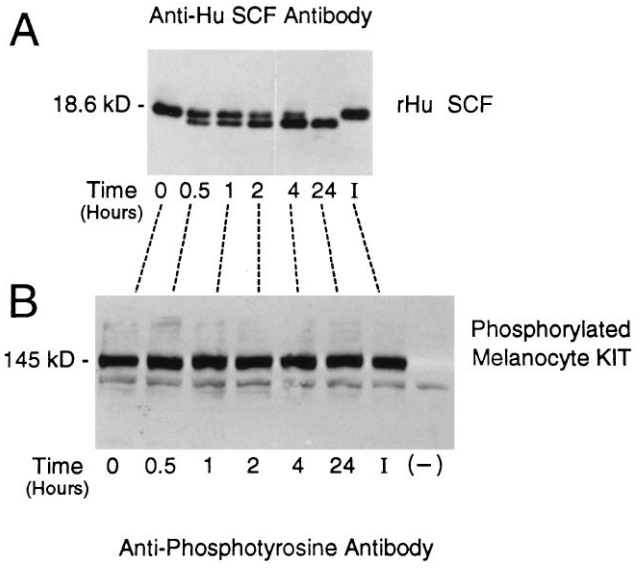

FIG. 2. Chymase hydrolysis of rHuSCF. (*A*) Western blot of the time course of digestion of rHuSCF by human chymase. The nonglycosylated rHuSCF^{165} produces a sharp band at 18.6 kDa. After digestion with 0.1 μM chymase for up to 24 h, a stable product of ≈17.9 kDa is produced. The last lane, marked "I," contains the rHuSCF^{165} and chymase, plus a specific chymase inhibitor. (*B*) Melanocyte KIT is phosphorylated on tyrosine in response to chymase digested rHuSCF^{165}. The appearance of a band at ≈145 kDa indicates that both the undigested and chymase digested SCF are biologically active and capable of inducing KIT phosphorylation *in vitro*. The lane marked "I" was treated with the inhibited chymase treated preparation. The lane on the far right, marked (−), contains melanocytes plus chymase, digestion buffer, and inhibitor, but no SCF. Blotting of the membrane with anti-KIT after stripping off the original antibodies confirmed that all lanes contained approximately equal amounts of KIT (data not shown).

to reproducibly demonstrate SCF cleavage in membrane preparations because the SCF was converted to a faster migrating form indistinguishable from the predicted chymase product, despite the addition of protease inhibitors.

The size of rHuSCF cleavage products was confirmed by mass spectrometry data obtained after their separation by reversed-phase chromatography (50 μl of rHuSCF, digested as described). Two absorption peaks were obtained at retention times of 25.5 and 65 min. Intact rHuSCF^{165} has a theoretical mass value of 18,649.5. The 25.5 min peak gave a protonated molecular ion (MH^+) mass of 698.5 (Fig. 3*A*), which matched essentially that expected for the C-terminal peptide (amino acids 159–165, theoretical MH^+ value of 698.4). The 65-min peak did not give a good ion signal with on-line analysis, probably because of suppression by ions of other components. Therefore, this material was collected and analyzed separately. Its average mass value was 17,980 (Fig. 3*B*), which is very close to the theoretical value of 17,969.7 for rHuSCF^{165} terminating at amino acid 158.

Chymase Cleaves Between SCF Amino Acids 158 and 159. Automated N-terminal sequencing of chymase-digested rHuSCF^{165} [2 μg, digested for 24 h, with rHuSCF^{165} at 100 μg/ml (about 5,400 nM) and chymase at 100 nM] yielded dual assignments through the first seven cycles and a single sequence thereafter. From the identified amino acids and the known sequence of rHuSCF^{165}, it was clear that the data represented the dual sequencing of the intact N terminus of rHuSCF (Met-Glu-Gly-Ile–) and a peptide sequence derived from the C terminus of rHuSCF^{165} (Met-Leu-Pro-Pro-Val-Ala-Ala, representing amino acids 159–165). We conclude that chymase specifically cleaves rHuSCF^{165} between amino acids 158 and 159.

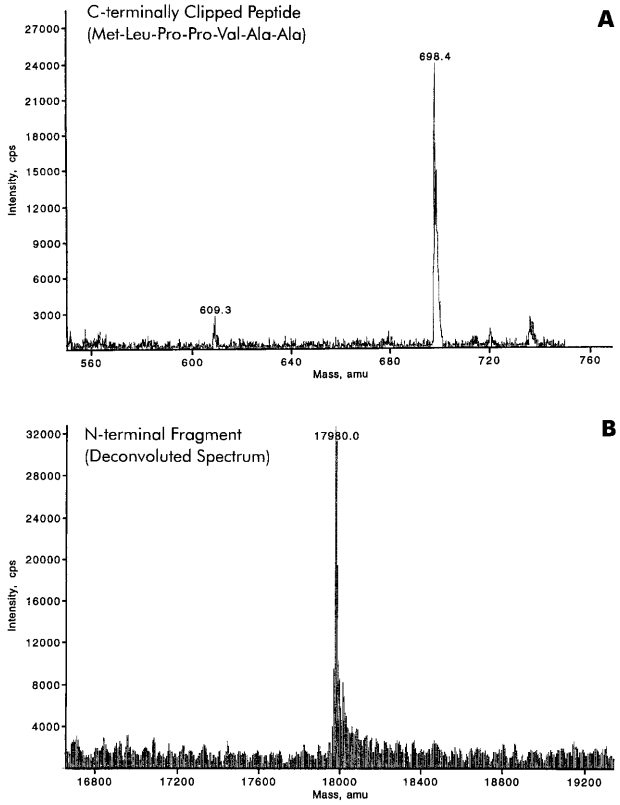

FIG. 3. Mass spectroscopy data confirm the predicted molecular weights of rHuSCF^{165} chymase digestion products. The molecular weights of the products of the chymase digestion of rHuSCF^{165} closely approximate the fragment sizes predicted for cleavage between amino acids 158 and 159. The smaller fragment (*A*) has a molecular weight of 698.5 Da, almost identical to that predicted for the C-terminal septa-peptide. The larger, stable product (*B*) has a molecular weight of 17,981.5 Da, essentially identical to that predicted for the N-terminal 158 amino acids of rHuSCF.

DISCUSSION

Our data show that chymase specifically cleaves SCF at a novel site encoded within exon 6 of the SCF gene, and that the resulting product retains the ability to stimulate KIT. This site is highly evolutionary conserved, being present in feline (our unpublished data), porcine (37), murine (2, 3, 38), canine (39), and rat (1) but not chicken SCF (40), a fact which suggests that the site may have biologic significance. The site of chymase cleavage is 7 residues upstream from the C terminus of soluble SCF found in supernatants of cell lines producing SCF. This finding is based on Western blot analyses, mass spectroscopy, and direct protein sequencing of cleavage products. Thus the cleavage site recognized by chymase, Phe-158–Met-159, is different from the site including Ala-164 and Ala-165, presumably processed in cell culture.

Human chymase has been shown to be a highly efficient converter of angiotensin I, a peptide of 10 residues, to angiotensin II, a peptide of 8 residues; cleavage of angiotensin I occurs preferentially between Phe-8–His-9 (28, 29). The high sensitivity for chymase processing of angiotensin I (k_{cat}/K_m = 2.5×10^6) was shown to be primarily based on recognition of the Pro–Phe sequence prior to His-9 and the absence of another Pro residue in the immediate residues around the cleavage site. The chymase cleavage site in SCF appears to have the same sequence properties as angiotensin I, suggesting that it should be susceptible to chymase hydrolysis. The efficiency of cleavage of SCF by chymase, however, appears slow ($k_{cat}/K_m \approx 2 \times 10^3$ $M^{-1}\cdot s^{-1}$) (28, 29). The difference in catalytic efficiency may be due to conformational constraints

in SCF, a protein, compared with angiotensin I, a peptide. Calculations based the numbers of mast cells in normal human skin and the amount of chymase per mast cell suggest that the chymase concentration in human mast cells may be in the range of 50 μM and that the complete release of chymase into tissues could produce local concentrations as high as 1 μM (41). At this upper limit of chymase, a significant amount of SCF could be solubilized from surrounding cells in a relatively short period of time. For example at 1 μM chymase, the $t_{1/2}$ for processing of SCF in the experiment depicted in Fig. 2 would be 6 min. Thus, despite the relatively slow kinetics of the reaction, the local release of high amounts of chymase from mast cells may have a role in affecting the level of soluble SCF in tissues.

Although chymase is clearly not the only protease capable of cleaving SCF, it is believed to be the first such enzyme to be identified. Because it is produced by a cell type that depends on SCF for its development and survival, our findings raise the possibility of a novel feedback loop in which degranulation of mast cells results in release of chymase activity with subsequent generation of soluble SCF. The soluble SCF may in turn affect other KIT positive cells within the microenvironment, as well as stimulating mast cell accumulation and perhaps further mediator release (5, 8, 12, 42–44). This sequence of events might thus constitute a mechanism for amplifying mast cell responses to various stimuli. This appears to be a novel method of growth which has some features of autocrine stimulation and some features of paracrine stimulation, and might be termed parautocrine stimulation. Interestingly, our analysis of exon 6 encoded sequences suggests that other enzymes released from inflammatory cells, such as mast cell tryptase, neutrophil elastase, and neutrophil cathepsin may also cleave SCF, raising the possibility that cleavage of SCF might be a general mechanism by which mast cells accumulate at sites of inflammation.

The relatively slow kinetics for chymase cleavage of SCF suggests that its *in vivo* effects might be most easily observable in situations where mast cell degranulation occurs chronically in a confined area and where there are elevated numbers of mast cells. Evidence for function of this paraautocrine loop exists in the human disease mastocytosis. Mastocytosis is a heterogeneous group of conditions characterized by increased numbers of mast cells within various tissues (45). In addition to increased mast cells in the dermis, skin lesions of mastocytosis show increased epidermal pigment suggesting activation of melanocytes, a known effect of SCF (6, 21). Furthermore, many features of cutaneous mastocytosis can be produced by exogenous soluble SCF (8, 11, 46), and local increases in soluble SCF have been found in lesions of cutaneous mastocytosis (21, 22). The colocalization of the mast cells and soluble SCF in these lesions implies that either the mast cells themselves are producing or inducing the soluble SCF, or that there is some basic alteration in the production of SCF by stromal cells. However, it appears likely that the accumulation of mast cells in some cases of mastocytosis is driven by somatic activating mutations of KIT, which should not directly involve the stromal cells or the melanocytes in cutaneous lesions (47, 48). Release of chymase by neoplastic mast cells could explain the generation of soluble SCF in these cases. In addition to melanogenesis, solubilized SCF could play a role in the mast cell accumulation in mastocytosis, because analysis of data published in two different studies including one patient with mastocytosis suggest that cells heterozygous for activating c-*KIT* mutations may be further stimulated by exogenous SCF (47, 49). Thus, mast cells that are moderately stimulated to proliferate because of heterozygous expression of mutated KIT may be further stimulated in those anatomic locations where they express chymase. The restricted distribution of chymase, in mast cells of the skin and gut submucosa, may explain the frequent involvement of these two organs in mastocytosis.

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