Proteolytic Activation of Pro-Macrophage-Stimulating Protein by Hepsin

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Abstract

Macrophage-stimulating protein (MSP) is a plasminogen-related growth factor and ligand for the receptor tyrosine kinase RON. The MSP/RON system promotes wound healing and invasive tumor growth and suppresses proinflammatory immune response. MSP binding to RON requires proteolytic conversion of the inactive single-chain form (pro-MSP) into the disulfide-linked α/β heterodimer. The pro-MSP cleavage sequence (Ser-Lys-Leu-Arg<sup>483</sup>-Val<sup>484</sup>) closely matches the substrate recognition sequences of hepsin, a type II transmembrane serine protease, that is overexpressed in several cancers. Here, we show that recombinant hepsin cleaves pro-MSP at the consensus site Arg<sup>483</sup>-Val<sup>484</sup> with superior efficiency compared with the known activators MT-SP1 and hepatocyte growth factor activator (HGFA). At least 50% of pro-MSP was processed within 1 hour at a hepsin concentration of 2.4 nmol/L and at a molar enzyme to substrate ratio of 1:500. An uncleavable single-chain variant of MSP weakly bound to a RON–Fc fusion protein, whereas hepsin-cleaved MSP bound with a K<sub>D</sub> of 10.3 nmol/L, suggesting that the high-affinity binding site in MSP β-chain was properly formed. LNCaP prostate cancer cells overexpressing hepsin on the cell surface efficiently activated pro-MSP, which was blocked by a specific anti-hepsin antibody. Incubation of pro-MSP with hepsin led to robust RON-mediated phosphorylation of mitogen-activated protein kinase, ribosomal S6 protein, and Akt in human A2780 ovarian carcinoma cells stably expressing RON protein. In macrophages, pro-MSP with hepsin induced chemotaxis and attenuated lipopolysaccharide-dependent production of nitric oxide. These findings suggest that the MSP/RON signaling pathway may be regulated by hepsin in tissue homeostasis and in disease pathologies, such as in cancer and immune disorders. Mol Cancer Res; 9(9); 1–12. ©2011 AACR.

Introduction

Macrophage-stimulating protein (MSP, also known as hepatocyte growth factor–like protein, HGFL) is a plasminogen-like growth factor that mediates its biological activities by activating the receptor tyrosine kinase RON [recepteur d’origine nantais, also known as macrophage stimulating receptor-1, (MSTR1)], a member of the MET proto-oncogene family (1). MSP shares high sequence and structural homology with HGF, the ligand for MET; similarly, their respective cognate receptors, RON and MET, also share high sequence and domain homology (2). The α-chain of MSP consists of an N-terminal PAN sequence followed by 4 kringle domains and is disulfide linked to the trypsin-like β-chain (3). MSP is constitutively expressed by hepatic parenchymal cells, as well as in lungs, adrenal glands, placenta, kidney, and pancreas (4, 5). It is secreted as an inactive single-chain precursor pro-MSP, which requires proteolytic cleavage at the Ser-Lys-Leu-Arg<sup>483</sup>-Val<sup>484</sup> bond to attain functional activity (5, 6). In HGF, cleavage at the corresponding Arg<sup>494</sup>-Val<sup>495</sup> bond results in distinct structural rearrangements within the HGF β-chain and the formation of a MET-binding site that is competent for signal transduction (7–9). Comparable conformational rearrangements centered at the “pseudo-active site” in the MSP β-chain are likely to occur on activation cleavage of pro-MSP (7). However, unlike HGF, where the high-affinity MET-binding site resides in the α-chain, the high-affinity RON-binding site for MSP is located on the mature β-chain that forms following pro-MSP cleavage (10). Several trypsin-like serine proteases, including MT-SP1 (also known as matrissipe) and HGF activator (HGFA; refs. 11–15), are known to activate pro-MSP, leading to typical cellular responses mediated by the RON signaling pathway (11, 12).

Hepsin is a cell surface–expressed trypsin-like protease and a member of the type II transmembrane serine protease family (16, 17). It consists of an N-terminal cytoplasmic...
domain, a transmembrane domain, and an extracellular portion composed of a scavenger receptor–like cysteine-rich domain and a C-terminal protease domain with a trypsin-like fold (18, 19). Hepsin was identified as one of the most highly upregulated genes in prostate cancer (20–25) and immunohistochemical staining revealed strong expression in late-stage tumors and metastatic bone lesions (26, 27). Studies with preclinical prostate cancer models suggested that hepsin may play a role in invasive cancer growth and cancer progression (28, 29). Moreover, gene expression analyses have also implicated hepsin in ovarian cancer (30), renal cell carcinoma (31, 32), and endometrial cancer (33). A putative function of hepsin in tumor progression could be related to its enzymatic activity toward the macro-molecular substrates pro-HGF (19, 34), pro-uPA (35), and laminin-332 (36). Additional substrates that have been identified are coagulation factors (37) and pro-prostasin (38). The hepsin cleavage sequences of these substrates are in good agreement with the consensus sequence obtained from substrate profiling by positional scanning of a synthetic combinatorial peptide library (19).

Here, we report that both recombinant soluble hepsin (sHepsin) and cell surface–expressed hepsin efficiently cleave human pro-MSP at the physiologic activation site. The hepsin-cleaved MSP was functionally competent for RON binding and in triggering cellular responses mediated by the RON signaling pathway. Finally, we detected significant coexpression of MSP and hepsin in a number of organs, suggesting a potential physiologic role of hepsin in regulating activity of MSP.

Materials and Methods

Cloning, expression, and purification of recombinant proteins

The extracellular domain of the human recombinant hepsin harboring a C-terminal His-tag (sHepsin) was expressed and purified as described (35). Recombinant RON (SEMA/IPT/TIG1 domains: Glu25-Met682) was made as Fc fusion protein by expressing it in Chinese hamster ovary (CHO) cells. RON–Fc was purified by affinity chromatography followed by size exclusion chromatography. Recombinant pro-MSP harboring a C-terminal His6 tag along with a C672A mutation, previously shown to yield better protein expression, was expressed in CHO cells as described (39). Secreted pro-MSP was purified by Ni-NTA affinity chromatography followed by size exclusion chromatography. Recombinant pro-MSP containing the Kunitz domain-1 (KD1) was expressed and purified as described (40, 41). Recombinant active human MSP was obtained from R&D Systems and used as reference material. Antibody25 (Ab25) inhibits hepsin enzymatic activity and was generated by using antibody phage display. Ab25 (IgG1) and Fab25 were expressed in CHO cells and Esherichia coli, respectively, and were purified according to standard procedures.

In vitro activation of pro-MSP by sHepsin, HGFA, and sMT-SP1

Pro-MSP (100 μg/mL = 1.25 mmol/L) was incubated for 1 hour at 37°C with different concentrations (100 nmol/L–97 pmol/L, 2-fold dilution series) of sHepsin, HGFA, and sMT-SP1 in a buffer containing 50 mmol/L Tris-HCl, pH = 8.0, 150 mmol/L NaCl, 0.05% Triton X-100, and 2 mmol/L CaCl2 for 1 hour at 37°C. In addition, 100 nmol/L of the proteases were preincubated for 15 minutes with 1 μmol/L of the hepsin-specific Fab25 or 1 μmol/L of KD1 (an inhibitor of hepsin, MT-SP1, and HGFA) before addition of pro-MSP. For time course experiments, 12.5 nmol/L sHepsin was incubated with 1.25 μmol/L of pro-MSP and aliquots were analyzed by SDS-PAGE and proteins stained with SimplyBlue Safe Stain (Invitrogen). The experiments were repeated 3 times. Similar experiments were carried out with 250 μg/mL of the scMSP mutant and hepsin (10 nmol/L). For the densitometric analysis of pro-MSP degradation, the pro-MSP band intensities were quantified using ImageJ (http://rsb.info.nih.gov/ij/index.html). The effective concentration to give 50% reduction (EC50) was determined by a 4-parameter fit (Kaleidagraph; Synergy Software).

sHepsin-cleaved MSP was prepared by incubating 100 μg/mL of pro-MSP with 15 nmol/L sHepsin for 1 hour, resulting in complete conversion to cleaved MSP, sHepsin was removed by adding molar excess of anti-hepsin Ab25 to the reaction mixture followed by Protein A-Sepharose chromatography. Thus purified MSP did not contain any residual hepsin as assessed by SDS-PAGE and enzymatic assays. A similar protocol was used to generate HGFA-cleaved MSP except that the anti-HGFA antibody Ab40 (42) was used to remove HGFA from the reaction mixture.

Pro-MSP activation by cell surface–expressed hepsin on LNCaP-34 cells

LNCaP-34 cells stably overexpressing human full-length hepsin were described previously (35). Confluent cultures in 24-well plates were incubated for 15 minutes at 37°C with 500 μL serum-free RPMI-1640 medium containing 1 mmol/L each of Ab25 or KD1 or Ac-KQLR chloromethyl ketone (Ac-KQLR-cmk; AnaSpec) or 10 nmol/L sHepsin. 125I-labeled pro-MSP, prepared as described for pro-HGF (34), was added (25 μg/mL) and incubated for 3 hours at 37°C. Aliquots were analyzed by SDS-PAGE (4%/20% gradient gel) followed by exposure to X-ray films. The intensities of the bands were quantified using ImageJ.
software. The loss of pro-MSP band intensity was used to determine the percentage of proteolytic activity.

**Binding of hepsin-activated pro-MSP to RON by surface plasmon resonance and ELISA**

For surface plasmon resonance measurements on a BIAcore-3000 instrument (GE Healthcare) rabbit anti-human IgG was immobilized (amine coupling) on CM5 biosensor chips and the RON–Fc fusion protein was captured to give approximately 250 response units (RU). Different concentrations of either sHepsin-activated MSP or reference MSP (R&D Systems) or scMSP were injected in HBS-P buffer (10 mmol/L HEPES, pH = 7.5, 150 mmol/L NaCl, 0.005% P20) at 25°C with a flow rate of 30 μL/min. Association rates (k_a) and dissociation rates (k_d) were obtained by using a simple one-to-one Langmuir binding model (BIA-Evaluation software) and the equilibrium dissociation constants (K_D) were calculated (k_d/k_a). For ELISA experiments, maxiSorp microtiter plates (Nunc) were coated overnight at 4°C with 2 μg/mL of rabbit anti-human IgG Fc-specific antibody (Jackson ImmunoResearch Laboratory) in 50 mmol/L sodium carbonate buffer, pH = 9.6. After blocking with assay buffer (PBS, pH = 7.4, 0.5% BSA, and 0.05% Tween-20, 15 ppm Proclin), 1 μg/mL RON–Fc was added and incubated for 1 hour. After washing with PBS, 0.05% polysorbate 20, MSP proteins were added and incubated for 1 hour. Bound MSP was detected using anti-His-HRP (Qiagen) and TMB/H_2O_2 substrate (KPL). The reaction was stopped with 1 mol/L H_3PO_4 and the absorbance at 450 nm was measured.

**Peritoneal macrophage chemotaxis and morphology change assay**

Murine peritoneal resident macrophages were obtained from C57BL/6 mice by washing the peritoneal cavity with 15 mL of serum-free RPMI-1640 medium. Cells were washed and resuspended in medium to a concentration of 1 × 10⁶ cells/mL. The macrophage chemotaxis assay was conducted using a QCM chemotaxis assay kit with a pore size of 5 μm (Millipore). To the bottom wells was added RPMI-1640 medium containing the following components: (a) 80 ng/mL pro-MSP, (b) 80 ng/mL scMSP, (c) 80 ng/mL MSP from R&D Systems, (d) 80 ng/mL pro-MSP and 10 nmol/L sHepsin, and (e) 80 ng/mL pro-MSP, 10 nmol/L sHepsin, and 100 nmol/L Ab25. The reaction mixtures (a–e) were preincubated at 37°C for 1 hour before adding to the bottom wells. Macrophage suspension (10⁵ cells/100 μL) was added to the upper wells. After incubation at 37°C for 4 hours, the migrated cells were collected using detachment buffer (Millipore) and

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**Figure 1.** A, activation of pro-MSP by sHepsin, sMT-SP1, and HGFA. Purified pro-MSP (100 μg/mL = 1.25 μmol/L) was incubated for 1 hour at 37°C with different concentrations (100 nmol/L–97 pmol/L) of recombinant soluble hepsin (sHepsin) or the known activators sMT-SP1 and HGFA. For control experiments, the proteases (100 nmol/L) were incubated with the KD1 (inhibitor of Hepsin, MT-SP1, and HGFA) or the hepsin-specific antibody Fab25. Products were separated by SDS-PAGE under reducing conditions. N-terminal sequencing identified the ~50-kDa band as MSP α-chain and the ~30-kDa band as MSP β-chain. Intensities of the pro-MSP band were quantified by densitometry and plotted against enzyme concentrations. The EC₅₀ values are the average ± SD of 3 independent experiments. B, activation of pro-MSP by cell surface-expressed hepsin. LNCaP-34 prostate cancer cells, which stably overexpress full-length hepsin, were incubated with [¹²⁵I]-pro-MSP alone or in combination with different inhibitors for 3 hours. In a control experiment, no significant proteolytic cleavage of pro-MSP was observed on incubation for 3 hours (lane 1), whereas addition of sHepsin (10 nmol/L) led to nearly complete (>92%) [¹²⁵I]-pro-MSP conversion (lane 2). Efficient pro-MSP processing (>80%) by LNCaP-34 cells was observed (lane 4) compared with the start of the experiment (lane 1). All 3 hepsin inhibitors (Ac-KQLR-cmk, KD1, and Ab25) effectively blocked pro-MSP processing (lanes 5–7).
were seeded at a density of 2 × 10^5 cells/mL) were cultured in serum-free RPMI-1640 medium overnight. Nonadherent cells were removed and 80 ng/mL each of pro-MSP, sHepsin-cleaved MSP, HGFA-cleaved MSP, MSP from R&D Systems, or scMSP were added. After 1 hour, morphologic changes were captured by phase contrast microscopy.

**Inhibition of nitric oxide synthesis by mature MSP**

Bone marrow cells were isolated from femurs of C57BL/6 mice as described (11). After washing the cell suspension with Dulbecco’s Modified Eagle’s Media (DMEM), red blood cells were lysed with erythrocyte lysis buffer. Cells were resuspended in macrophage differentiation medium (DMEM with glutamine, 10% FBS, 1 × Pen/Strep, and 50 ng/mL mCSF-1) and added to 24-well plates. Medium was changed the next day and subsequently every second day. After 6 days, the mature macrophages were incubated for 24 hours at 37°C in 300 μL/well of serum-free medium with or without 1 μg/mL of lipopolysaccharide (LPS; Sigma) and containing the following: (a) 10 ng/mL pro-MSP, (b) 10 ng/mL pro-MSP and 1 nmol/L sHepsin, (c) 10 ng/mL pro-MSP, 1 nmol/L sHepsin, and 500 nmol/L Ab25, and (d) 10 ng/mL MSP (R&D Systems). Nitric oxide (NO) production was quantified by measuring the concentration of nitrite in diluted aliquots of culture medium by use of the Griess Reaction Kit (Molecular Probes).

**Phosphorylation of signaling proteins downstream of RON**

A2780 human ovarian carcinoma cells engineered to express human RON (A2780-RON; unpublished data) express human RON (A2780-RON; unpublished data) were transfected with 20 pmol of either RON-specific siRNA 5′-GGGCGACAGAAUGAGAGUTtt-3′ (Ambion/Applied Biosystems) or nontargeting control siRNA (Dharmacon/Thermo Scientific) using RNAi-Max (Invitrogen). Forty-eight hours following transfection, cells were serum starved for an additional 24 hours and incubated with indicated concentrations of pro-MSP, scMSP, MSP, and pro-MSP with sHepsin (10 nmol/L). After 1-hour incubation, cells were washed once with cold PBS followed by incubation on ice in lysis buffer (10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 10% glycerol, 0.1% Triton X-100, 5 mmol/L β-glycerophosphate, 2 mmol/L NaF, 1 mmol/L sodium orthovanadate, and protease and phosphatase inhibitors; Sigma Life Sciences). Lysates were clarified by centrifugation at 14,000 g, subjected to SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes, and incubated overnight with anti-RON C20 (Santa Cruz Biotechnology), anti-phospho-MAPK (Cell Signaling Technology), anti-phospho-S6 (Cell Signaling Technology), or anti-phospho-Akt (Cell Signaling Technology) antibodies. RON phosphorylation was analyzed by immunoprecipitating RON from cell lysates using anti-RON antibody, 1A2.2 (Genentech, Inc.), coupled to agarose beads. Immunoprecipitates were washed 3 times in cold lysis buffer, resuspended in 4 × SDS-PAGE sample buffer (Invitrogen), and analyzed after immunoblotting with anti-phospho-tyrosine pTyr-100 antibody (Cell Signaling Technology). The secondary antibodies used were IRDye800-conjugated goat anti-mouse IgG (Rockland) and AlexaFluor 680 goat anti-rabbit IgG (Invitrogen). Protein bands were visualized and quantified on the Odyssey Infrared Imaging System (LI-COR Biosciences) and the experiments were repeated 3 times.

**Computational gene expression analysis**

Microarray data on the Affymetrix HG-U133A and HG-U133B GeneChips were obtained from the GeneLogic database. We extracted samples that were classified as normal or as having noncancer disease and used the signal intensity values from the Microarray Analysis Suite Version 5 Software. We used the following microarray probes to measure gene expression: MSP, 205614_x_at; Hepsin, 204934_s_at; HGFA, 207027_at; MT-SP1, 202005_at; and RON, 205455_at. For each tissue type, we generated expression profiles to show the mean ± SD of gene expression values. For the 4 tissue types having the highest mean expression of MSP, liver, kidney, pancreas, and small intestine, we generated scatter plots to show the relations of expression of MSP against that of other genes. All plots were generated using the R statistical package.

**Results**

**In vitro activation of pro-MSP by recombinant hepsin**

Substrate profiling of hepsin by the use of a synthetic combinatorial library determined (P/K)-(K/Q)-(T/L/N)-R as the P4-P1 (nomenclature according to Schecter and Berger; ref. 43) consensus sequence (19), which is in good agreement with the identified cleavage site sequences from macromolecular substrates of hepsin (refs. 34–37; Table 1). The consensus sequence and particularly the specific hepsin recognition sequences of laminin-332 (SQLR/L) and pro-HGF (KQLR/V) bear a close resemblance to the cleavage sequence of pro-MSP (SKLR/V). Therefore, we hypothesized that hepsin could be a pro-MSP activator. To examine this hypothesis, we expressed pro-MSP in CHO cells but found it partially converted into the 2-chain form (MSP). Because MSP, but not pro-MSP, binds to its receptor RON, we further purified pro-MSP by incubating it with RON-Fc for 16 hours at 4°C followed by an affinity chromatographic purification step. The resulting pro-MSP was of high purity and contained only small amounts of activated MSP (Supplementary Fig. S1A). The soluble form of hepsin (dHepsin) comprising the extracellular portion cleaved pro-MSP in a concentration- and time-dependent manner at 37°C. dHepsin (12.5 nmol/L) converted...
more than 50% of pro-MSP (1.25 μmol/L) within 20 minutes and complete conversion into the α/β heterodimer was achieved within 1 hour at a molar enzyme to substrate ratio of 1:100 (Supplementary Fig. S1B). N-terminal sequencing identified the approximately 60-kDa band as MSP a-chain (19QRSPLN) and the approximately 30-kDa band as MSP b-chain (484VVGGHPG), indicating that sHepsin processed pro-MSP at the consensus cleavage site Arg483-Val484.

We compared the enzymatic activity of sHepsin with the 2 known pro-MSP–converting proteases sMT-SP1 and HGFA. sHepsin, sMT-SP1, and HGFA cleaved pro-MSP in a concentration-dependent fashion, and their activities were completely inhibited by KD1, the N-terminal Kunitz domain of their physiologic inhibitor HAI-1 (Fig. 1A). The anti-hepsin antibody Fab25 specifically inhibited pro-MSP processing by sHepsin but not by sMT-SP1 or HGFA (Fig. 1A). The relative pro-MSP–converting potencies of the 3 proteases were quantified by measuring the disappearance of the pro-MSP band by densitometry. The results showed that sHepsin (EC₅₀ = 2.4 ± 0.3 nmol/L) was 5-fold and 7-fold more efficient than sMT-SP1 (EC₅₀ = 11.7 ± 1.3 nmol/L) and HGFA (EC₅₀ = 17.7 ± 1.9 nmol/L), respectively. Prolonged incubation of pro-MSP with sHepsin over a 24-hour period did not result in additional cleavage products (data not shown). Consistent with this result, an uncleavable single-chain form of pro-MSP generated by mutating the Arg483 residue to Glu483 (scMSP) was resistant to cleavage by sHepsin during a 24-hour reaction period (data not shown).

Pro-MSP activation by cell surface–expressed hepsin

To determine pro-MSP processing by cell surface–expressed full-length hepsin, we used the prostate cancer cell line LNCaP-34, which was engineered to stably overexpress hepsin on the cell surface (35). Incubating LNCaP-34 cells with [¹²⁵I]labeled pro-MSP over a 3-hour period showed that more than 80% of pro-MSP was converted to the 2-chain α/β heterodimer form. The effect is similar to what was observed when pro-MSP was treated with 10 nmol/L sHepsin in the LNCaP-34 cell cultures (Fig. 1B), which resulted in more than 92% conversion of pro-MSP compared with the pro-MSP only control (lane 1). Cleavage of pro-MSP was inhibited by 3 different inhibitors—KD1, which inhibits hepsin, MT-SP1, and HGFA (40); KQLR-cmk, an irreversible peptide inhibitor mimicking the pro-HGF cleavage sequence KQLR|V (19, 44); and Ab25 (Fig. 1B). Although KD1 and KQLR-cmk are not selective inhibitors, the inhibition observed for them is similar as that for the selective hepsin inhibitor Ab25. Thus, any activation by other proteases, in particular MT-SP1 that is present on LNCaP-34 cells (35), is minimal.

Table 1. Cleavage sequence of hepsin substrates

<table>
<thead>
<tr>
<th>Hepsin substrates</th>
<th>Cleavage sequence</th>
</tr>
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<tbody>
<tr>
<td>Pro-MSP</td>
<td>SKLR</td>
</tr>
<tr>
<td>Pro-HGF</td>
<td>KQLR</td>
</tr>
<tr>
<td>Laminin-332</td>
<td>SQRL</td>
</tr>
<tr>
<td>Pro-uPA</td>
<td>PRFK</td>
</tr>
<tr>
<td>FVII</td>
<td>PGGR</td>
</tr>
<tr>
<td>Prostasin</td>
<td>PQAR</td>
</tr>
<tr>
<td>PS-SCL (consensus)</td>
<td>(P/K)-(K/Q)-(T/L/N)-R</td>
</tr>
</tbody>
</table>

NOTE: | indicates cleavage site.
Abbreviations: SCL, synthetic combinatorial library; PS, profiling of substrate.

Binding of sHepsin-cleaved MSP to RON

Cleavage of pro-MSP at the Arg⁴⁸³-Val⁴⁸⁴ bond leads to the formation of a high-affinity binding site on the MSP β-chain that is absent in pro-MSP. Therefore, proper processing of pro-MSP, leading to the generation of the high-affinity binding site for RON can be monitored by measuring MSP binding to RON. In ELISA assays,
sHepsin-cleaved MSP bound to immobilized RON-Fc in a concentration-dependent manner with half-maximal binding (EC$_{50}$) of 0.25 nmol/L, whereas uncleavable scMSP showed 500-fold decreased binding (EC$_{50}$ = 125 nmol/L; Fig. 2A). Surface plasmon resonance experiments (BIAcore) with sHepsin-cleaved MSP gave a $k_a$ of $3.47 \times 10^6$ (mol/L)$^{-1}$ s$^{-1}$, $k_d$ of $3.59 \times 10^{-2}$ s$^{-1}$ and a calculated $K_D$ of 10.3 nmol/L (Fig. 2B), which was almost identical to the $K_D$ of 9.1 nmol/L measured for the commercially obtained MSP. Neither pro-MSP nor the cleavage site mutant scMSP showed any detectable binding to RON up to a concentration of 1 $\mu$mol/L (data not shown). We observed a minor discrepancy in the results for the binding of pro-MSP to RON in BIAcore and ELISA assays, which we attribute to sample heterogeneity. Nonetheless, the overall results show that pro-MSP processing by sHepsin unmasked the high-affinity receptor-binding site on the MSP $\beta$-chain.

MSP-mediated activation of signaling

The biological effects of sHepsin-cleaved MSP were first assessed by monitoring phosphorylation of RON and downstream signaling proteins. Incubation of human A2780 ovarian cancer cells overexpressing RON with pro-MSP, scMSP, or sHepsin alone did not result in any appreciable phosphorylation of RON or the downstream mitogen-activated protein kinase (MAPK), the ribosomal S6 protein, or the protein kinase Akt (Fig. 3A and B). However, coincubation of A2780-RON cells with pro-MSP and sHepsin resulted in robust phosphorylation of RON, MAPK, S6, and Akt to levels that were comparable with treatment with active MSP (Fig. 3A and B). Furthermore, siRNA-mediated suppression of RON expression completely attenuated the phosphorylation of these downstream signaling molecules, suggesting that the effect was mediated through RON (Supplementary Fig. S2).

Peritoneal macrophage morphology change and chemotaxis assay

MSP induces mouse resident peritoneal macrophages to assume a more flat and spread-out morphology similar to what is seen with other chemoattractants (6, 45). Therefore, we examined whether processing of pro-MSP by sHepsin produced an active MSP capable of eliciting these responses. sHepsin-cleaved MSP was added to primary mouse peritoneal macrophages in culture. After 1-hour incubation, peritoneal macrophages treated with MSP/sHepsin underwent distinct changes in cell shape, assuming a flat morphology with elongated protrusions (Fig. 3C), unlike the cells treated with pro-MSP alone, which had a more spherical morphology. The effect of sHepsin-activated MSP was comparable with that of the MSP from a commercial source. No cell shape changes were observed in the medium control or with the addition of scMSP or sHepsin (Fig. 3C). However, weak morphologic changes were observed in pro-MSP control wells, perhaps because of baseline activation of pro-MSP by some of the known

![Figure 3](image-url)

**Figure 3.** A, representative immunoblot analysis of RON, phospho-MAPK, phospho-S6, phospho-Akt, and phospho-RON in human A2780 ovarian carcinoma cells. Cells were treated with pro-MSP, scMSP, MSP (from commercial source), or pro-MSP with 10 nmol/L sHepsin at the indicated concentrations for 1 hour. Phosphorylation of MAPK, S6, and Akt were detected by immunoblotting cell lysates using phospho-specific antibodies. Phosphorylation of RON was detected by first immunoprecipitating (IP) for RON followed by immunoblotting using phospho-tyrosine antibody. B, relative signal intensities of phospho-MAPK and phospho-S6. The numbers in parentheses represent the concentration of pro-MSP, scMSP, or MSP (from commercial source) in nanogram per milliliter. The values are mean ± SD of 3 experiments. C, peritoneal macrophage morphology change assay. On stimulation with sHepsin-cleaved MSP, peritoneal macrophages underwent distinct changes in cell shape, showed by protrusion and elongation. The effect of hepsin-activated MSP was comparable with that of a commercially available MSP; sHepsin alone, pro-MSP alone, and uncleavable scMSP had no appreciable effect.
pro-MSP activators like MT-SP1, which are also expressed in macrophages (11).

In addition, macrophages were coincubated with pro-MSP and sHepsin, which resulted in a significant increase \((P < 0.001)\) in peritoneal macrophage migration (Fig. 4A) that was comparable with MSP from a commercial source. The promigratory effect was due to the generation of active MSP by sHepsin, as neither pro-MSP nor uncleavable scMSP by themselves increased cell migration (Fig. 4A). In accord with this, the addition of hepsin-neutralizing antibody (Ab25) completely inhibited the increased promigratory activity found with pro-MSP and sHepsin (Fig. 4A).

### Inhibition of nitric oxide synthesis

In epithelial cells and macrophages, MSP/RON signaling can function as a negative regulator of NO production (46). MSP is capable of blocking the increase in macrophage-inducible nitric oxide synthase mRNA and its associated increase in the production of NO in response to stimuli, including LPS (47). The ability of sHepsin to generate MSP that actively suppresses NO production was examined in a cell culture system in which primary mouse bone marrow macrophages were exposed to LPS. Exposure to LPS resulted in a dramatic increase in macrophage NO production as measured by nitrite concentration in medium, whereas this response remained unchanged by the addition of pro-MSP alone (Fig. 4B). However, the addition of sHepsin to pro-MSP in the culture medium led to a significant attenuation of NO production, comparable to the effect by mature MSP from a commercial source (Fig. 4B) and in agreement with the approximately 50% reduction \((P < 0.0002)\) of NO production by MSP reported in the literature (11). Inhibition of sHepsin by Ab25 reversed NO production back to control levels, suggesting that reduction of NO synthesis was entirely mediated by sHepsin-dependent activation of pro-MSP.

### Gene expression profiles of MSP and hepsin in comparison to HGFA and MT-SP1

Tissue distribution of MSP expression in normal and disease tissues (Fig. 5A) showed that MSP is expressed most highly in liver, followed by kidney, pancreas, and small intestine. Comparison of this tissue expression profile with that of hepsin, HGFA, MT-SP1, and RON (Fig. 5A) revealed a similar tissue distribution for hepsin, which was also highly expressed in liver, kidney, and pancreas but not in small intestine. HGFA was also expressed at highest levels in liver, but not in other tissues, whereas MT-SP1 and RON were expressed predominantly in colorectal, small intestine, and stomach samples.

To evaluate these gene expression relationships in more detail, we generated scatter plots of MSP expression against that of the other genes in the liver, kidney, pancreas, and small intestine (Fig. 5B). These scatter plots showed a strong correlation of expression between MSP and hepsin among normal and disease samples of kidney, liver, and pancreas but not of small intestine. Coexpression was also observed between MSP and HGFA in liver samples and between MSP and both MT-SP1 and RON in small intestinal samples.

### Discussion

MSP in its latent form (pro-MSP) has no biological activity and its maturation via proteolytic processing is an important regulatory step in the MSP/RON signaling pathway. This is
akin to the related HGF/MET system in which biologically active HGF is generated by an obligatory pro-HGF cleavage reaction. Hepsin activates pro-HGF by cleavage at KQLR\_V (19, 34). The scissile peptide sequence of pro-MSP, SKLR\_V, has strong similarity to that of pro-HGF as well as to the SQLR\_L cleavage sequence of the recently identified hepsin substrate laminin-332 (36), suggesting that pro-MSP could potentially be a hepsin substrate. Here, we provide several lines of evidence to show that hepsin is an efficient pro-MSP activator that generates the biologically active 2-chain α/β-heterodimeric MSP signaling molecule.

Using the highly purified single chain pro-MSP, both sHepsin and cell surface–expressed full-length hepsin are capable of processing pro-MSP at the consensus cleavage site. The proteolytic activity of sHepsin on pro-MSP was specific, as it was completely inhibited by hepsin inhibitors, such as KD1, Ac-KQLR-cmk, and most importantly, a neutralizing anti-hepsin antibody (Ab25). Additional evidence for the specificity of the cleavage reaction came from experiments with the cleavage site mutant scMSP, which remained intact on prolonged treatment with high concentrations of sHepsin. The pro-MSP cleavage site sequence recognized by hepsin is consistent with a preference for arginine at P1, leucine at P2, and lysine at P3 positions (19). On the basis of the published structure of hepsin with bound Ac-KQLR-cmk (pro-HGF sequence, PDB 1Z8G; ref. 19),

Figure 5. A. MSP expression in normal and diseased tissues. MSP is most highly expressed in liver, followed by kidney, pancreas, and small intestine. Comparison of this tissue expression profile with that of hepsin, HGFA, MT-SP1, and RON reveals a similar tissue distribution for hepsin, which is also highly expressed in liver, kidney, and pancreas but not in small intestine. HGFA is also expressed at highest levels in liver, but not in other tissues, whereas MT-SP1 and RON are ubiquitously expressed with highest expression in colorectal, small intestine, and stomach samples.
we built a model with the pro-MSP sequence SKLR (Supplementary Fig. S3). The replacement of a P4-Lys/P3-Gln with a P4-Ser/P3-Lys should have minimal effects, as the modeled SKLR peptide also orients its side chains in a very similar position as that of the KQLR peptide. In addition, there are potential hydrogen bond (distance < 3 Å) interactions of the SKLR residues P4-Ser and P3-Lys with the hepsin residues Gln175b and Tyr146 (Supplementary Fig. S3). These additional interactions may confer favorable effects on the preference of substrates with a serine at P4 and lysine at P3 positions.

Cleavage of pro-MSP at the Arg483-Val484 bond leads to the formation of the receptor-binding site on the MSP β-chain that is not present on pro-MSP (3). In accordance, sHepsin-cleaved MSP was capable of binding to its receptor with high affinity as determined by surface plasmon resonance and ELISA experiments, suggesting that the cleaved MSP is functionally competent. This was further established in a set of RON-dependent cellular activity assays, namely, the phosphorylation of downstream signaling proteins in RON-expressing A2780 cells, the change in chemotaxis and morphology in peritoneal macrophages, and the attenuation of LPS-induced NO production in bone marrow–derived macrophages. Consistently, RON-mediated cellular responses were elicited only when cells were exposed to the combination of pro-MSP and sHepsin but not to individual pro-MSP, scMSP, or sHepsin treatments. These results strongly suggest that pro-MSP processing generates a functional MSP signaling molecule with an activity indistinguishable from MSP from a commercial source. A recent report showed that recombinant human hepsin was unable to cleave pro-MSP, yet was able to cleave the internally quenched fluorescence peptide encompassing the pro-MSP cleavage sequence SKLR-VVGG (P4-P4' ref. 48). Although there is no straightforward explanation for these discrepant results, it may be possible that the quality and the source of the pro-MSP used by Beliveau and colleagues (48) could be a reason for the different findings.

The in vitro reaction conditions, specifically the high substrate to enzyme ratio and the short reaction time, suggested that hepsin is a highly efficient pro-MSP activator. This view was further substantiated by a comparison with 2 recently identified pro-MSP activators MT-SP1 and HGFA, suggesting that hepsin has superior pro-MSP converting activity. A caveat is that the assays used only extracellular portions of MT-SP1 and hepsin, both of which are integral cell surface proteases of the TTSP family. Experiments with LNCaP-34 cells, which express full-length forms of both hepsin and MT-SP1 (35), indicated that pro-MSP processing was entirely due to hepsin, because no processing was observed in the presence of hepsin-specific inhibitor Ab25. Although these results are consistent with the potent pro-MSP processing activity of sHepsin, they do not imply that cell surface expressed MT-SP1 lacks pro-MSP convertase activity; because LNCaP-34 cells were engineered to over-express hepsin, contributions by basal levels of MT-SP1 may have been masked.

In addition to pro-MSP, hepsin was shown to activate pro-HGF and pro-uPA, and to cleave laminin-332 (19,
pro-MSP

MT-SP1

Hepsin

HGFA

Cell proliferation, migration and differentiation

Figure 6. Model of protease/inhibitor system regulating growth factor signaling. The activation of the MSP/RON and HGF/MET signaling pathways is dependent on the availability of the biologically active forms of MSP and HGF. The 3 trypsin-like serine proteases Hepsin, HGFA, and MT-SP1 can activate the biologically inactive latent growth factors (pro-MSP and pro-HGF) by enzymatic cleavage at the consensus Arg-Val bond to generate the active α/β heterodimers MSP and HGF. The enzymatic activities of the proteases are, in turn, regulated by the cell surface–expressed Kunitz domain inhibitors HAI-1 and HAI-2.

34, 36). The hepsin-mediated processing of these substrates activates enzymatic cascades and/or initiates biological pathways that pertain to tumor growth and progression. Hepsin-activated pro-uPA activates plasminogen to plasmin, which then degrades extracellular matrix proteins directly or indirectly by activating latent forms of matrix metalloproteases (49, 50). Degradation of stromal components and basement membranes are critical for tumors to invade and metastasize, and it constitutes a hallmark for increased tumor aggressiveness. Similarly, hepsin enzymatic activity toward the basement membrane constituent laminin-332, which generates promigratory laminin-332 fragments, as well as the hepsin-mediated activation of the HGF/MET and MSP/RON signaling pathways, may promote tumor invasion and progression. For instance, hepsin is highly upregulated in prostate cancer epithelial cells (20, 28), which also express RON (51, 52). RON was found to regulate the production of angiogenic chemokines promoting tumor growth and angiogenesis (52, 53). Therefore, the increased expression of hepsin in prostate cancer could activate RON-dependent signaling in cancer cells to promote cancer progression. In vivo studies using mouse prostate tumor models support a role of hepsin in basement membrane degradation and in tumor invasion and metastasis (28, 29).

The plasminogen-like growth factors MSP and HGF share the same domain architecture and activation mechanism and initiate intracellular signaling pathways that lead to proliferation, migration, and differentiation (Fig. 6). The fact that the single-chain precursors, pro-HGF and pro-MSP, have no biological activity strongly suggests that the activating proteases are critical in regulating the MSP/RON and HGF/MET pathways. It is intriguing that both pro-MSP and pro-HGF are activated by the 3 trypsin-like serine proteases hepsin, MT-SP1, and HGFA and that all 3 proteases are inhibited by the same 2 Kunitz domain inhibitors, HAI-1 and HAI-2 (refs. 40, 41, 54; Fig. 6). This could mean that they are components of the same biological systems involving growth factor–mediated cellular responses such as tissue repair and tumorogenesis. Given the assumption that latent growth factors are abundantly present in the extracellular space (55–57), this protease/inhibitor system could control the availability of biologically active growth factors in the microenvironment. Except for HGFA, the proteases, inhibitors, and growth factor receptors (RON and MET) are integral cell surface–expressed proteins, which may allow for highly localized reactions and their regulation by inhibitors.

Recently, gene expression profiling used to identify MT-SP1 as a pro-MSP activator showed that both MSP and MT-SP1 expression correlated in normal and disease tissues (11). In this report, we detected a strong correlation of MSP expression with hepsin expression in the liver, kidney, and pancreas that was superior to the corresponding correlations observed between MSP and HGFA or MSP and MT-SP1. Both hepsin and MSP are produced by hepatocytes in the liver (58, 59) and renal tubule cells in the kidney (58, 60), which were recently shown to increase MSP production during the regenerative phase in a mouse renal injury model (60). In light of the potent pro-MSP convertase activity for hepsin in vitro, the coexpression results suggest that hepsin may regulate pro-MSP activation in tissue homeostasis or after tissue injury. The biochemical linkage between hepsin with the MSP/RON system presented in this study is likely to form the basis for further investigations directed at understanding the biological regulation of the MSP/RON pathway by proteases under normal and pathophysiologic conditions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Proteolytic Activation of Pro-Macrophage-Stimulating Protein by Hepsin


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