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Activation of macrophage-stimulating protein by human airway trypsin-like protease

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ABSTRACT

Macrophage-stimulating protein (MSP) circulates as a proform protein and requires proteolytic processing for activation. Respiratory ciliated cells express the MSP receptor, recepteur d'origine nantais (RON), at the apical surface, which reportedly has an important role in ciliary function. Like RON, human airway trypsin-like protease (HAT) is also expressed at the apical surface of ciliated cells. Here we show that HAT cleaves proMSP at the physiological activation site, Arg483-Val484. MSP processed by HAT could induce chemotactic responses and morphological changes of peritoneal macrophages. In human respiratory epithelial cells, knock down of HAT expression reduced proMSP processing and RON autophosphorylation. We suggest that HAT is important for MSP-RON signaling in the respiratory tract.

Structured summary of protein interactions:
HAT cleaves **proMSP** by protease assay (View interaction)

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1. Introduction

Macrophage-stimulating protein (MSP) was originally identified as a plasma protein that induces chemotactic responses of macrophages [1]. Mature MSP autophosphorylates its specific receptor tyrosine kinase recepteur d'origine nantais (RON) [2]. RON is expressed not only in macrophages, but also in many types of epithelial cells, and MSP-RON signaling is now believed to be involved in various pathophysiological settings [2]. In the respiratory tract, RON is expressed in the ciliated respiratory epithelium and is localized at the apical surface [3]. Activation of RON by MSP induces an increased ciliary beat frequency [3]. As ciliary motility is critical for mucociliary transport and cilia dysfunction can cause chronic respiratory tract infections, MSP-RON signaling is an important regulatory system in the respiratory tract. In fact, biologically significant concentrations of MSP were observed in bronchioalveolar lavage fluid (1.3–5.8 ng/mL) [3] and in induced sputum of normal individuals (around 8.4 ng/mL) [4].

MSP belongs to the kringle protein family, whose members contain multiple copies of a highly conserved triple disulfide loop structure known as the kringle domain. Mature MSP is a disulfide-linked heterodimer with a molecular weight of 80–95 kDa, consisting of a ~60 kDa α chain and a ~30 kDa β chain [2]. MSP is produced by the liver and circulates in the plasma as a single-chain precursor (proMSP) that has no biological activity until the protein is cleaved into α and β chains at the Arg483-Val484 bond [2]. The amount of proMSP is relatively high in the serum, with concentrations around 8 μg/mL [4]. Several serum- and cell-derived proteases have been identified as candidate convertases for proMSP processing. Among the serum convertases, hepatocyte growth factor activator (HGFA) is the most efficient activator [5], while for cellular convertases, matriptase was reported to be an important activator [6]. Matriptase is a type-II transmembrane protein expressed primarily in epithelial cells [7]. Recently, another type-II transmembrane protease, hepsin, was identified as a cellular MSP activator in invasive prostatic cancer cells [8]. Each of these proteases is a trypsin-like serine protease.

Similar to matriptase and hepsin, human airway trypsin-like protease (HAT), encoded by the *TMPRSS11D* gene, belongs to the type-II transmembrane serine protease superfamily [7]. HAT was

Abbreviations: MSP, macrophage-stimulating protein; RON, recepteur d'origine nantais; HGFA, hepatocyte growth factor activator; HAT, human airway trypsin-like protease; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PC50%, processing concentration 50%; siRNA, small interfering RNA

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initially identified in the human trachea and bronchi [9]. In the respiratory tract, HAT is expressed by ciliated epithelial cells [10]. Unlike matriptase, which is localized on the basolateral surface of epithelial cells, HAT is localized at the basal part of cilia on the apical surface [10]. Since RON is also localized at the apical surface of ciliated cells [3], we hypothesized that HAT may be a proMSP activator that transduces signaling through RON on the apical surface of ciliated cells. This study aims to elucidate the processing activity of HAT towards proMSP.

2. Materials and methods

2.1. Antibodies and recombinant proteins

Anti-HAT mouse polyclonal antibody was purchased from Sigma (St. Louis, MO). Anti-human MSP goat and anti-phosphorylated RON (Tyr1238/Tyr1239) rabbit polyclonal antibodies were from R&D Systems (Minneapolis, MN). Anti-RONβ (C-20) rabbit polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human HAT (active form) and MSP were from R&D Systems. Preparation of recombinant proMSP protein with a 5 × His tag at the C teminus and active-form HGFA was described previously [5,11].

2.2. Activation of proMSP

ProMSP was incubated at 37 °C with various concentrations of HAT in 20 μL reactions containing 20 mM Tris–HCl (pH 7.6), 50 to 150 mM NaCl, 0.05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) for the indicated time periods. ProMSP processing activity was determined by immunoblot analysis [5]. The specific activity for proMSP processing was expressed as the enzyme concentration required for the conversion of 50% of 5 nM proMSP to its mature form, and was designated as processing concentration 50% (PC50%). To assess the time course of cleavage by HAT, proMSP (5 nM) was incubated with HAT (0.5 nM) at 37 °C for 0 to 8 h. Immunoblots for MSP were performed as described previously [5].

2.3. N-terminal amino acid sequencing of cleaved proMSP

ProMSP (413 nM) was incubated with 41.3 nM HAT in a 40 μL reaction containing 20 mM Tris–HCl, 150 mM NaCl, 0.05% CHAPS (pH 7.6) at 37 °C for 8 h. The reaction mixture was run on SDS–PAGE, after which the proteins were transferred to an Immobilon membrane (Millipore, Bedford, MA) and stained with 0.1% Coomassie Brilliant Blue in a water/methanol/acetic acid solution (4.5:4.5:1, v/v). The cleaved MSP protein band was cut and processed for N-terminal amino acid sequencing by automated Edman degradation using the Procise 494 HT Protein Sequencing System (Applied Biosystems, Foster City, CA).

2.4. Bioassays of MSP

Peritoneal resident macrophages were obtained from C57BL/6 mice as described previously [5]. Briefly, cells were washed and resuspended in RPMI1640 medium. The macrophage chemotaxis assay was performed using a polycarbonate membrane (pore size, 5 μm). The bottom wells were filled with medium containing proMSP treated or not treated with HAT at 37 °C for 2 h. The recombinant active-form MSP was used as a positive control. After incubation at 37 °C for 3.5 h, the cells on the upper surface were wiped off and the membranes were fixed and stained with hematoxylin. Migration was quantified by counting the number of cells on the lower surface in ten randomly selected fields (200-fold magnification). Statistical analyses were carried out using the Statview 5.0 program (SAS, Cary, NC). To test the effect of MSP on macrophage morphology, murine peritoneal macrophages were cultured in serum-free RPMI1640 overnight. Non-adherent cells were then removed and proMSP (1.25 nM) pretreated or not pretreated with HAT was added to the culture medium. After an additional incubation at 37 °C for 1 h, morphological changes were evaluated by phase-contrast microscopy [5].

2.5. Silencing of HAT in bronchial epithelial cells

Normal human bronchial epithelial cells (NHBE) were purchased from Clonetics (San Diego, CA). Cells were cultured at 37 °C in serum-free bronchial epithelial cell growth medium (Clonetics). The culture flasks were pre-coated with 0.03 mg/mL bovine collagen type 1 (KOKEN, Tokyo, Japan). Small interfering RNAs (siRNA) were used for silencing the *TMPRSS11D* gene encoding HAT. The sequences of HAT stealth siRNA and its scrambled control (Invitrogen) were 5′-UAAUAAGCCAAUUUGCUGCAGCCUG-3′ and 5′-AAACAUCUUGAAGUCGAAGUUACGU-3′, respectively. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours after the transfection, cellular proteins were extracted with lysis buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol) with protease inhibitor cocktail (Sigma), 100 mM NaF and 1 mM Na_3VO_4, and used for immunoblotting.

3. Results

3.1. Processing of proMSP by HAT

The effect of recombinant HAT on the processing of recombinant human proMSP was tested. Incubation of proMSP with HAT at 37 °C led to proMSP processing in a time-dependent manner and generated a ~60 kDa band, presumably the α-chain of mature MSP as detected by an anti-MSP antibody that recognizes the α-chain (Fig. 1A). Generation of a ~30 kDa band, presumably the β-chain, was also detected by anti-His tag antibody (Fig. 1B). Cleavage site analysis was performed after separating the HAT cleavage products by SDS–PAGE under reducing conditions. The N-terminal amino acid sequence of the 30 kDa product was determined to be Val-Val-Gly-Gly-His, indicating that HAT cleaved proMSP at the normal processing site, Arg483-Val484. The processing activity of HAT was dose-dependent and influenced by NaCl concentration (Fig. 1C). Similar to HGFA, the physiological activator of pro-MSP [5], HAT showed more efficient pro-MSP processing at low NaCl concentrations (50 mM). The concentration of HAT required to activate 50% of 5 nM proMSP (PC50%) after 4 h at 37 °C was 0.04 nM, which was comparable to or a little less efficient than that of HGFA (PC50%, 0.03 nM), but was much more efficient than factor XIa (PC50%, 0.5 nM; data not shown) [5]. The processing was suppressed at higher NaCl concentrations (Fig. 1C).

3.2. Biological activity of MSP processed by HAT

The biological activity of MSP after HAT processing was determined using murine peritoneal macrophages. After incubation of proMSP with HAT, the processed products significantly induced macrophage migration, with an activity comparable to that of commercially available recombinant mature human MSP α/β-heterodimer (Fig. 2A). HAT alone did not detectably induce the chemotactic response. We also examined the effect of HAT processing on the culture morphology of mouse peritoneal macrophages. MSP processed by HAT induced an elongated, migratory morphology of macrophages within 1 h, showing an effect similar to that produced by recombinant mature MSP (Fig. 2B). On the other hand, murine alveolar macrophages lacking stem cell-derived tyrosin

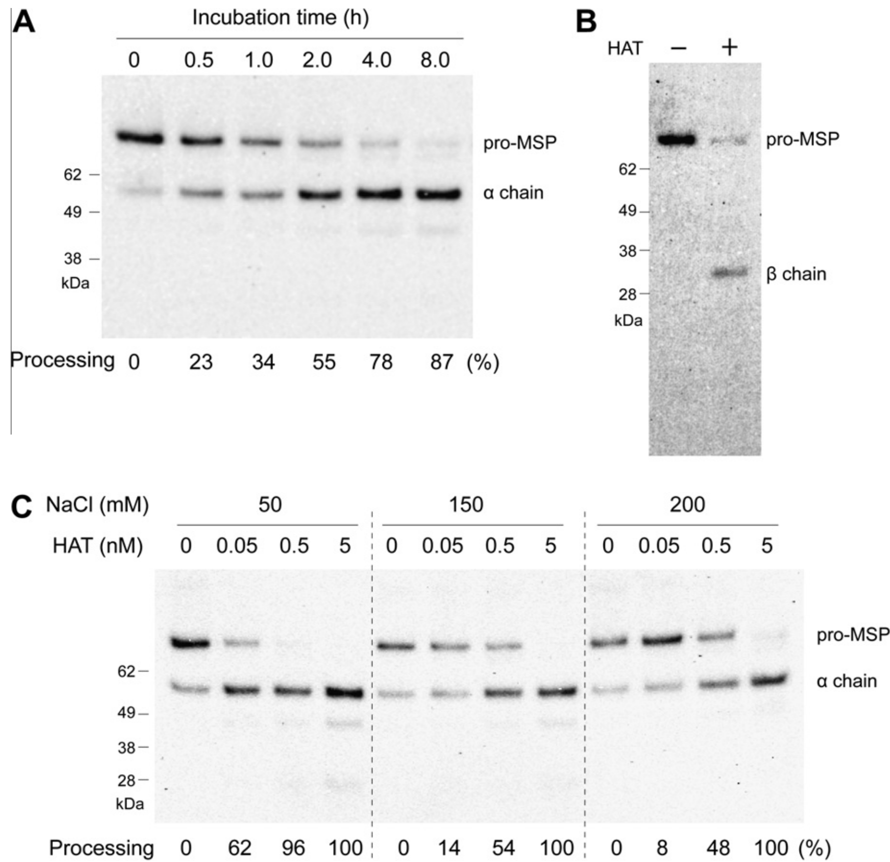

Fig. 1. Immunoblot analyses of proteolytic cleavage of His-tagged (C-terminus) human proMSP recombinant protein by human HAT. (A) Time-dependent processing of proMSP (5 nM) by HAT (0.5 nM) in 20 mM Tris buffer (pH 7.6), 150 mM NaCl, 0.05% CHAPS. Anti-MSP α chain antibody was used for detection. (B) Immunoblot of proMSP with or without HAT treatment (8 h) as described above using anti-His tag antibody. (C) Dose-dependent processing of proMSP (5 nM) by HAT (0–5 nM, 4 h treatment) and effect of NaCl concentration. The reaction mixtures were incubated at 37 °C and the processed products analyzed by immunoblot using anti-MSP α chain antibody.

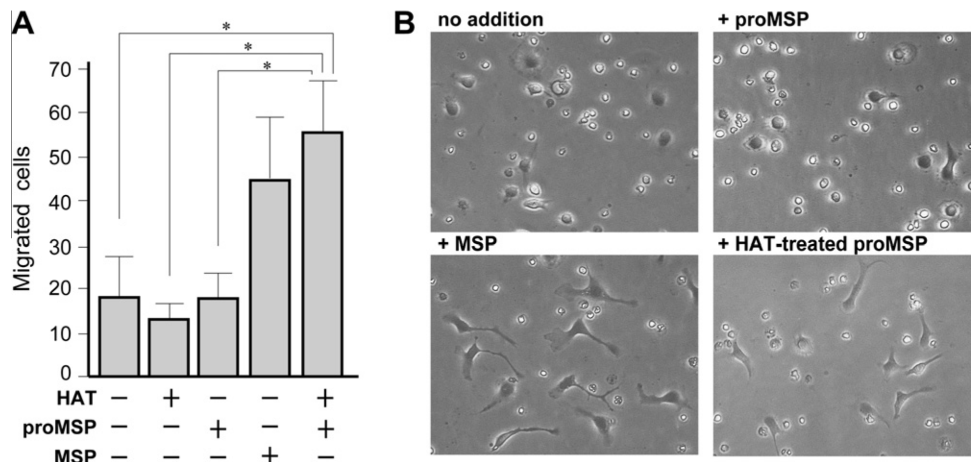

Fig. 2. Biological activity of MSP processed by HAT. (A) Results of chemotaxis assays. Murine peritoneal resident macrophages (1×10^5 cells) were placed in the upper well of a Boyden chamber and incubated for 3.5 h at 37 °C. The bottom well contained proMSP (1.25 nM) with or without HAT (0.125 nM) pretreatment (4 h) or recombinant active MSP (1.25 nM). Values are mean ± standard deviation from triplicate experiments. $^*p < 0.001$ (Mann–Whitney U test). (B) Morphology of macrophages treated with proMSP (1.25 nM), MSP (1.25 nM) or proMSP (1.25 nM) pretreated with HAT (0.125 nM) for 4 h.

kinase (STK), the RON counterpart in mice, did not respond to processed proMSP (data not shown).

3.3. Cellular HAT is involved in proMSP processing in human respiratory epithelial cells

We next sought to determine whether cellular HAT is in fact involved in RON activation in human respiratory epithelium. For this purpose, we used primary cultured normal human bronchial epithelial cells namely, NHBE, which concomitantly express HAT and RON as determined by RT-PCR (Fig. 3A). Notably, NHBE cells did not express significant levels of the cellular proMSP activators, such as matriptase and hepsin. Moreover, low but distinct levels of MSP expression were also observed. Consequently, we observed steady-state RON phosphorylation in these cells in our culture conditions even in the absence of serum (Fig. 3B). Therefore, we

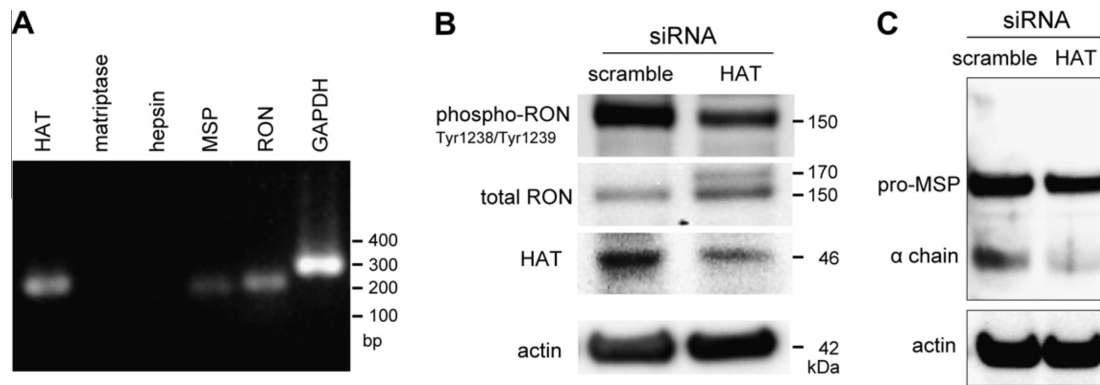

Fig. 3. Reduced RON autophosphorylation and proMSP processing after silencing of HAT in NHBE cells. (A) RT-PCR analysis for the expression of cellular activators of proMSP (HAT, matriptase, hepsin), MSP and RON. (B) Effects of HAT siRNA on RON autophosphorylaton. Same blot membrane was used to detect phosphorylated RON, total RON and HAT. In total RON, 150-kDa and 170-kDa bands represent β chain of mature two-chain form RON and single-chain proRON proteins, respectively. (C) Effects of HAT siRNA on processing of endogenous proMSP. Cellular extracts were prepared 3 days after siRNA transfection.

attempted to knock down cellular HAT by siRNA targeting of HAT. Transient transfection of HAT siRNA reduced HAT expression by 60% in NHBE cells, and knock down of HAT resulted in the reduction of RON phosphorylation (Fig. 3B). In fact, the processing of endogenous proMSP was reduced by 50% after HAT silencing (Fig. 3C).

4. Discussion

ProMSP is known to be proteolytically converted to its mature active form by proMSP convertases [2,5]. This activation step serves as a critical regulatory mechanism in MSP-induced physiological and pathophysiological tissue responses. After proteolytic cleavage, mature MSP stimulates macrophages expressing RON by inducing RON autophosphorylation [2]. Epithelial cells also frequently express RON [2,3], and thus it is reasonable to postulate that MSP has substantial roles in epithelial biology. In this study, we show that HAT, a type II transmembrane serine protease predominantly expressed by respiratory ciliated epithelium [7,9,10], efficiently activates human proMSP. As RON is also expressed by ciliated epithelial cells and a certain amount of MSP is present in bronchioalveolar lavage fluid [3], we suggest that HAT may be involved in activating proMSP and promoting MSP/RON signaling in the respiratory tract. Moreover, this study also revealed that low levels of MSP are expressed by cultured human normal bronchial epithelial cells, which may create an autocrine loop of MSP/RON.

Matriptase, a type-II transmembrane serine protease widely expressed by epithelial cells, has been reported to be an important cellular activator of proMSP [6]. As with respiratory ciliated cells, in polarized epithelial cells, the plasma membrane is divided by tight junctions into apical and basolateral portions, with matriptase localized on the basolateral surface [7]. Given the apical localization of RON in ciliated cells [3], it is conceivable that a proMSP convertase other than matriptase resides at the apical surface. The polarized expression of HAT on the apical surface [9] supports our hypothesis that HAT may indeed be a physiological convertase of proMSP in the respiratory tract.

Regarding the role of MSP/RON signaling in the respiratory tract, only a limited amount of information is available at present. MSP-induced RON signaling increases ciliary beat frequency, which would be important for protecting the respiratory tract from infectious diseases [3]. In inflammatory processes, MSP/RON signaling appears to have both stimulatory and inhibitory effects on macrophages. While MSP inducing macrophage spreading, migration, phagocytosis and cytokine production, it inhibits lipopolysaccharide (LPS)-induced production of inflammatory mediators [1,2,12]. In the lung, MSP differently affects human alveolar macrophages from smoker and non-smoker patients, and significantly enhances respiratory burst in alveolar macrophages from smokers, suggesting that MSP may enhance cigarette smoke toxicity [13].

In summary, we demonstrated for the first time that HAT is a potent activator of proMSP. Although proMSP activation can be mediated by various proteases [5,6,8], we suggest that HAT is the most convincing activation protease for proMSP in the airway epithelium due to its specific localization at the apical surface of respiratory ciliated cells, where RON is also expected to be localized.

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