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Characterization of Free α- and β-Chains of Recombinant Macrophage-Stimulating Protein

Wataru Yoshikawa,*[,1] Hisanori Hara,*[,2] Toyohiro Takehara,*[,3] Manabu Shimonishi,*[,4] Harumi Sakai,* Norihide Shimizu,* Shin Shimizu,* Ming-Hai Wang,†[,5] Michio Hagiya,*[,6] Alison Skeel,† and Edward J. Leonard†[,7]

**Toyobo Co., Ltd., 2-1-1 Katata, Ohtsu, 520-02 Japan; and †Immunopathology Section, National Cancer Institute, Frederick Maryland 21702*

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Human serum macrophage-stimulating protein (MSP) induces motile activity of murine resident peritoneal macrophages and is a growth and motility factor for epithelial cells. It belongs to the plasminogen-related family of kringle proteins, and is secreted as a single-chain, 78-kDa, biologically inactive pro-MSP. Proteolytic cleavage of pro-MSP at a single site yields active MSP, a disulfide-linked $\alpha\beta$-chain heterodimer. However cleavage of recombinant pro-MSP yielded not only the disulfide-linked heterodimer, but also free α- and β-chains, indicating that some of the recombinant molecules lacked an $\alpha\beta$-chain disulfide. We purified the free chains for characterization. The β-chain of MSP has three extra cysteines, Cys_{527}, Cys_{562}, and Cys_{672}, which are not found in the plasminogen β-chain. Disulfide bond analysis showed a Cys_{527}–Cys_{562}, but also a Cys_{588}–Cys_{672}. Coopting Cys_{588} by Cys_{672} prevented the expected formation of a disulfide between α-chain Cys_{468} and β-chain Cys_{588}. Concomitant studies determined structures of oligosaccharides at the three Asn-linked glycosylation sites of MSP. The oligosaccharides at the three Asn loci are heterogeneous; 11 different sugars were identified, all being sialylated fucosyl biantennary structures. We also located the pro-MSP signal peptide cleavage site at Gly_{18}-Gln_{19} and the scissile bond for formation of mature MSP at Arg_{483}–Val_{484}.

Macrophage-stimulating protein (MSP)[8] was originally identified as a serum protein that stimulates macrophage motility (1, 2). MSP is also a growth or motility factor for other cell types, including epithelia (3–5), megakaryocytes (6), and bone marrow-derived osteoclast-like cells (7).

Human MSP purified from plasma is a disulfide-linked heterodimer comprising α- and β-chains with molecular masses (based on amino acid composition) of 53 and 25 kDa, respectively (8, 9). It is a member of a family of proteins characterized by multiple copies of a highly conserved triple disulfide loop (kringle). These proteins are typically proteolytic enzymes, which are secreted as single-chain precursors that acquire enzymatic activity after proteolytic cleavage. MSP does not have enzymatic activity, but has retained the activation mechanism, by which biologically inactive pro-MSP is cleaved to make the active disulfide-linked heterodimer. Pro-MSP is synthesized by hepatocytes (10) and is released into the circulating blood, where concentrations in the low nanomolar range are found (11). Pro-MSP is cleaved and activated at extravascular sites by trypsin-like proteases (11), as well as by an enzyme on the plasma membrane of murine macrophages (12). The MSP gene was cloned by screening a human genomic library with a probe coding for a krin-

The first two authors of this paper contributed equally to the research.

[1] Present address: Tetsuseikai Hospital, Esebi-Cho, Shijou-nawate, Osaka 575 Japan.

[2] Present address: Nippon Glaxo Ltd., 43 Wadai, Tsukuba, Ibaragi, 300-42 Japan.

[3] Present address: Amgen Ltd., 2-31-1 Nihonbashi Hamacho, Tokyo 103 Japan.

[4] Present address: National Cardiovascular Center, Research Institute, 5-125 Fujisirodai, Suita, Osaka 565 Japan.

[5] Present address: Department of Medicine, University of Colorado School of Medicine, Denver Health and Medical Center, 700 Delaware Street, Denver, CO 80204.

[6] Present address: Takara Shuzo Co., 3-4-1 Seta, Ohtsu 520-21 Japan.

[7] To whom correspondence should be addressed at NCI-FCRDC, Bldg. 560, Rm. 12-71, Frederick, MD 21702. Fax: 301-846-6145.

[8] Abbreviations used: MSP, macrophage-stimulating protein; HGF/SF hepatocyte growth factor; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; FCS, fetal calf serum; TFA, trifluoroacetic acid; ESI, electronspray ionization.

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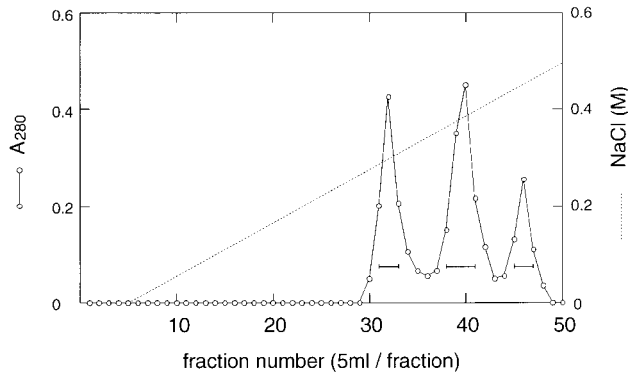

FIG. 1. Separation on CM-Sepharose FF of components derived from purified pro-MSP after cleavage by human kallikrein. Fractions indicated by bars (peaks 1–3, in order of elution) were pooled for further analysis.

gle region of a related protein (prothrombin) (13), and the cDNA was cloned independently from a hepatocarcinoma cell line, Hep G2 (9). The MSP cDNA encodes a 711-amino-acid protein, which has a 45% sequence similarity to its closest family member, hepatocyte growth factor (HGF/SF) (9). The cell receptor for HGF/SF is the Met gene product (14), a typical protein tyrosine kinase growth factor receptor. The Met homologue, RON, (15), is the receptor for MSP (16).

We established a high-production expression system for recombinant pro-MSP. When recombinant pro-MSP was cleaved by kallikrein at the activation site to produce the expected disulfide-linked $\alpha\beta$-chain heterodimer, SDS–PAGE under nonreducing conditions showed not only the disulfide-linked heterodimer, but also free α- and β-chains. The basis for the unlinked α- and β-chains was formation of an aberrant intrachain disulfide in the β-chain, confirming a similar finding by Wahl *et al.* (17). We purified the free α- and β-chains, which enabled us to locate the β-chain disulfides, to determine the site for signal peptide cleavage, to identify the scissile bond that is cleaved to form mature MSP, and to obtain data on the N-linked oligosaccharides of MSP.

EXPERIMENTAL PROCEDURES

Materials. Human serum MSP was purified as described (8). The Chinese hamster ovary (CHO) cell line deficient in dihydrofolate reductase (DHFR) activity was a gift from Dr. A. Chasin (Columbia University, New York, NY) (18). Calf liver pyroglutamate aminopeptidase (EC 3.4.19.3) was from Boehringer-Mannheim. Lysylendopeptidase (EC 3.4.21.50) and thermolysin (EC 3.4.24.4) were from Wako Pure Chemical (Osaka, Japan). *N*-Glycosidase (EC 3.5.1..52 and EC 3.2.2.1) was from Genzyme.

Production of recombinant pro-MSP. A blunt-ended MSP cDNA fragment was inserted into the unique *Eco*RV site of an expression vector, pEVSV, in which expression is controlled by the SV40 early promoter. The vector also contains the HBV poly(A) additional signal for efficient processing of transcripts, and the mouse DHFR cDNA for selection of transformants and amplification of the MSP gene. CHO cells were transfected using Lipofectin reagents (GIBCO). For the selection of transfected cells, we used nucleotide-free minimum essential medium-α with 10% fetal calf serum (FCS), from which nucleotides were removed by extensive dialysis. Colony-forming cells were expanded and the expression of pro-MSP was quantified by sandwich enzyme-linked immunosorbent assay (19). Recombinant protein production was amplified over a period of 2–3 months by sequential passage of cells in the selection medium containing increasing amounts of methotrexate (20) up to a final concentration of 2 mg/ml. Selected transfectants were grown in RPMI 1640 with 10% FCS. Subsequently, the medium was replaced by RPMI 1640 with 1% FCS, and 50 mg/L of leupeptin to minimize cleavage of pro-MSP by proteolytic enzymes.

Purification of recombinant pro-MSP. The conditioned medium was diluted with an equal volume of distilled water and the pH was adjusted to 5.5. The medium was passed through a 0.22-mm filter and applied to a S-Sepharose FF column (5.0 × 20 cm) equilibrated with a pH 5.8, 10 mM sodium citrate, 10 mM sodium phosphate buffer. The column was washed with 2 L of the same buffer and pro-MSP was eluted with 1 L of 0.5 M NaCl, buffered to pH 8.5 with 20 mM Tris–HCl. The eluate was pooled and applied on a 2.6 × 9.4-cm column of anti-MSP monoclonal antibody linked to Affi-Gel 10. After washing the column with 500 ml of 10 mM Tris–HCl buffer, pro-MSP was eluted with 100 ml of 0.1 M glycine–HCl (pH 2.5) and immediately neutralized with 2 M Tris–HCl buffer (pH 8.3).

Cleavage of pro-MSP by kallikrein and purification of the products. One milligram of human kallikrein purified from human plasma (American Diagnostica Inc., Greenwich, CT) was immobilized on Affi-Gel 10 and packed into a 1.0 × 2.5-cm column. Pro-MSP solution was passed through the kallikrein column at a flow rate of 5.0 ml/h at 37°C. The eluate was adjusted to pH 5.0 and applied to a 1.6 × 15-cm CM-Sepharose column. The column was washed with pH 5.0, 10 mM Na-acetate buffer and proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer.

Preparation of S-carboxymethyl proteins. Proteins in 1 M Tris–HCl, 5 M guanidine hydrochloride, and 0.2% EDTA were incubated in the presence of 2-mercaptoethanol at 50°C for 15 h, and then incubated with monoiodoacetic acid at 30°C for 1 h in the dark. The resulting S-carboxymethyl protein was separated by reverse-phase HPLC on Phenyl 5PW-RP, 4.6 × 75mm (TOSOH). Solvent A was 0.1% trifluoracetic acid (TFA) and solvent B was isopropanol:acetonitrile (1:1) containing 0.1% TFA. The column was preequilibrated with solvent A. After the sample was injected, materials were eluted with a linear gradient from 0 to 100% solvent B in 50 min. Flow rate was 1 ml/min. Recovered fractions were lyophilized.

Amino acid analysis and sequencing. A Hitachi L-8500 high-performance amino acid analyzer was used. Amino acid sequence was determined by Edman degradation with an Applied Biosystems

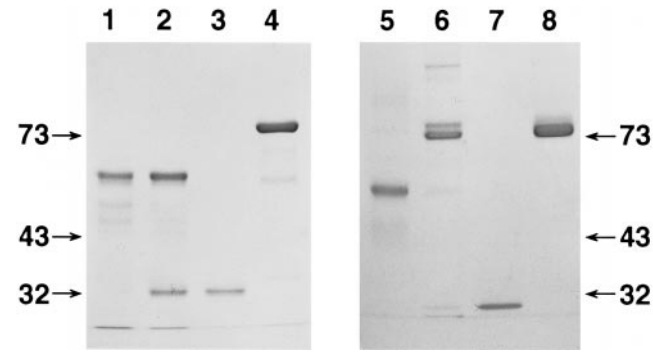

FIG. 2. SDS–PAGE of pooled fractions from peaks 1–3 shown in Fig. 1. Lanes 1–4, reducing conditions; lanes 5–8, nonreducing. Lanes 1 and 5, peak 1; lanes 2 and 6, peak 2; lanes 3 and 7, peak 3; lanes 4 and 8, recombinant pro-MSP. Molecular masses (kDa) of protein standards are shown in margins.

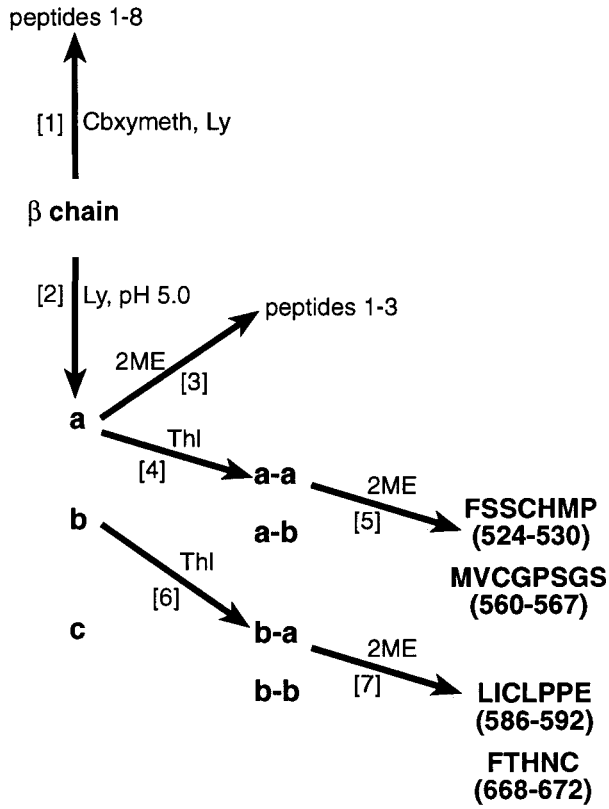

FIG. 3. Flow diagram of partial digestion of MSP β-chain to establish disulfide bond locations: a representative result locating C_{527}–C_{562} and C_{588}–C_{672}. [1] Digestion with lysylendopeptidase and S-carboxymethylation yielded eight peptides (with disulfides reduced) comprising the entire β-chain. They were isolated and sequenced to establish locations of cysteines. [2] Digestion of β-chain with lysylendopeptidase at pH 5.0 yielded three disulfide-linked peptide complexes, **a, b,** and **c.** [3] Reduction of complex **a** showed that it comprised peptides 1–3 of the eight obtained in step 1. [4] Partial digestion of complex **a** with thermolysin yielded two disulfide-linked complexes, **a–a** and **a–b.** [5] Reduction of **a–a** yielded the two peptide sequences shown, establishing the C_{527}–C_{562} linkage. [6] Digestion of complex **b** with thermolysin yielded disulfide-linked complexes, **b–a** and **b—b.** [7] Reduction of **b–a** yielded the two peptide sequences that establish the C_{588}–C_{672} linkage.

477A protein sequencer and Shimadzu PPSQ-10 protein sequencer. For removal of α-chain pyroglutamic acid prior to amino acid sequencing, 500 pmol of *S*-carboxymethyl α-chain in 200 μl of 0.1 M phosphate buffer (pH 8.0) containing 10 mM EDTA, 5 mM dithiothreitol, and 5% glycerol was incubated at 37°C for 8.5 h with 3 μg calf liver pyroglutamate aminopeptidase. Products were separated by reverse-phase HPLC on Phenyl 5PW-RP.

Limited proteolysis. Limited proteolysis of *S*-carboxymethyl protein was carried out by incubation with lysylendopeptidase in 0.1 M Tris–HCl buffer (pH 9.5) at 37°C for 15 h. For identification of disulfide bonds, liimited proteolysis with lysylendopeptidase and thermolysin was at pH 5.0. For deglycosylation after limited proteolysis, *S*-carboxymethyl α- and β-chains were heated at 100°C for 2 min and then incubated with *N*-glycosidase at 37°C for 15 h. Peptide fragments produced by limited proteolysis were separated by reverse-phase HPLC on a μBondasphere 5 μ C18 3.9 × 150-mm column (Waters).

HPLC-ESI mass spectrometry. Peptide fragment mixtures, with or without deglycosylation, were analyzed by ESI mass spectrometry (Finnigan MAT TSQ7000 ESI Mass spectrometer).

RESULTS

Cleavage of recombinant pro-MSP by human kallikrein and characterization of the products. After pro-MSP was cleaved on a column of immobilized human kallikrein, chromatography on CM-Sepharose yielded three well-separated peaks (Fig. 1). SDS–PAGE of the three peaks is shown in Fig. 2. Peak 2 appeared to be mature $\alpha\beta$-chain MSP, since it migrated as an approximately 80-kDa protein under nonreducing conditions (lane 6), and as 55- and 30-kDa proteins under reducing conditions (lane 2). Biological activity of the peak 2 protein, determined as a chemoattractant for macrophages (8), was approximately equivalent to that of native MSP purified from human serum (data not shown). Peaks 1 and 3 had SDS–PAGE mobilities corresponding to molecular masses of about 55 kDa (lanes 1 and 5) and 30 kDa (lanes 3 and 7), respectively. Since the N-terminus of peak 1 was blocked, treatment of peak 1 with pyroglutamate aminopeptidase was required for Edman degradation, which showed that the N-terminus of peak 1 began with Gln_{19} of the α chain of MSP. This was confirmed by HPLC-ESI mass spectrometry (c.f. Fig. 4). N-terminal amino acid sequence analysis of peak 3 showed that it began with Val_{484}. Thus, human kallikrein cleaved only at Arg_{483}–Val_{484}, the cleavage site for formation of α- and β-chains. The coexistence of free α-chain, free β-chain, and the disulfide-linked heterodimer after cleavage at this site indicates that recombinant pro-MSP is heterogeneous: Some molecules have an $\alpha\beta$-chain disulfide linkage, and others do not.

Analysis of free β-chain for disulfide bond positions. Wahl *et al.* (17) reported that the presence of free α- and β-chains among recombinant MSP molecules was due to formation of a β-chain Cys_{588}–Cys_{672} disulfide, which prevented formation of the expected $\alpha\beta$-chain Cys_{468}–Cys_{588}. Their conclusion was based on the fact that replacement of Cys_{672} by Ala prevented formation of the mismatched disulfide and resulted in a normal $\alpha\beta$-chain disulfide. After independently obtaining the same mutagenesis result, we established the presence of Cys_{588}–Cys_{672} by direct localization of β-chain disulfides. Disulfide bond positions in the β-chain were established by a series of limited digestions, peptide separation by HPLC, and N-terminal sequencing, as outlined in the legend to Fig. 3. The direct demonstration of Cys_{588}–Cys_{572} confirmed the conclusion that coopting Cys_{588} by Cys_{672} prevented $\alpha\beta$-chain linkage between Cys_{468} and Cys_{588}. Our analysis also established the presence of Cys_{527}–Cys_{562}, which is not found in serine protease-like domains of other proteins in the plasminogen-related family.

N-linked oligosaccharide analysis. Purification of separate α- and β-chains provided material for further analysis of these subunits. MSP is a glycoprotein with three possible N-linked glycosylation sites, two in the

TABLE I

Assignment of Oligosaccharide Composition to Glycopeptide Mass Signals

Glycosylation site	Glycopeptide mass	Oligosaccharide mass[a]	Candidate oligosaccharide (mass)[b]
Asn72	12334	2352	$NANA_2Gal_2Man_3GlcNAc_4Fuc_1$ (2350)
	12042	2060	$NANA_1Gal_2Man_3GlcNAc_4Fuc_1$ (2059)
	11895	1913	$NANA_1Gal_2Man_3GlcNAc_4$ (1913)
	11752	1770	$Gal_2Man_3GlcNac_4Fuc_1$ (1768)
Asn296	6223	2353	$NANA_2Gal_2Man_3GlcNAc_4Fuc_1$ (2350)
	6077	2207	$NANA_2Gal_2Man_3GlcNAc_4$ (2204)
	5931	2061	$NANA_1Gal_2Man_3GlcNAc_4Fuc_1$ (2059)
	5785	1915	$NANA_1Gal_2Man_3GlcNAc_4$ (1913)
	5638	1768	$Gal_2Man_3GlcNAc_4Fuc_1$ (1768)
	5491	1621	$Gal_2Man_3GlcNAc_4$ (1622)
Asn616	4939	2350	$NANA_2Gal_2Man_3GlcNAc_4Fuc_1$ (2350)
	4648	2059	$NANA_1Gal_2Man_3GlcNAc_4Fuc_1$ (2059)
	4357	1768	$Gal_2Man_3GlcNAc_4Fuc_1$ (1768)
	4284	1695	$NANA_1Gal_1Man_3GlcNAc_3Fuc_1$ (1694)
	4121	1532	$NANA_1Gal_1Man_2GlcNAc_3Fuc_1$ (1532)
	3993	1404	$Gal_1Man_3GlcNAc_3Fuc_1$ (1403)
	3830	1241	$Gal_1Man_2GlcNAc_3Fuc_1$ or $Man_3GlcNAc_3Fuc_1$ (1241)

[a] Oligosaccharide mass: glycopeptide mass minus deglycosylated peptide mass. The masses of the three deglycosylated Asn-containing peptides in the order listed are 9982, 3870, and 2589 Da.
[b] Residue masses: NANA, 291 Da; Man, Gal, 162 Da; Glc, 203 Da; Fuc, 146 Da.

α-chain at Asn_{72} and Asn_{296} and one in the β-chain at Asn_{615}. To deduce the composition and structure of these N-linked oligosaccharides, we generated peptide fragments of S-carboxymethylated α- and β-chains with or without prior treatment with *N*-glycosidase. Peptide fragments were separated, sequenced, and analyzed by HPLC-ESI mass spectrometry. The three Asn-containing peptide fragments after deglycosylation showed single mass spectra, with molecular masses of 9982, 3870, and 2589 Da. In contrast, spectra of the corresponding peptides before deglycosylation showed multiple peaks, the molecular masses of which are listed in the second column of Table I. A representative result is shown in Fig. 4. These data indicate that the N-linked oligosaccharides at the three Asn loci are heterogeneous. The multiple structures were deduced by comparison of their molecular masses with the masses of a series of candidate oligosaccharides (Table I). Four different sugars are bound to Asn_{72}, six to Asn_{296}, and eight to Asn_{615}. Five of the eight Asn_{615} sugars are unique to this β-chain site. By two-dimensional sugar mapping, the oligosaccharides were sialylated fucosyl biantennary structures identical to those found in HGF (21) (data not shown).

DISCUSSION

Purification of the products of kallikrein-treated pro-MSP provided us with biologically active disulfide-linked $\alpha\beta$-chain MSP and also free α- and β-chains. This gave us the opportunity to characterize MSP and its components and to compare it with the closely related HGF/SF. From the sequence of the MSP cDNA, Yoshimura *et al.* predicted that the signal peptide would terminate at Gly_{18} and that the N-terminus of the α chain would begin with Gln_{19} (9). The present work confirmed this and showed that the N-terminus is pyroglutamate, as it is for HGF (22). Other similarities to HGF include the same scissile Arg–Val bond for conversion to the two-chain active form of the protein (23) and similar multiple N-linked oligosaccharides (21).

We also used the purified α- and β-chains to determine the region of MSP that binds to its receptor. In contrast to the similarities between MSP and HGF noted above, there is a surprising difference in the primary receptor binding site, which is in the α-chain for HGF (24) and in the β-chain for MSP (25). We estimated a K_d of 1.7 nM for binding of the β-chain to the RON receptor, higher than the K_d of 0.6–0.8 nM for binding of the $\alpha\beta$-chain heterodimer to RON. Could free β-chain be an MSP inhibitor *in vivo?* Although excess β-chain can partially inhibit binding of MSP to RON, a concentration of 100 nM was required to partially inhibit autophosphorylation of RON induced by a physiologically relevant concentration of 5 nM MSP (25). This would not occur *in vivo,* since the total concentration of pro-MSP in human plasma (including any molecules with a possibly mismatched disulfide) is only 5 nM (11).

Inasmuch as biological activity of HGF and MSP requires both α- and β-chains, we suggested that

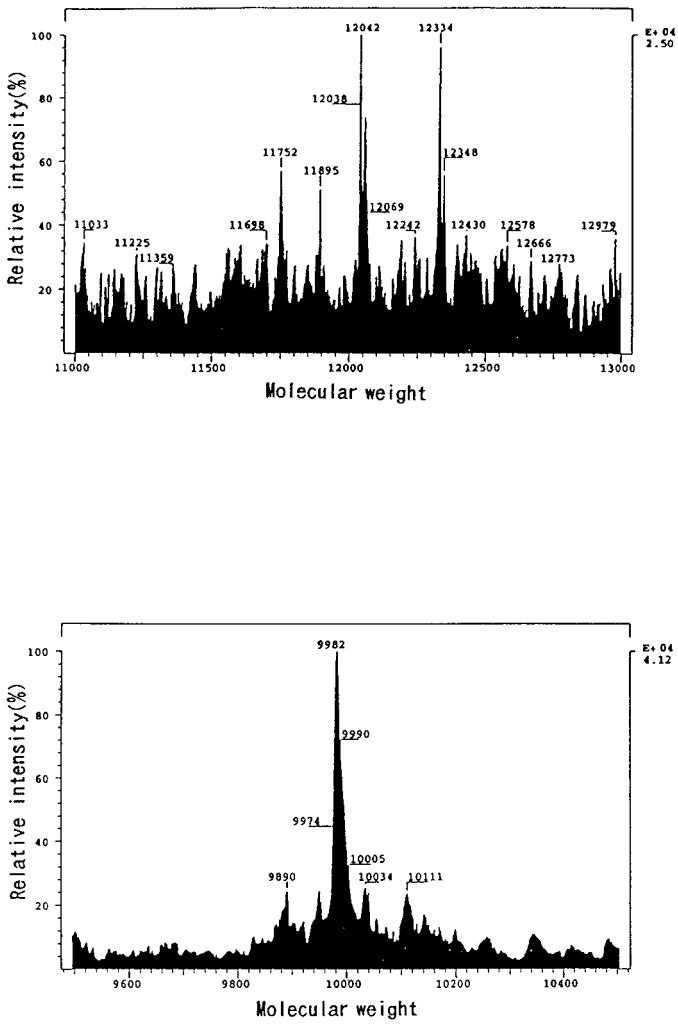

FIG. 4. Reconstructed ESI-mass spectra of MSP α-chain glycopeptide Gln19–Lys103, derived from S-carboxymethylated α-chain after digestion with lysylendopeptidase. (Top) Multiple peaks reflecting heterogeneity of oligosaccharides linked to Asn72 of this peptide. (Bottom) Result with deglycosylated peptide.

dimerization of these typical protein tyrosine kinase receptors is mediated by two binding sites on the ligands (26), a model that has been confirmed for induction of receptor dimerization by growth hormone (27). For MSP, there would be a high-affinity binding site on the β-chain and a low-affinity site on the α-chain. We have obtained support for this model by detection of α-chain binding to receptor and identification by mutagenesis residues critical for β-chain binding (A. Danilkovitch *et al.*, unpublished data).

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