Macrophage Stimulating Protein (MSP) Binds to Its Receptor via the MSP β Chain*

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Macrophage stimulating protein (MSP) is a 78-kDa disulfide-linked heterodimer belonging to the plasminogen-related kringles protein family. MSP activates the RON receptor protein-tyrosine kinase, which results in cell migration, shape change, or proliferation. A structure-activity study of MSP was performed using pro-MSP, MSP, MSP α and β chains, and a complex including the first two kringles and IgG Fc (MSP-NK2). Radiiodinated MSP and MSP β chain both bound specifically to RON. The Kₐ of 1.4 nm for MSP β chain is higher than the reported Kₐ range of 0.6–0.8 nm for MSP. Pro-MSP, MSP α chain, and MSP-NK2 did not bind. Only MSP stimulated RON autophosphorylation. Although the β chain bound to RON and partially inhibited MSP-induced RON phosphorylation in kidney 293 cells, it did not induce RON phosphorylation. Pro-MSP, MSP α chain, or MSP-NK2 failed to activate RON, consistent with their inability to bind to the RON receptor. Functional studies showed that only MSP induced cell migration, and shape change in resident macrophages, and growth of murine keratinocytes. Our data indicate that the primary receptor binding domain is located in a region of the MSP β chain, in contrast to structurally similar hepatocyte growth factor, in which the receptor binding site is in the α chain. However, full activation of RON requires binding of the complete MSP disulfide-linked αβ chain heterodimer.

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The abbreviations used are: MSP, macrophage stimulating protein; HGF, hepatocyte growth factor; SF, scatter factor; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; MDCK, Madin-Darby canine kidney.

but is devoid of enzymatic activity due to amino acid substitutions in the catalytic triad. MSP belongs to the kringles protein family that includes plasminogen (2) and hepatocyte growth factor/scatter factor (HGF/SF) (3, 4). MSP is synthesized mainly by liver cells (5, 6), circulates in blood as a biologically inactive single chain precursor (7), and is cleaved by members of the kallikrein family (8, 9) or by trypsin-like enzymes located on macrophage surfaces (7). Recent functional studies have revealed that in addition to induction of macrophage shape change, chemotactic migration (10), and phagocytosis of C3bi-coated erythrocytes (1), MSP has other activities. These include inhibition of expression of inducible nitric oxide synthase mRNA in endotoxin or cytokine-stimulated macrophages (11), induction of interleukin-6 production and differentiation of megakaryocytes (12), suppression of colony formation of human bone marrow cells induced by Steel factor plus granulocyte macrophage-stimulating factor (13), increase in beat frequency of nasal epithelium cilia (14), and stimulation in vitro of proliferation of certain epithelial cell lines (15–17).

The receptor for MSP was recently identified as the human RON gene product (18), a transmembrane receptor protein-tyrosine kinase cloned from a human keratinocyte cDNA library (19). The murine STK gene cloned from hematopoietic stem cells of bone marrow is the homologue of human RON (20, 21). The RON gene encodes a 190-kDa heterodimeric protein composed of a 40-kDa extracellular α chain and a 150-kDa transmembrane β chain with intrinsic tyrosine kinase activity (21). This property places the product of the RON/STK gene into a subfamily of receptor tyrosine kinases that includes proto-oncogene MET and SEA (22, 23). These receptors share many unique structural properties including a putative proteolytic cleavage site, similar location of cysteine residues in their extracellular domain, and two conserved tyrosines in the C-terminal tail (19, 20, 22, 23). Studies of the signaling pathways of RON have shown that tyrosine-phosphorylated RON associates in vivo with intracellular signal transducers, including Grb-2-Sos and phosphatidylinositol 3-kinase (17, 24).

In this work, we initiated a structure-activity study of MSP to identify functionally important domains that interact with the RON receptor. Five purified recombinant proteins were used, including pro-MSP, MSP, MSP α and β chains, and the MSP N terminus (including the first two kringles) fused to human IgG Fc. We report the binding capacity of MSP and its subunits to RON receptor in intact cells. We also analyzed the capacity of MSP and its subunits to induce receptor phosphorylation and consequent cellular responses.

MATERIALS AND METHODS

Reagents—Human mature plasma MSP was purified as described (1). Human recombinant single chain pro-MSP was derived from CHO...
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Mice peritoneal resident macrophages were obtained from C3H/HeN mice by lavage of the peritoneal cavity with 15 ml of sterile RPMI 1640 medium containing 0.5% fetal bovine serum (8). To see the effect of MSP subunits and their different combinations were added. After incubation at 37 °C for 45 min, cells were photographed.

Cell Migration Assay—The assay was done as described (18). Bottom wells of a chemotaxis chamber were filled with triplicate with 30 μl of RPMI 1640 medium containing different amounts of MSP or MSP subunits and then covered with a polycarbonate membrane coated with mouse collagen IV. Upper wells were filled with 45 μl of cell suspension (2 × 10⁶/ml in RPMI 1640 medium). To see the effect of MSP subunits on MSP-induced migration, cells were first mixed with 5 or 30 nM of MSP α or β chain or MSP-NK2 and then added to top wells. After a 3-h incubation at 37 °C, the chamber was disassembled, and the membranes were dried in air. The migrated cells were stained and counted with an image analyzer. The results were expressed as the percentage of input cells that migrated.

Cell Proliferation Assay—The experiments were performed as described (15). BK-1 cells at a concentration of 10⁶/ml of a serum-free medium (equal volumes of keratinocyte serum free-medium, Eagle’s minimum essential medium, and CHO-SM medium) were seeded at 100 μl/well in a 96-well culture plate. MSP, MSP α or β chains, MSP-NK2, or their different combinations were added. Cells without stimulation served as control. After incubation for 5 days, cells were stained and lysed in 1% SDS buffer. Color intensity was measured at 570 nM in an enzyme-linked immunosorbent assay plate reader. Absorbance was converted into cell number by reference to a standard curve derived from stained cell concentration.

RESULTS

Absorption of Free MSP β Chain by MDCK-RE7 or 3T3/STK Cells—In the course of studying pro-MSP conversion into mature MSP, we noticed that about 30% of our metabolically [35S]-labeled recombinant pro-MSP lacked the disulfide link between its α and β chain, which resulted in free α and β chain after specific cleavage by kallikrein of the pro-MSP R-V bond at the αβ chain junction (data not shown). We took advantage of

FIG. 1. SDS-PAGE of recombinant human pro-MSP, human serum MSP, recombinant MSP α chain, MSP β chain, and MSP-NK2 (fused with IgG Fc). Proteins (1.5–4 μg) were dissolved in sample buffer with or without 2-mercaptoethanol and separated in 10% polyacrylamide gel and stained with Coomassie Blue.

Cells—Madin-Darby canine kidney (MDCK) cells transfected with a human RON cDNA (clone RE7) (18), NIH3T3 cells transfected with murine STK cDNA (21), and CHO-K1 cells transfected with human MSP cDNA (clone 18) (8) were described (18). Human kidney 293 cells were from ATCC (Rockville, MD). Murine keratinocyte cell line (clone RE7) (18), NIH3T3 cells transfected with human RON cDNA (clone RE7) (18), NIH3T3 cells transfected with human MSP cDNA and purified in two steps by Excellulose GF-5 desalting column (Pierce) equilibrated with phosphate-buffered saline containing 0.2% leupeptin, 20 μg/ml aprotinin, and 50 μg/ml soybean trypsin inhibitor). Lysate proteins were precipitated with monoclonal antibody ID2 to RON or rabbit anti-STK serum coupled to protein G-Sepharose beads. Samples were dissolved in sample buffer with 2-mercaptoethanol, separated on a 7.5% polyacrylamide gel by SDS-PAGE, and transferred to Immobilon-P (Millipore). Membranes were blocked with 1% bovine serum albumin in 0.15 M NaCl, 0.5% Tween 20. After incubation with 0.2 μg/ml anti-phosphotyrosine antibody overnight, followed by goat anti-mouse IgG conjugated with horseradish peroxidase. The horseradish peroxidase reaction was developed with ECL detection reagents. In some experiments, the membrane was treated with SDS-2-mercaptoethanol erasure buffer and reprobed with rabbit anti-RON serum as described (18).
this finding to determine if free α or β chain binds to the MSP receptor (human RON or murine STK) using an absorption assay. When 35S-labeled pro-MSP was cleaved by kallikrein and then equilibrated with RON-expressing MDCK-RE7 or NIH3T3/STK cells or nontransfected control cells, the decrease in intensity of the β chain lines is due to absorption of free β chain by transfected cells.

Assay for Binding of 125I-pro-MSP, MSP, and Its Subunits to Cells Expressing RON or STK—We tested for binding of radiolabeled pure MSP and its subunits to murine keratinocyte BK1 and MK308 cells, which express 10,000–15,000 STK receptors/cell (15). Fig. 3 shows that in both cell lines, specific binding of 125I-MSP was inhibited in a concentration-dependent manner by unlabeled MSP β chain but not by MSP α chain. On a molar basis, MSP is more potent than the free β chain as a competitive inhibitor of labeled β chain binding. Pro-MSP did not compete with MSP for RON, as previously reported (15). Binding of 125I-MSP β chain to MDCK-RE7 cells is shown in Fig. 4. Binding of the MSP β chain to the RON receptor was specific; either unlabeled MSP or MSP β chain inhibited binding of 125I-MSP β chain. From Fig. 4, we estimated a Kd for binding of the MSP β chain of about 1.7 nM, higher than the Kd values of 0.6 to 0.8 for binding of MSP to the RON receptor (17). On the other hand, we did not detect specific binding of the MSP α chain to the RON receptor (data not shown). The relatively low binding of 125I-MSP-NK2 to the cell surface was unaffected by unlabeled MSP or MSP-NK2, indicating that the interaction of labeled MSP-NK2 with MDCK-RE7 cells is nonspecific (Fig. 5).

Induction of RON Tyrosine Phosphorylation by MSP and Its Subunits—We next studied tyrosine phosphorylation of RON induced by pro-MSP, MSP, and its subunits in kidney 293 and MDCK-RE7 cells. After precipitation of proteins with ID2 anti-RON, Western blot with monoclonal antibody 4G10 to phosphorylated Tyr showed that only MSP-induced tyrosine phosphorylation of the 150-kDa RON β chain (Fig. 6A). No phosphorylated proteins were observed in cells treated with pro-MSP, MSP α chain, or MSP-NK2 protein. Interestingly, although MSP β chain binds to RON, it failed to induce RON autophosphorylation, indicating that MSP β chain alone is insufficient to activate RON. Experiments were also designed to study if MSP α chain, MSP β chain, or NK2 protein could modulate RON phosphorylation. Fig. 6B shows that high con-
centrations of MSP β chain could partially inhibit MSP-induced tyrosine phosphorylation of RON. No inhibition was observed by MSP α chain or MSP-NK2 protein.

Effect of MSP Subunits on MSP-induced Cell Shape Change and Migration—To see if MSP subunits can induce cell shape change or migration, mouse peritoneal resident macrophages were used. Fig. 7 shows that MSP subunits can induce cell shape change or migration, mouse peritoneal resident macrophages did not induce morphological changes in resident macrophages. In combination with MSP, none of these three subunits inhibited the biological effect of MSP on macrophages. Likewise, except for a statistically insignificant effect of MSP β chain, the addition of MSP subunits to macrophages did not inhibit their migration toward MSP as a chemoattractant (data not shown).

Effect of MSP Subunits on MSP-stimulated Cell Proliferation—BK-1 keratinocytes were used to assess if MSP α chain, MSP β chain, or MSP-NK2 protein at high concentration could affect MSP-induced cell proliferation. Table I shows that only MSP increased cell number after 5 days in culture, compared with the medium control. In combination experiments, none of the fragments affected MSP-induced proliferation, except for a small inhibition by 100 nM MSP β chain.

DISCUSSION

We have presented several lines of evidence that the MSP β chain binds to RON. 1) Metabolically labeled free β chain, but not α chain, was specifically absorbed by cells expressing the RON receptor. 2) 125I-β chain bound to RON in intact cells in a specific and saturable manner. 3) Not only unlabeled MSP but also β chain competitively inhibited binding of 125I-MSP to RON in intact cells. Thus, in contrast to the β chain of HGF/SF, which does not bind to its receptor (Met) (27), the β chain of MSP appears to contain the primary binding site for the RON receptor. The β chain is the serine protease domain of kringle chain, fused to IgG Fc. We found that neither MSP-NK2 nor MSP α chain bound to RON on intact cells including kidney 293 and MDCK Fc. We cannot explain the reported activity of MSP-NK2, especially because the source of the MSP-NK2 was the same. However, our β chain data combined with the fact that free α chain does not bind to RON support the conclusion that MSP binds to its receptor via the β chain.

We have shown that although the MSP β chain binds to RON, it does not cause biological activity or induce phosphorylation of the receptor, except for a small amount at high ligand concentrations. It is generally accepted that ligand binding to growth factor receptors is associated with receptor oligomerization and autophosphorylation (30). Receptor oligomerization may be mediated by interaction of ligand pairs. In this context, our results indicate that receptor oligomerization requires an intact αβ chain disulfide-linked heterodimeric ligand. Although HGF differs from MSP in that the primary binding pocket, the corresponding locations of which are Gly in HGF. The reported activity of MSP-NK2, especially because the source of the MSP-NK2 was the same. However, our β chain data combined with the fact that free α chain does not bind to RON support the conclusion that MSP binds to its receptor via the β chain.

There is one report that in RON cDNA-transfected COS-1 cells, MSP-NK2 stimulated RON phosphorylation (16). MSP-NK2 is a recombinant protein comprising the first two kringle chains of HGF/SF, 2 M. Miller, A. Danilkovitch, and E. J. Leonard, unpublished data.

**Fig. 5. Absence of specific binding of **125I-MSP-NK2 to MDCK-RE7 cells. Equilibration conditions were as described for the experiment illustrated in Fig. 4. Nonspecific binding was determined by equilibration with a 30-fold excess of unlabeled MSP-NK2 or MSP. The results from one of two experiments are shown.

**Fig. 6. Induction of autophosphorylation of the RON receptor by pro-MSP, MSP and its subunits.** Cells (3 × 10⁶/ml) were incubated at 37 °C for 15 min with 5 nM protein in 1 ml of serum-free RPMI 1640 medium. Lysates were immunoprecipitated with monoclonal anti-RON antibody. Samples were loaded on 7.5% polyacrylamide gel under reducing conditions. After transfer of proteins to Immobilon-P, the membrane was probed with antibodies to phosphotyrosine (4G10), and developed with ECL. A, MDCK-RE7 cells. B, kidney 293 cells. The Control lane is for cells stimulated with 5 nM HGF/SF.
site resides in the α chain, two or three amino acid substitutions in the β chain are sufficient to reduce biological activity to less than 2% that of wild type HGF (31). Thus, for both MSP and HGF the αβ chain heterodimer is required to fully activate receptor II. The authors suggest that ligand binding to receptor II is stabilized by interaction between the two receptor domains near their C terminus. If this model applied to the RON receptor, the candidate region for binding to receptor I might be a cluster of β chain surface loop arginines; a single arginine on the N domain hairpin loop of the α chain (29) might mediate binding to receptor II. The Arg cluster density for the corresponding regions of HGF is reversed, which is consistent with receptor binding by the α chain. This model would account for primary binding by MSP β or HGF-α, and the requirement for binding by the αβ chain heterodimer for optimal receptor activation. The model should be readily testable by mutagenesis studies.

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