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 pro-inflammatory 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-Arg483↓Val484) closely matches the substrate recognition sequences of hepsin, a type II transmembrane serine protease, which is overexpressed in several cancers. Here we demonstrate that recombinant hepsin cleaves pro-MSP at the consensus site Arg483-Val484 with superior efficiency compared to the known activators MT-SP1 and HGFA. At least 50% of Pro-MSP was processed within 1 h at a hepsin concentration of 2.4 nM and at a molar enzyme: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 $K_D$ of 10.3 nM, 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 attenuation of lipopolysaccharide-dependent production of nitric oxide. These findings suggest the MSP/RON signaling pathway may be regulated by hepsin in tissue homeostasis and in disease pathologies such as in cancer and immune disorders.

Keywords: Hepsin, MSP/RON, HGF/MET, cancer, plasminogen-related growth factors
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 nantaise, also known as macrophage stimulating receptor-1, MSTR1), a member of the MET-proto-oncogene family (1). MSP shares high sequence and structural domain homology with hepatocyte growth factor (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 module followed by four 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↓Val bond to attain functional activity (5, 6). In HGF, cleavage at the corresponding Arg↓Val 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 upon 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 matriptase) and HGF activator (HGFA) (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 a 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 towards the macromolecular 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 physiological 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 co-expression of MSP and hepsin in a number of organs, suggesting a potential physiological role of hepsin in regulating MSP’s activity.
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 on Superdex-S200 column. Most of the MSP present in the pro-MSP preparation was removed by incubation with RON-Fc followed by a protein-A column purification and pro-MSP was collected in the flow-through (Suppl. Fig. S1A and Fig. 1A). To generate a non-activatable form of pro-MSP the P1 residue Arg483 was mutated to Glu (scMSP) using Quickchange mutagenesis (Stratagene). Thus, scMSP actually contains two mutations: R483E and C672A. The protein was expressed and purified as described for wild-type pro-MSP. The ‘serum-form’ of HGF activator (HGFA) (Val³⁷³ - Ser⁶⁵⁵), the MT-SP1 protease domain (sMT-SP1) as well as the Kunitz domain-1 (KD1) derived from HGF activator inhibitor type-1 (HAI-1) were 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 E. 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 μM) was incubated for 1 h at 37°C with different concentrations (100 nM – 97 pM, 2-fold dilution series) of sHepsin, HGFA and sMT-SP1 in a buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Triton-X100 and 2 mM CaCl₂ for 1 h at 37°C. In addition, 100 nM of the proteases were pre-incubated for 15
min with 1 µM of the hepsin-specific Fab25 or 1 µM of KD1 (an inhibitor of Hepsin, MT-SP1 and HGFA) before addition of pro-MSP. For time course experiments, 12.5 nM sHepsin was incubated with 1.25 µM of pro-MSP and aliquots were analyzed by SDS-PAGE and proteins stained with SimplyBlue Safe Stain (Invitrogen, Carlsbad, CA). The experiments were repeated three times. Similar experiments were performed with 250 µg/ml of the scMSP mutant and hepsin (10 nM). For the densitometric analysis of pro-MSP degradation, the pro-MSP band intensities were quantified using ImageJ from the National Institutes of Health (http://rsb.info.nih.gov/ij/index.html). The effective concentration to give 50% reduction (EC$_{50}$) was determined by a four parameter fit (Kaleidagraph, Synergy Software, Reading, PA).

sHepsin-cleaved MSP was prepared by incubating 100 µg/ml of pro-MSP with 15 nM sHepsin for 1 h 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 min at 37°C with 500 µl serum-free RPMI-1640 medium containing 1µM each of Ab25 or KD1 or Ac-KQLR chloromethyl ketone (Ac-KQLR-cmk; Anaspec, San Jose, CA) or 10 nM sHepsin. $^{125}$I-labeled pro-MSP, prepared as described for pro-HGF (34), was added (25 µg/ml) and incubated for 3 h 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 Health Care, NJ) 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 concentration of either sHepsin-activated MSP or reference MSP (R&D Systems) or scMSP were injected in HBS-P buffer (10 mM Hepes pH 7.5, 150 mM 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, Roskilde, Denmark) were coated overnight at 4°C with 2 μg/ml of rabbit anti-human IgG Fc specific antibody (Jackson ImmunoResearch Laboratory, West Grove, PA) in 50 mM 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 incubated for 1h. After washing with PBS, 0.05% polysorbate 20, MSP proteins were added and incubated for 1 h. Bound MSP was detected using anti-His-HRP (Qiagen, Valencia, CA) and TMB/H$_2$O$_2$ substrate (KPL, Gaithersburg, MD). The reaction was stopped with 1 M H$_3$PO$_4$ 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 $1 \times 10^6$ cells/ml. The macrophage chemotaxis assay was performed using a QCM chemotaxis assay kit with a pore size of 5 μm (Millipore). To the bottom wells was added RPMI-1640 medium containing: (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 nM sHepsin, (e) 80 ng/ml pro-MSP, 10 nM sHepsin and 100 nM Ab25. The reaction mixtures (a-e) were pre-incubated at 37 °C for 1 h before adding to the bottom wells. Macrophage suspension ($10^5$ cells/100 μl) was added to the upper wells. After incubation at 37 °C for 4 h, the migrated cells were collected using detachment buffer (Millipore) and quantified by incubation for 15 min.
with lysis buffer and CYPRO dye followed by fluorescence measurements (RFU, relative fluorescence units) on a microplate reader (Spectramax-M5, Molecular Devices) with excitation at 480 nm and emission at 520 nm.

To examine the morphological changes, peritoneal macrophages (1 × 10^6 cells/ml) were cultured in serum-free RPMI-1640 medium overnight. Non-adherent 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 h morphological changes were captured by phase-contrast microscopy.

**Inhibition of NO synthesis by mature MSP**

Bone marrow cells were isolated from femurs of C57BL/6 mice as described (11). After washing the cell suspension with DMEM, red blood cells were lysed with erythrocyte lysis buffer. Cells were resuspended in macrophage differentiation medium (DMEM with glutamine, 10% FBS, 1X 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 matured macrophages were incubated for 24 h at 37°C in 300 µl/well of serum-free medium with or without 1 µg/ml of lipopolysaccharide (LPS) (Sigma) and containing: (a) 10 ng/ml pro-MSP, (b) 10 ng/ml pro-MSP and 1 nM sHepsin, (c) 10 ng/ml pro-MSP, 1 nM sHepsin and 500 nM Ab25, (d) 10 ng/ml MSP (R&D). 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) were seeded at a density of 2 x 10^5 cells/ well into a 12-well tissue culture plate. For siRNA experiments, RON expression was reduced in A2780-RON cells by transfecting 20 pmol of either RON-specific siRNA 5'-GGGCGACAGAAAUGAGAGUtt-3' (Ambion / Applied Biosystems) or non-targeting control siRNA (Dharmacon / Thermo Scientific) using RNAi-Max (Invitrogen). Forty eight hours following transfection, cells were serum-starved for an additional 24 h and...
incubated with indicated concentrations of pro-MSP, scMSP, MSP, and pro-MSP with sHepsin (10 nM). After 1 h incubation, cells were washed once with cold PBS followed by incubation on ice in lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 5 mM β-glycerophosphate, 2 mM NaF, 1 mM sodium orthovanadate, and protease and phosphatase inhibitors (Sigma Life Sciences)). Lysates were clarified by centrifugation at 14,000g, subjected to SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes, and incubated overnight with either 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), coupled to agarose beads. Immunoprecipitates were washed three times in cold lysis buffer, resuspended in 4X 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, Gilbertsville, PA) and Alexa-Fluor 680 goat anti-rabbit IgG (Invitrogen). Protein bands were visualized and quantified on the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) 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 (Gaithersburg, MD) database. We extracted samples that were classified as normal or as having non-cancer 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 and standard deviation of gene expression values. For the four 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 (43)) consensus sequence (19), which is in good agreement with the identified cleavage site sequences from macromolecular substrates of hepsin (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 Chinese Hamster Ovary (CHO) cells, but found it partially converted into the 2-chain form (MSP). Since, MSP but not pro-MSP binds to its receptor RON, we further purified pro-MSP by incubating it with RON-Fc for 16 h 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 (Suppl. Fig. S1A). The soluble form of hepsin (sHepsin) comprising the extracellular portion cleaved pro-MSP in a concentration- and time-dependent manner at 37°C. sHepsin (12.5 nM) converted >50% of pro-MSP (1.25 µM) within 20 minutes and complete conversion into the α/β heterodimer was achieved within 1 h at an enzyme:substrate molar ratio of 1:100 (Suppl. Fig. S1B). N-terminal sequencing identified the ~60 kDa band as MSP α-chain (\(^{19}\)QRSPLN) and the ~30 kDa band as MSP β-chain (\(^{484}\)VVGGHPG), indicating that sHepsin processed pro-MSP at the consensus cleavage site Arg\(^{483}\)-Val\(^{484}\).

We compared the enzymatic activity of sHepsin with the two 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 three proteases were quantified by measuring the disappearance of the pro-MSP band by densitometry. The results showed that sHepsin (EC\(_{50} = 2.4 \pm 0.3\) nM) was 5-fold and 7-fold more efficient than sMT-SP1 (EC\(_{50} = 11.7 \pm 1.3\) nM) and HGFA (EC\(_{50} = 17.7 \pm\)
1.9 nM), respectively. Prolonged incubation of pro-MSP with sHepsin over a 24 h 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 Arg\(^{483}\) residue to Glu\(^{483}\) (scMSP) was resistant to cleavage by sHepsin during a 24 h 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 \(^{125}\)I-labeled pro-MSP over a 3 h period showed that >80% of pro-MSP was converted to the two-chain \(\alpha/\beta\) heterodimer form. The effect is similar to what was observed when pro-MSP was treated with 10 nM sHepsin in the LNCap cell cultures (Fig. 1B), which resulted in >92% conversion of pro-MSP compared to the pro-MSP only control (lane 1). Cleavage of pro-MSP was inhibited by three different inhibitors – KD1, which inhibits hepsin, MT-SP1, and HGFA (40), KQLR-cmk, an irreversible peptide inhibitor mimicking the pro-HGF cleavage sequence KQLR\(\downarrow\)V (19, 44), and Ab25 (Fig. 1B). While KD1 and KQLR-cmk and are not selective inhibitors, the inhibition observed for them is similar 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.

Binding of sHepsin-cleaved MSP to RON

Cleavage of pro-MSP at the Arg\(^{483}\)-Val\(^{484}\) bond leads to the formation of a high affinity binding site on the MSP \(\beta\)-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 nM, while uncleavable scMSP showed 500-fold decreased binding (EC\(_{50}\) 125 nM) (Fig. 2A). Surface-plasmon resonance experiments (Biacore) with sHepsin-cleaved MSP gave a \(k_a\) of 3.47 x 10\(^6\) M\(^{-1}\)s\(^{-1}\), \(k_d\) of 3.59 x 10\(^{-2}\) s\(^{-1}\) and a calculated \(K_D\) of 10.3 nM (Fig. 2B), which was almost identical to the \(K_D\) of 9.1 nM.
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 μM (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 β-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, B). However, co-incubation of A2780 cells with pro-MSP and sHepsin resulted in robust phosphorylation of RON, MAPK, S6, and Akt to levels that were comparable to treatment with active MSP (Fig. 3A,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 (Suppl. 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 chemo-attractants (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 h 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 morphological changes were observed in pro-MSP control wells, perhaps due to baseline activation of pro-MSP by some of the known pro-MSP activators like MT-SP1, which are also expressed in macrophages (11).

In addition, macrophages were co-incubated with pro-MSP and sHepsin, which resulted in a significant increase (p<0.001) in peritoneal macrophage migration (Fig. 4A) that was comparable to MSP from a commercial source. The pro-migratory effect was due to the generation of active MSP by sHepsin, since 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 pro-migratory 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 nitric oxide (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 lipopolysaccharide (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, while 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. Co-expression 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 demonstrate that hepsin is an efficient pro-MSP activator that generates the biologically active two-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 since 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 upon prolonged treatment with high concentrations of sHepsin. The pro-MSP cleavage site sequence recognized by hepsin is consistent with a preference for Arg at P1, Leu at P2 and Lys at P3 positions (19). Based on the published structure of hepsin with bound Ac-KQLR-cmk (pro-HGF sequence, PDB 1Z8G) (19), we built a model with the pro-MSP sequence SKLR (Suppl. Fig. S3). The replacement of a P4-Lys/P3-Gln with a P4-Ser/P3-Lys should have minimal effects, since 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 (Suppl. Fig. S3). These additional interactions may confer favorable effects on the preference of substrates with a Ser at P4 and Lys 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).
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′) (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 et al (48) could be a reason for the different findings.

The in vitro reaction conditions, specifically the high substrate: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 two 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 since no processing was observed in the presence of the hepsin-specific inhibitor Ab25. While 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; since LNCaP-34 cells were engineered to overexpress 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, 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 and basement membranes are critical for tumors to invade and metastasize, and it constitutes a hallmark for increased tumor aggressiveness. Similarly, hepsin enzymatic activity towards the basement membrane constituent laminin-332, which generates pro-migratory 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 three trypsin-like serine proteases hepsin, MT-SP1, and HGFA and that all three proteases are inhibited by the same two Kunitz domain inhibitors, HAI-1 and HAI-2 (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 tumorigenesis. 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 co-expression 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 patho-physiological conditions.

Acknowledgments
We thank Kaushiki Mahapatra for running the phosphorylation assay, Jose Zavala for animal husbandry, Greg Bennett and Daniel Tran for their help with 125I-labeling of pro-MSP and Wendy Sandoval for N-terminal amino acid sequencing.
References


Table 1.

<table>
<thead>
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<th>Hepsin substrates</th>
<th>Recognition sequence</th>
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<tr>
<td>Pro-MSP</td>
<td>SKLR↓V</td>
</tr>
<tr>
<td>Pro-HGF</td>
<td>KQLR↓V</td>
</tr>
<tr>
<td>Ln332</td>
<td>SQLR↓L</td>
</tr>
<tr>
<td>Pro-uPA</td>
<td>PRFK↓I</td>
</tr>
<tr>
<td>FVII</td>
<td>PQGR↓I</td>
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<tr>
<td>Pro-prostasin</td>
<td>PQAR↓I</td>
</tr>
<tr>
<td>PS-SCL (consensus)</td>
<td>(P/K) - (K/Q) - (T/L/N) - R</td>
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↓ indicates cleavage site
Figure Legends

**Figure 1. (A)** Activation of pro-MSP by sHepsin, sMT-SP1 and HGFA. Purified pro-MSP (100 µg/ml = 1.25 µM) was incubated for 1 h at 37°C with different concentrations (100 nM – 97 pM) of recombinant soluble hepsin (sHepsin) or the known activators sMT-SP1 and HGFA. For control experiments the proteases (100 nM) 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 ~60 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 h. In a control experiment, no significant proteolytic cleavage of pro-MSP was observed upon incubation for 3 h (lane 1), while addition of sHepsin (10 nM) 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 to the start of the experiment (lane 1). All three hepsin inhibitors (Ac-KQLRcmk, KD1, Ab25) effectively blocked pro-MSP processing (lanes 5-7).

**Figure 2.** Binding of sHepsin-cleaved MSP to RON. **(A)** ELISA measuring binding of plate-immobilized RON-Fc to sHepsin-cleaved MSP (filled circles; EC50 0.25 nM) and to uncleavable scMSP (filled triangles; EC50 125 nM). **(B)** Surface plasmon resonance experiments. Anti-Fc antibody coupled to the CM5 chip was used to capture RON-Fc on the biosensor. Binding curves of sHepsin-cleaved MSP were obtained by fitting the experimental data (grey lines) to a 1:1 binding model (black lines). The calculated K_D was 10.3 nM.
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 nM sHepsin at the indicated concentrations for 1 h. Phosphorylation of MAPK, S6, Akt were detected by immunoblotting cell lysates using phospho-specific antibodies. Phosphorylation of RON was detected by first immunoprecipitating for RON, followed by immunoblotting using phospho-tyrosine antibody. (B) Relative signal intensities of phospho-MAPK and phospho-S6. The numbers in parenthesis represent the concentration of pro-MSP, scMSP, or MSP (from commercial source) in ng/ml. The values are mean ± SD of three experiments. (C) Peritoneal macrophage morphology change assay. Upon stimulation with sHepsin-cleaved MSP, peritoneal macrophages underwent distinct changes in cell shape, demonstrated 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.

Figure 4. (A) Peritoneal macrophage chemotaxis assay. Co-incubation of pro-MSP and sHepsin led to a significant increase in cell migration comparable to mature MSP from a commercial source (= MSP). Pre-treatment with a neutralizing anti-hepsin antibody (Ab25) inhibited migration. sHepsin alone, pro-MSP alone and uncleavable scMSP did not stimulate migration. SFM, serum-free medium. After 4 h incubation at 37 °C, the migrated cells were collected and quantified by fluorescence measurements on a microplate reader. The values are mean ± SD of three experiments. *p < 0.001 vs SFM control; Student’s t test). (B) MSP-mediated inhibition of nitric oxide synthesis. Treatment of mouse bone-marrow derived macrophages with LPS dramatically increases NO production quantified by the nitrite concentration in culture medium. Nitrite levels remained unchanged after addition of pro-MSP alone or sHepsin alone, but were reduced by about 50% when sHepsin was added to pro-MSP or with MSP from a commercial source (= MSP). Addition of anti-hepsin antibody Ab25 to the pro-MSP/sHepsin mixture restored nitrite levels to LPS control levels. SFM, serum-free medium. The values are mean ± SD of three experiments. *p<0.002 vs SFM+LPS; Student’s t test).
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, while MT-SP1 and RON are ubiquitously expressed with highest expression in colorectal, small intestine, and stomach samples. (B) Expression of hepsin correlates well with MSP in liver, kidney and pancreas (normal – green, diseases – blue) and expression of HGFA correlates well with MSP only in liver. MT-SP1 and RON shows minor correlation with MSP expression in these tissues.

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 three 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 HGF activator inhibitor-1 (HAI-1) and HAI-2.
Figure Legends – Supplementary

Figure S1. (A) SDS-PAGE (reducing conditions) of recombinant pro-MSP (Rec. pro-MSP) expressed in CHO cells and of purified pro-MSP stained with SimplyBlue Safe Stain. Contaminating MSP (α- and β-chain) was removed from recombinant pro-MSP preparations by affinity chromatography using RON-Fc fusion protein. (B) Time-dependent proteolytic processing of purified pro-MSP (1.25 μM) by sHepsin (12.5 nM) in buffer containing 50 mM Tris, 150 mM NaCl, 0.05% Triton-X100 and 2 mM CaCl₂, at 37°C. Samples taken at indicated time points were analyzed by SDS-PAGE (reducing conditions; 4-20% gradient gel). At this enzyme:substrate molar ratio (1:100) >95% processing occurred within 1 h of incubation.

Figure S2. RON mediates downstream phosphorylation of MAPK, S6, and Akt. Immunoblot analysis of human A2780 ovarian carcinoma cells that has been transfected with RON-specific siRNA (+) or non-targeting control siRNA (-). Cells were then treated with 20 ng/ml pro-MSP, with or without 10 nM sHepsin, for 1 h prior to cell harvest and immunoblotting.

Figure S3. A molecular model of SKLR peptide (P4 – P1 residues of pro-MSP) and the hepsin active site based on the structure of hepsin in complex with Ac-KQLR-cmk (cartoon representation in grey, catalytic triad and the Ac-KQLR inhibitor as lines in yellow). The replacement of a P4-Lys/P3-Gln of KQLR peptide with a P4-Ser/P3-Lys of SKLR peptide might have minimal effect as the modeled SKLR peptide (stick representation, magenta) appears to orient its side chains in a very similar position as that of the KQLR peptide. Additionally, the modeled SKLR peptide has the potential to form hydrogen bond interactions with Gln175b and Tyr146 (green).
Figure -1

[A diagram showing protein bands and EC₅₀ values for different samples.]

Figure -2

[A graph showing protein concentration versus absorbance and a response curve over time.]
Figure -3

A

<table>
<thead>
<tr>
<th>Control</th>
<th>Pro-MSP</th>
<th>\ ( \text{scMSP} )</th>
<th>MSp</th>
<th>Pro-MSP-sHepsin (10 nM)</th>
<th>sHepsin (10 nM)</th>
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<td>20</td>
<td>100</td>
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<tr>
<td>( \alpha)-RON</td>
<td>( \alpha)-pMAPK</td>
<td>( \alpha)-pS6</td>
<td>( \alpha)-pAkt</td>
<td>( \alpha)-Actin</td>
<td></td>
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</table>

Cell Lysates

| \( \alpha\)-pTyr                  | \( \alpha\)-RON            |

IP: \( \alpha\)-RON

B

Fold change over control

<table>
<thead>
<tr>
<th>Control</th>
<th>Pro-MSP</th>
<th>\ ( \text{scMSP} )</th>
<th>MSp</th>
<th>Pro-MSP-sHepsin (10 nM)</th>
<th>sHepsin (10 nM)</th>
</tr>
</thead>
</table>

C

Medium (serum-free) | Pro-MSP | scMSP

sHepsin | MSP-sHepsin | MSP
Figure -5

A

B

Gene expression (log2 scale, arbitrary unit)

Other gene expression

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Figure – 6

![Diagram showing the relationship between pro-HGF, HGF, HGF/Met, Hepsin, HGFA, MT-SP1, HAI-1, HAI-2, Cell proliferation, migration, differentiation, pro-MSP, MSP, MSP/RON.]
Molecular Cancer Research

Proteolytic activation of pro-macrophage-stimulating protein by hepsin

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