Vol. 188, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1992 Pages 292-297

DELETION MUTAGENESIS OF STEM CELL FACTOR DEFINES THE C-TERMINAL SEQUENCES ESSENTIAL FOR ITS BIOLOGICAL ACTIVITY

Mitsuo Nishikawa[1], Arinobu Tojo[1], Kenji Ikebuchi[2], Kanako Katayama[2], Nobuyoshi Fujii[2], Keiya Ozawa[1] and Shigetaka Asano[1,2]

[1]Department of Hematology and Oncology, and [2]Department of Blood Transfusion and Cell Culture, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan

Received August 31, 1992

SUMMARY: We constructed a series of murine stem cell factor (mSCF) cDNAs which were sequentially truncated at the 3' termini. The resultant six mutant cDNA encode N-terminal 183, 179, 162, 149, 142 and 133 amino acid residues of the mature mSCF protein fused to the heterogeneous C-terminal peptides derived from the linker sequences. Each mutant cDNA was transiently expressed in COS cells, and the cultured supernatant was assayed for its ability to support the growth of a human factor-dependent cell line, TF-1 and to enhance colony formation by murine hematopoietic progenitor cells. The results showed that as few as N-terminal 142 but not 133 amino acid residues of mSCF remained biologically active *in vitro*, suggesting that the region of 9 amino acids from Asp^{134} to Ser^{142} containing a Cys^{138}-mediated disulfide bond may contribute to the C-terminal end of the active subdomain of mSCF.

Stem cell factor (SCF) or *c-kit* ligand is a recently described cytokine which is produced by stromal cells and has potentially a wide range of target cells including hematopoietic stem cells, germ cells, neural cells and melanocytes[1]. Especially in mice with certain genetic defects in the SCF gene (*Sl*) or the *c-kit* gene (*W*), macrocytic anemia and mast cell depletion are common, indicating the importance of *c-kit* ligand-receptor interaction in normal hematopoiesis[2]. It has been shown that the SCF gene generates two alternate mRNA species and results in two SCF isoforms which are originally synthesized as transmembrane proteins[3]. However, one isoform can be secreted after proteolytic cleavage at Ala^{164} or at Ala^{165} in the spacer region encoded by the alternatively spliced exon[3]. Both the natural and recombinant forms of soluble SCF reveal potent activities in colony-forming assays to amplify the action of many other cytokines including interleukins and colony-stimulating factors[4, 5], although membrane-bound SCF may play a central role *in vivo*[3]. Bazan recently showed structural homology between SCF and macrophage colony-stimulating factor (M-CSF) and predicted the common tertiary structure of both factors[6]. The other groups previously analyzed the structure of M-CSF and presented the results compatible with this hypothetical model[7]. Here we have performed deletion mutagenesis of mSCF cDNA and defined the C-terminal sequences essential for its biological activity *in vitro*.

0006-291X/92 $4.00

MATERIALS AND METHODS

Cloning and Deletion Mutagenesis of mSCF cDNA. To isolate a full-length mSCF coding sequence, first-strand cDNA was synthesized from total RNA isolated by an acid guanidine phenol-chloroform procedue[8] from the murine stromal cell line ST2[9]. The cDNA was then amplified by the polymerase chain reaction[10] with a pair of primers (sense; 5'-CTGGAGCTCCAGAACAGCTA-3' and antisense; 5'-ATCTAGACTTCT GAAACTCTCTC-3'). The amplified cDNA fragment, extending from nucleotide 113 to 1096 of the published mSCF cDNA sequence[11] and flanked by restriction sites for SacI and XbaI (indicated by underlines), was cloned between the corresponding sites of pUC19. DNA sequencing of obtained clones revealed one base pair mutation upon comparison with the reported sequence, resulting in one amino acid change from Glu to Ala at residue 2 of the mature mSCF protein. For the sequential deletion of 3' end of mSCF cDNA, at first, universal translation terminator (Pharmacia, Uppsala, Sweden) was ligated into HincII site of pBluescriptSK+ (Stratagene, CA), and the resultant plasmid was termed as pBUT. mSCF cDNA was inserted between BamHI and HindIII site of pBUT after converting SacI site of the cDNA to BamHI site. After digestion with SalI and SphI, pBUTmSCF was treated by exonuclease III (TaKaRa, Kyoto, Japan) at 37°C in 5mM Tris-HCl (pH8.0), 10mM NaCl, 5mM $MgCl_2$ and 1mM 2-mercaptoethanol. After every 200 sec, an aliquot of the reaction mixture was transferred to another tube containing an equal volume of 4mM sodium acetate (pH4.5), 10mM NaCl, 2mM $ZnCl_2$ and10% glycerol ,then placed on ice. After heating at 65°C for 5 min, they were treated with mung bean nuclease (TaKaRa, Kyoto, Japan) at 37°C for 60 min. The plasmid DNA was purified after electrophoresis, filled-in by Klenow, self-ligated and transformed into *E.coli* XL-1 Blue. Desired deletion mutants were selected on the basis of sequencing data.

Expression and Metabolic Labeling of SCF in COS Cells. The individual mutant cDNA was inserted into a modified version of pcDSRα[12] and the plasmid DNA was prepared for transfection. COS7 cells seeded in 6-well plates (Costar, MA) were transfected with 5 μg of plasmid DNA by the DEAE-dextran method as described previously[13] and then maintained in 1 ml of IMDM (GIBCO, NY) with 10% fetal calf serum (FCS). The 72 hrs cultured supernatant was taken for biological assays. For metabolic labeling, COS cells 72 hrs after transfection were rinsed with phophate-buffered saline (PBS), then incubated with 100 μCi/ml [^{35}S]-methionine (ICN, CA) in 1 ml of methionine-free RPMI 1640 (GIBCO, NY) containing 1% dialyzed FCS. After 3 hrs, the supernatant was taken and a 100 μl aliquot was subjected to 0.1% SDS-15% polyacrylamide gel electro-phoresis (PAGE)[14], followed by autoradiography.

TF-1 Proliferation Assay. TF-1 cells were routinely cultured in IMDM with 10% FCS and 10 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF)[15]. Prior to an assay, exponentially growing cells were washed free of GM-CSF and resuspended in opti-MEM (GIBCO, NY) with 0.1% FCS. 5 x 10^4 cells were incubated in 100 μl of opti-MEM-0.1% FCS containing serial 2-fold dilution of the COS7 supernatant in 96-multiwell-plates (Nunc, IL). After 48 hrs, the growth of TF-1 cells was determined by the colorimetric MTT method[16].

Hemopoietic Progenitor Cell Assay. BDF1 mice were injected i.v. with 150 mg/kg 5-FU 4 days before sacrifice. Single cell suspensions were prepared from pooled spleens. The nucleated cells were plated at a density of 1 x 10^6 cells/ml in 1.2% methylcellulose/α-MEM (GIBCO, NY) in the presence of 100 U/ml recombinant murine interleukin-3 (IL3) and 2 U/ml recombinant human erythropoietin (EPO; KIRIN, Tokyo, Japan)[17]. The COS7 supernatant was added to a final concentration of 10%. Colonies with diameters of greater than 0.5 mm were counted under an inverted microscope after 8, 11 and 14 days of culture, respectively.

RESULTS

We selected a total of 6 mutant cDNAs which were truncated at the sequences encoding the extracellular domain of mSCF. Figure 1 lists the C-terminal amino acid sequences of the 6 mutant SCF. Each mutant has several unrelated amino acids derived from the linker sequences of pBUT. [^{35}S]-methionine labeled proteins secreted from COS cells transfected with the

```
mSCF183 :  GCC CCT GCA AGC TTA TCG ATA CCG TCG CTT AAT TAA
           Ala Pro Arg Ser Leu Ser Ile Pro Ser Glu Asn end
               183

mSCF179 :  AAA GCC CAA GCT TAT CGA TAC CGT CGC TTA ATT AAT TAA
           Lys Ala Gln Ala Tyr Arg Tyr Arg Arg Leu Ile Asn end
               179

mSCF162 :  CCC CCG CTT ATC GAT ACC GTC GCT TAA
           Pro Pro Leu Ile Asp Thr Val Ala end
               162

mSCF149 :  GAT TCC AAG CTT ATC GAT ACC GTC GCT TAA
           Asp Ser Lys Leu Ile Asp Thr Val Ala end
               149

mSCF142 :  TCT TCA AGC TTA TCG ATA CCG TCG CTT AAT TAA
           Ser Ser Ser Leu Ser Ile Pro Ser Glu Asn end
               142

mSCF133 :  GCA TCT CAA GCT TAT CGA TAC CGT CGC TTA ATT AAT TAA
           Ala Ser Gln Ala Tyr Arg Tyr Arg Arg Leu Ile Asn end
               133
```

Figure 1. Amino acid sequences near the C-terminus of individual mSCF derivative. The C-terminal amino acids corresponding to mature wild-type mSCF protein are numbered. Nucleotide sequences derived from the plasmid pBUT are underlined.

truncated mSCF cDNA as well as the wild-type were analysed by SDS-PAGE and fluorography (Figure 2). The wild-type cDNA yielded soluble SCF which migrated with an apparent molecular mass of 25 kd (Figure 2A, lane 1), the same size as soluble SCF previously identified in BRL-3A[4], BALB/c-3T3[18] and COS7 cells transfected with the full-length mSCF cDNA[3]. As expected, every mutant SCF molecule was also secreted and migrated with an

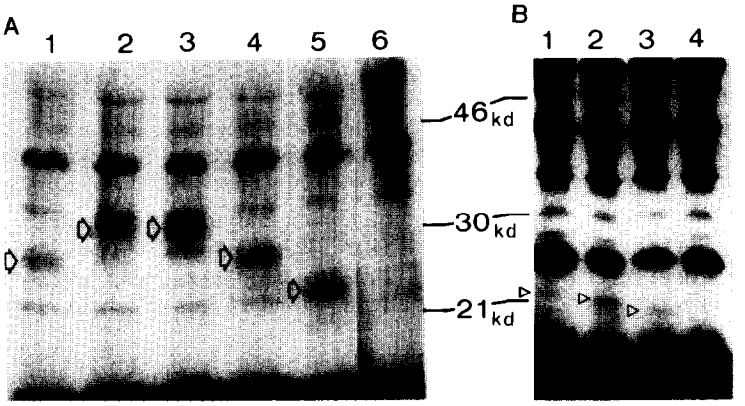

Figure 2. Fluorograph of metabolically labeled supernatants from COS7 cells transfected with the mutated mSCF constructs. Samples were prepared as described in MATERIALS AND METHODS, and electrophoresed under reducing conditions using 15% polyacrylamide gel. Figures A and B indicate separately performed electrophoresis. A: lanes 1, wild-type mSCF; 2, mSCF183; 3, mSCF179; 4, mSCF162; 5, mSCF149; 6, mock. B: lanes 1, mSCF149; 2, mSCF142; 3, mSCF133; 4, mock.

molecular masses ranging from 33 to 20 kd (Figure 2A, lane 2-5 and Figure 2B , lane 1-3). Considering the modifications of SCF protein by glycosylation, the individual molecular size was not conflicting with that expected from the corresponding cDNA sequence. Interestingly, both $mSCF^{183}$ and $mSCF^{179}$ migrated more slowly than the cleaved wild-type mSCF, indicating that proteolysis around Ala^{164} of mSCF requires membrane-association of the molecule, as is the case for M-CSF[19].

Biological activitiy of the COS7 supernatant containing each mSCF derivative was studied in two independent assays. TF-1 cells were well-characterized by their absolute dependency on multiple cytokines for continuous growth and survival[14]. *E.coli*-derived $mSCF^{164}$ also has been shown to sustain the survival of TF-1 cells in a dose-dependent manner (data not shown). When TF-1 cells were incubated with the COS7 supernatants containing mSCF derivatives, all preparations except for $mSCF^{133}$ could support their viability in a concentration-dependent fashion (Figure 3). The relatively high baseline values may be explained by the presence of FCS which includes cross-reactive bovine SCF. The wild-type mSCF used here has a substitution of Ala^2 to Glu^2 of the reported sequence, which appears a silent change in the overall activity of mSCF. Comparison of the dose-response curve shown here with that obtained for *E.coli*-derived $mSCF^{164}$ suggested that each active COS7 supernatant would contain 100 ng/ml equivalent of soluble $mSCF^{164}$ (data not shown). The similar results on SCF activity of each mutant were obtained in a colony-forming assay using 5-FU treated

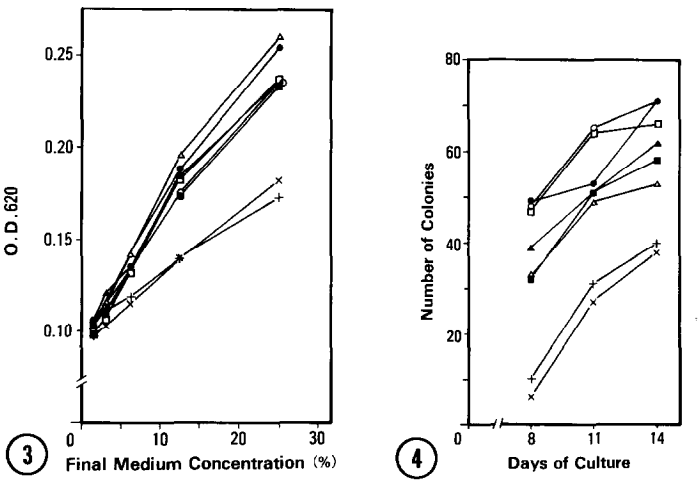

Figure 3. TF-1 supporting activity of mSCF derivatives. TF-1 cells (5×10^4) were incubated in a 0.1 ml volume with serial 2-fold dilution of the COS7 supernatant. After 48 hrs, the MTT assay was performed according to Mosmann[16]. Results are the means of triplicate determinations from one of two experiments. Each symbol indicates as follows: wild-type mSCF (○), $mSCF^{183}$ (●), $mSCF^{179}$ (△), $mSCF^{162}$ (▲), $mSCF^{149}$ (□), $mSCF^{142}$ (■), $mSCF^{133}$ (+), mock (×).

Figure 4. Synergistic activity of mSCF derivatives. 5-FU treated murine spleen cells (1×10^6) were plated in a 1.0 ml volume containing 10% COS7 supernatant in the presence of 100 U/ml IL3 and 2 U/ml EPO. After 8, 11 and 14 days of culture, colonies with diameters of greater than 0.5 mm were counted. Results were the means of quadriplicate determinations from one of two experiments. Each symbol indicates the same as described in Figure 3.

murine spleen cells (Figure 4). This assay revealed the synergistic activity of mSCF with IL3 and EPO to stimulate the growth of high proliferative potential colony-forming cells. Only $mSCF^{133}$ could not show such a synergistic activity. These data clearly demonstrate that as short as N-terminal 142 amino acid residues of the mature mSCF protein have the *in vitro* activity comparable with the cleaved wild-type mSCF even in the presence of the heterogeneous C-terminal tag.

DISCUSSION

The present study aimed to define the minimum N-terminal sequences of mSCF required for its *in vitro* activity. It has already been evidenced that the N-terminal 164 amino acid residues are enough to reveal *in vitro* and *in vivo* SCF activity [20]. Bazan demonstrated the structural similarity between SCF and M-CSF extracellular domain which can be dissected into three components; cytokine, variable spacer and membrane tether subdomains[5]. He predicted that the minimal cytokine subdomain of SCF will be contained within the N-terminal 148 residue encoded by exon 2-5 [5], based on the result that receptor-binding M-CSF derivatives consist of as few as N-terminal 145-147 amino acids[6]. In this respect, our results are compatible with his prediction. Recently it has been shown that there are two disulfide bridges linking the Cys residues 4-89 and 43-138 in soluble SCF molecules[21]. These bridges are predicted to fold 4 α–helix bundles of the SCF cytokine subdomains[5]. Therefore, it is very likely that loss of the Cys^{138} residue results in the disruption of the SCF tertiary structure.

Interestingly, the unrelated C-terminal 6-10 peptides fused to the mSCF deletion mutants did not interfere with SCF activity, supporting the proposed model of the discrete subdomain organization in the SCF extracellular region. This unusual finding raises the possibility that the SCF cytokine subdomain will be applicable as a stem cell-targetted carrier protein for certain bioactive peptides such as interleukin 2/6-pseudomonas exotoxin chimera[22]. Utilities of soluble SCF are now extensively studied both *in vitro* and *in vivo* assays[23]. *In vivo* administration of a large amount of $mSCF^{164}$ was reported to partially repair the hemopoietic defects in *Sl/Sld* mice[22]. Nonetheless, membrane-bound SCF is essential for normal hematopoiesis since similar hematological abnormalities are still observed in *Sld/Sld* mice which can only produce soluble SCF. Future efforts should be directed to elucidate the physiological significance of soluble SCF.

ACKNOWLEDGMENTS

We appreciate A. Miyajima, T. Kitamura (DNAX institute) and S. Nishikawa (Kumamoto University) for kind gifts of rmIL3, TF-1 cell line and ST2 cell line, respectively. We would also thank Schering-Plough Co. and KIRIN Brewery Co. for a supply of rhGM-CSF and $rmSCF^{164}$, respectively. This work was supported by a grant-in-aid from the Ministry of Education, Science and Culture of Japan and by a Reserch Grant from the Foundation for Promotion of Cancer Research in Japan.

REFERENCES

1. Witte, O.N. (1990) Cell 63, 5-6
2. Broxmeyer, H.E., Maze, R., Miyazawa, K., Carow, C., Hendrie, P.C., Cooper, S., Hangoc, G., Vadhan-Raj, S., and Lu, L. (1991) Cancer Cells 3, 480-487
3. Flanagan, J.G., Chan, D.C., and Leder, P. (1991) Cell 64, 1025-1035
4. Zsebo, K.M., Wypych, J., McNiece, I.K., Lu, H.S., Smith, K.A., Karkare, S.B., Sachdev, R.K., Yuschenkoff, V.N., Birkett, N.C., Williams, L.R., Satyagal, V.N., Tung, W., Bosselman, R.A., Mendiaz, E.A., and Langley, K.E. (1991) Cell 63, 195-201
5. McNiece, I.K., Langley, K.E., and Zsebo, K.M.(1991) Exp. Hematol. 19, 226-231
6. Bazan, J.F.(1991) Cell 65, 9-10
7. Takahashi, M., Hirato, T., Takano, M., Nishida, T., Nagamura, K., Kamogashira, T., Nakai, S., and Hirai, Y.(1989) Biochem. Biophys. Res. Commun. 161, 892-901
8. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159
9. Nishikawa, S.I., Ogawa, M., Nishikawa, S., Kunisada, T., and Kodama, H. (1988) Eur. J. Immunol. 18, 1767-1771
10. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S., Higuchi, R., Horn, G.T., Mils, K.B., and Erlich, H.A.(1988) Science 239, 487-491
11. Anderson, D.M., Lyman, S.D., Baird, A., Wignall, J.M., Eisenman, J., Rauch, C., March, C.J., Scott, Boswell, H., Gimpel, S.D., Cosman, D., and Williams, D.E. (1990) Cell 63, 235-243
12. Takebe, Y., Seiki, M., Fujisawa, J.I., Hoy, P., Yokota, K., Arai, K.I., Yoshida, M., and Arai, N. (1988) Mol. Cell. Biol. 8, 466-472
13. Cosman, D., Cerretti, D.P., Larsen, A., Park, L., March, C.J., Dower, S., Gillis, S., and Urdal, D.L. (1984) Nature 312, 768-771
14. Laemmli, U.K. (1970) Nature 227, 680-685
15. Kitamura, T., Tange, T., Terasawa, T., Chiba, S., Kuwaki, T., MIyagawa, K., Piao, Y.F., Miyazono, K., Urabe, A., and Takaku, F. (1989) J. Cell. Physiol. 140, 323-334
16. Mosmann, T. (1983) J, Immunol. Methods 65, 55-63
17. Ikebuchi, K., Wong, G., Clark, S.C., Ihle, J.N., Hirai, Y., and Ogawa, M. (1987) Proc. Natl. Acad. Sci. USA. 84, 9035-9039
18. Nocka, K., Buck, J., Levi, E., and Besmer, P. (1990) EMBO J. 9, 3287-3294
19. Rettenmier, C.W., Roussel, M.F., Ashmun, R.A., Ralph, P., Price, K., and Sherr, C.J. (1987) Mol. Cell. Biol. 7, 2378-2387
20. Zsebo, K.M., Williams, D.A., Geissler, E.N., Broudy, V.C., Martin, F.H., Atkins, H.L., Hsu, R.Y., Birkett, N.C., Okino, K.H., Murdock, D.C., Jacobsen, F.W., Langley, K.E., Smith, K.A., Takeishi, T., Cattanach, B.M., Galli, S.J. and Suggs, S.V. (1990) Cell 63, 213-224
21. Lu, H.S., Clogston, C.L., Wypych, J., Fausset, P.R., Lauren, S., Mendiaz, E.A., Zsebo, K.M., Langley, K.E. (1991) J. Biol. Chem. 266, 8102-8107
22. Pastan, I., and FitzGerald, D. (1991) Science 254, 1173-1177
23. Ulich, T.R., Castillo, J.D., Yi, E.D., Yin, S., McNiece, I., Yung, Y.P., and Zsebo, K.M. (1991) Blood 78, 645-655