Role of Macrophage-stimulating Protein and Its Receptor, RON Tyrosine Kinase, in Ciliary Motility

Osamu Sakamoto,*‡ Atsushi Iwama,* Ryoichi Amitani,|| Toyohiro Takehara,¶ Naoto Yamaguchi,* Taro Yamamoto,‡ Keisuke Masuyama,§ Tohru Yamanaka,‡ Masayuki Ando,‡ and Toshio Suda*

**Department of Cell Differentiation, Institute of Molecular Embryology and Genetics, ‡First Department of Internal Medicine, and §Department of Otorhinolaryngology, Kumamoto University School of Medicine, Kumamoto 860, Japan; ||Department of Infection and Inflammation, Chest Disease Research Institute, Kyoto University, Kyoto 606, Japan; ¶Pharmaceuticals Research Center, Toyobo Co., Ltd., Ohtsu, Shiga 520-02, Japan*

Abstract

Macrophage-stimulating protein (MSP) is an 80-kD serum protein with homology to hepatocyte growth factor (HGF). Its receptor, RON tyrosine kinase, is a new member of the HGF receptor family. The MSP–RON signaling pathway has been implicated in the functional regulation of mononuclear phagocytes. However, the function of this pathway in other types of cells has not been elucidated. Here we show that in contrast to the HGF receptor, which was expressed at the basolateral surface, RON was localized at the apical surface of ciliated epithelia in the airways and oviduct. In addition, MSP was found in the bronchoalveolar space at biologically significant concentrations. MSP bound to RON on normal human bronchial epithelial cells with a high affinity (K_d = 0.5 nM) and induced autophosphorylation of RON. Activation of RON by MSP led to a significant increase in ciliary beat frequency of human nasal cilia. These findings indicate that the ciliated epithelium of the mucociliary transport apparatus is a novel target of MSP. Ciliary motility is critical for mucociliary transport. Our findings suggest that the MSP–RON signaling pathway is a novel regulatory system of mucociliary function and might be involved in the host defense and fertilization. (*J. Clin. Invest.* 1997. 99:701–709.) Key words: macrophage-stimulating protein • RON • receptor tyrosine kinase • ciliated epithelium • ciliary motility

Introduction

Receptor tyrosine kinases (RTKs)[1] play a critical role in pleiotropic cell functions by transmitting extracellular signals such as peptide hormones and growth factors (1–3). All RTKs are composed of three major domains: an extracellular ligand binding domain, a single membrane-spanning domain, and a cytoplasmic domain that contains a tyrosine kinase catalytic domain (4, 5). After ligand binding, RTKs undergo dimerization leading to activation of their intrinsic tyrosine kinase activity and autophosphorylation. Signaling molecules are recruited to phosphorylated tyrosines on the receptor. This recruitment leads to further activation of cytoplasmic signaling molecules, which transduce receptor-generated signals (2, 6, 7).

The RON/STK (stem cell-derived tyrosine kinase) receptor tyrosine kinase is part of a subfamily of RTK that includes the hepatocyte growth factor (HGF) receptor and c-Sea (8). The human RON gene was cloned from human keratinocytes (9), and the murine counterpart, the STK gene, was derived from mouse hematopoietic stem cells (10). RON/STK as well as the HGF receptor is synthesized as a single-chain precursor and then cleaved into a mature disulfide-linked heterodimer composed of an extracellular α-chain and a transmembrane β-chain with intrinsic tyrosine kinase activity (11, 12).

RON/STK has been identified as the receptor for macrophage-stimulating protein (MSP; 11, 13, 14). MSP is an 80-kD serum protein that belongs to a family characterized by the presence of a highly conserved triple disulfide loop structure (kringle domain). The family includes prothrombin, plasminogen, urokinase, and HGF, among which MSP shows high homology with HGF (45% identity at the amino acid level; 15–18). MSP is synthesized by hepatocytes (15, 17, 19), secreted into the circulation, and maintained in the serum at a relatively high concentration (20). MSP has been shown to act on mononuclear phagocytes by activating the RON/STK receptor (12). MSP exerts multiple biological activities on peritoneal resident macrophages, such as induction of shape change and motility (21), direct chemotactic attraction (22), stimulation of ingestion of complement-coated erythrocytes (16), and inhibition of endotoxin- or cytokine-induced expression of inducible nitric oxide synthase mRNA (23). MSP also stimulates the bone resorbing activity of osteoclasts (24).

Recently it has been shown that RON/STK is expressed relatively late in development in several tissues, including the central and peripheral nervous systems, digestive tract, skin, and lung (25, 26). In contrast to the relatively restricted expression of RON/STK, the HGF receptor is broadly expressed and mediates pleiotropic biological functions such as cell growth, motility, and morphogenesis (27, 28). This difference in expression profiles of the RON/STK and HGF receptors suggests that these receptors mediate distinct functions. To date, however, the biological significance of RON/STK expression in cells other than mononuclear phagocytes has not been clarified.

Address correspondence to Toshio Suda, M.D., Department of Cell Differentiation, IMEG, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860, Japan. Phone: 81-96-373-5328; FAX: 81-96-373-5332; E-mail: sudato@gpo.kumamato-u.ac.jp

Received for publication 9 July 1996 and accepted in revised form 12 December 1996.

1. *Abbreviations used in this paper:* BALF, bronchoaveolar lavage fluid; CBF, ciliary beat frequency; HRP, horseradish peroxidase; HGF, hepatocyte growth factor; MSP, macrophage-stimulating protein; NHBE cell, normal human bronchial epithelial cell; RTK, receptor tyrosine kinase; STK, stem cell-derived kinase.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/97/02/0701/09 $2.00
Volume 99, Number 4, February 1997, 701–709

To explore the function of the MSP–RON signaling pathway, we investigated specific cell types that express RON. In this paper, we show that RON is expressed on the ciliated epithelia of the mucociliary transport apparatus. In addition, we show that MSP stimulates ciliary motility in these cells by activating RON.

Methods

Reagents. Recombinant human MSP and a rabbit polyclonal anti–human MSP antibody were provided by Toyobo Co., Ltd. (Ohtsu, Shiga, Japan). Mouse monoclonal anti–human MSP antibody (MSP89-2S) was obtained from American Type Culture Collection (ATCC HB-10522) (Rockville, MD). A synthetic polypeptide corresponding to the COOH-terminal 20 amino acids of human RON (amino acid residues 1381 to 1400) and a rabbit polyclonal antibody against this synthetic polypeptide were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibody DO-24, directed against the extracellular domain of the human HGF receptor and monoclonal antiphosphotyrosine antibody (clone 4G10) were from Upstate Biotechnology, Inc. (Lake Placid, NY). Donkey anti–rabbit IgG conjugated with horseradish peroxidase (HRP) and sheep anti–mouse IgG conjugated with HRP were from Amersham International (Little Chalfont, UK). Goat anti–rabbit IgG conjugated with FITC was from GIBCO BRL (Gaithersburg, MD). The enhanced chemiluminescence (ECL) detection system was from Amersham. Protein A-Agarose was from BioRad Laboratories (Richmond, CA).

Cells and cell cultures. Normal human bronchial epithelial (NHBE) cells were purchased from Clonetics (San Diego, CA). Transformed human bronchial epithelial cell lines, BET-1A and BEAS-2B cells (29) were from ATCC. These cells were cultured at 37°C in serum-free bronchial epithelial cell growth medium (BE-GM; Kurabo, Osaka, Japan) supplemented with growth factors according to the manufacturer's instructions. The surface of the culture dishes was coated with human placental collagen (Sigma Chemical Co., St. Louis, MO) as described (30). Third or fourth passage NHBE cells were used.

Northern blot analysis. Northern analysis was performed using a Human Multiple Tissue Northern (MTN) Blot (Clontech, Palo Alto, CA), containing 2 μg of poly(A)$^+$ RNA per lane, according to the manufacturer's instructions. A human RON cDNA probe, corresponding to nucleotides 2,979 to 3,478 of the published sequence (9), was [α-^{32}P]-labeled and hybridized to the blot. Autoradiographs were quantified by densitometric analysis with the BAS2000 image analyzer (Fuji Photo Film Co., Tokyo, Japan) using MacBAS software. Loading was normalized by probing with a β-actin probe, and expression of RON mRNA was evaluated by the densitometric ratio (RON signal/β-actin signal).

Immunohistochemistry and periodic acid schiff (PAS) staining. Normal human lung, bronchus, and oviduct were obtained from surgical samples. Nasal mucosa was obtained from biopsy samples. These studies were approved by the Institutional Review Board of Kumamoto University. Immediately after the tissues were obtained, they were snap frozen in liquid nitrogen or mounted in OCT 4583 embedding compound (Miles Laboratories Inc, Elkhart, IN) and then frozen in liquid nitrogen. Serial cryostat sections (6-μm thick) cut in a cryomicrotome (MICROM, Heidelberg, Germany) were air dried, and fixed in acetone for 10 min at 4°C. After washing with PBS three times, cryostat sections were treated with 5 mM orthoperiodic acid solution for 10 min to block endogenous peroxidase activity as described (31). The sections were stained with a rabbit polyclonal antibody against RON (0.1-0.5 μg/ml) or a mouse monoclonal antibody against HGF receptor (DO-24, 1:2,000 dilution) by the indirect immunoperoxidase method using HRP-conjugated anti–rabbit IgG or HRP-conjugated anti–mouse IgG, respectively. Peroxidase activity was visualized using 3,3′-diaminobenzidine (Dojindo, Kumamoto, Japan) as a substrate. To confirm the specific reactivity of anti-RON with the RON protein, one of the serial sections was incubated with a mixture of anti-RON antibody and an excess molar ratio of antigen peptide, then processed as described above. Goblet cells in bronchial epithelia were identified by PAS staining as described (32).

Immunoelectron microscopy. Fresh frozen sections of bronchial tissue were fixed in acetone for 10 min at 4°C. After washing with PBS three times, immunostaining was performed as described above. After immunostaining, the sections were fixed with 0.1% glutaraldehyde for 10 min, followed by postfixation with 1.0% osmium tetroxide for 2 h, dehydration in a graded series of ethanols, and embedding in Epon 812 (E. Fullan, Inc., Latham, NY). Ultrathin sections were cut by a MT-7000 (RMC, Tucson, AZ) and observed with a HU-12A electron microscope (Hitachi Ltd., Tokyo, Japan).

FACS analysis of NHBE cells. NHBE cells were detached from culture dishes using 5 mM EDTA/PBS (pH 7.4), and analyzed by FACS. Briefly, the detached NHBE cells were washed with PBS and incubated with 70% ethanol on ice for 1 h to permeabilize and fix. After washing with staining medium containing 5% FBS and 0.01% sodium azide in PBS, the cells were stained with anti-RON (0.5 μg/ml), followed by FITC-conjugated anti–rabbit IgG. The cells were washed and suspended in staining medium containing propidium iodide. Stained cells were analyzed by FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). The specific staining for RON in the cells was confirmed by antigen-peptide blocking.

^{125}I-MSP binding assay. MSP was radio-iodinated by the Bolton-Hunter method as described by Wang et al. (13). NHBE cells were grown on a 48-well culture plate to subconfluency and washed with binding buffer (RPMI 1640 medium, 25 mM Hepes, 2 mg/ml BSA, pH 7.2). After equilibration in the binding buffer at 4°C for 30 min, the buffer was replaced by 100 μl of ice-cold binding buffer containing various amounts of ^{125}I-labeled MSP. Nonspecific binding was determined in parallel tubes with a 50-fold excess of unlabeled MSP. After incubation at 4°C for 3 h, the cells were washed five times with ice-cold binding buffer and then solubilized with 0.5 ml of 1% Triton X-100, 10% glycerol, 25 mM Hepes, 1 mg/ml BSA, pH 7.2 by incubation for 40 min. Radioactivity was measured in a gamma counter. All binding experiments were performed in triplicate.

In vivo tyrosine phosphorylation assays, immunoprecipitation, and Western blotting. NHBE, BET-1A, and BEAS-2B cells were stimulated with 4 nM human MSP at 37°C for 10 min. After washing with ice-cold Hepes buffered saline containing 1mM sodium orthovanadate, the cells were then solubilized with lysis buffer (50 mM Hepes [pH 7.4], 1% Triton X-100, 10% glycerol, 10 mM sodium pyrophos-

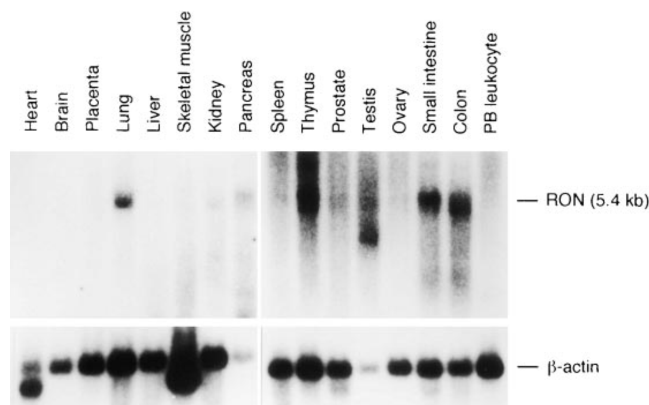

Figure 1. Northern blot of RON mRNA in human adult tissues. A [α-^{32}P]-labeled RON cDNA probe was prepared and hybridized to the blot. The blots were exposed for 4 d. Expression of RON mRNA was detected as a 5.4-kb band. The densitometric ratio (RON signal/β-actin signal) was quantitated as follows; lung (0.38), pancreas (0.67), thymus (0.74), prostate (0.18), testis (1.09), small intestine (0.77), and colon (0.82).

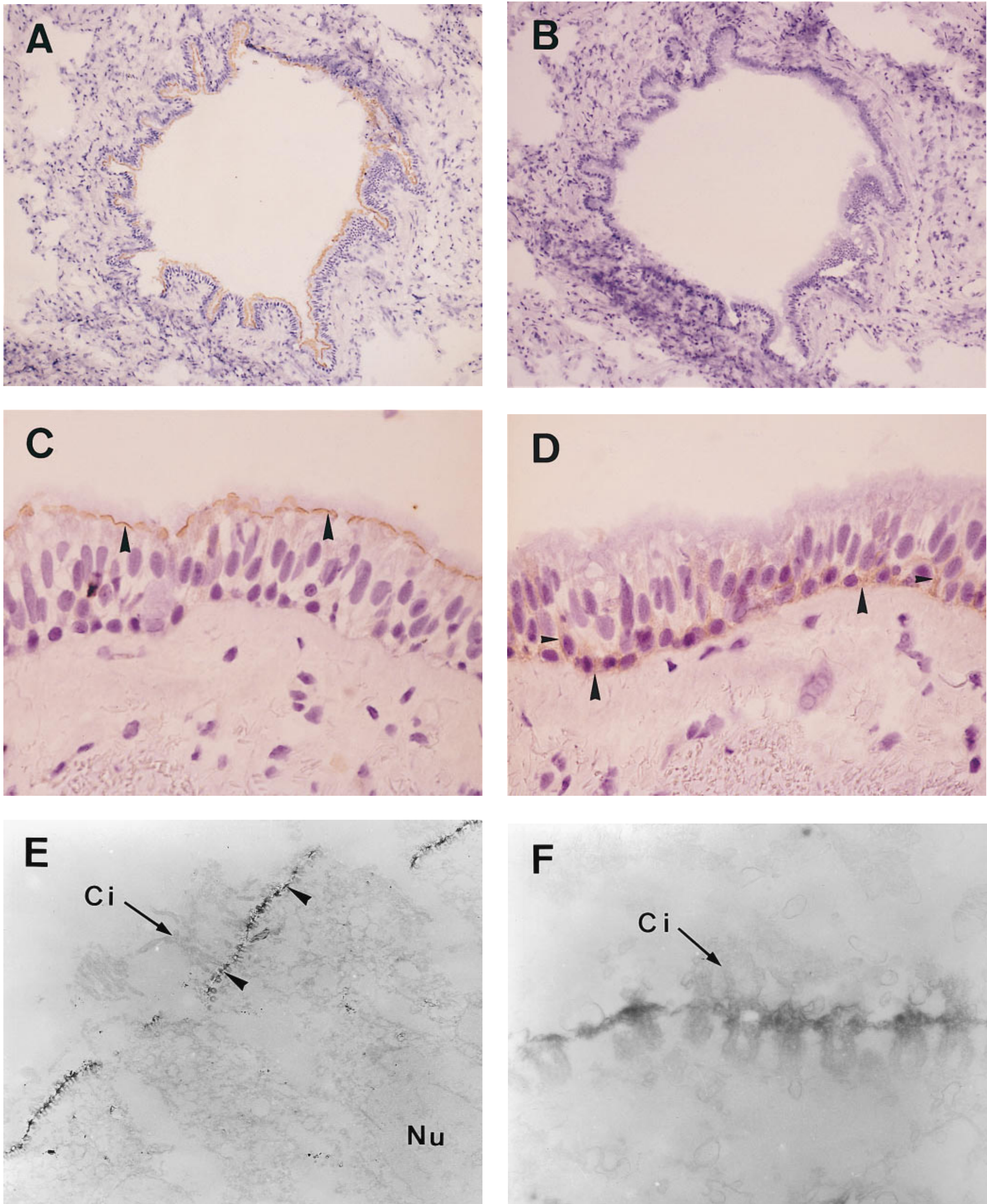

Figure 2. (*A* and *B*) Immunolocalization of RON in the human lung. (*A*) The apical surface of the bronchiolar ciliated epithelium showed positive staining. (*B*) The positive staining was completely blocked in the presence of an excess of antigen peptide (*A* and *B*, ×25). (*C* and *D*) Immunolocalization of RON and HGF receptor in the human bronchial epithelium. (*C*) RON was localized on the apical cell membrane of the ciliated epithelium (*arrowheads*), whereas (*D*) HGF receptor was localized on the basolateral cell membrane (*arrowheads*; *C* and *D*, ×132). (*E* and *F*) Immunoelectron microscopic demonstration of an anti-RON reaction in the human bronchial epithelium. The electro-dense reaction products were observed on the apical cell membrane beneath the cilia of the ciliated epithelium (*arrowheads*; *E*, ×3,000; *F*, ×20,000). Ci, Cilia (*arrow*); Nu, Nucleus.

phate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium orthovanadate, 50 μg/ml aprotinin, 1 mM PMSF, 100 μM leupeptin, 25 μM pepstatin A), and proteins in cell lysates were immunoprecipitated with anti-RON. Immune complexes were collected on protein A-agarose. Immunoprecipitates were boiled for 3 min at 100°C in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer with 5% 2-mercaptoethanol (2-ME), then resolved by SDS–PAGE with 7.5% polyacrylamide as described by Laemmli (33). After electrophoresis, proteins were transferred to a PVDF membrane (Nihon Millipore Ltd, Yonezawa, Japan). Tyrosine phosphorylation of RON was evaluated with an antiphosphotyrosine antibody, 4G10. To detect RON, the membrane was stripped and reprobed with anti-RON. Specific binding was detected using the ECL system.

Quantification of MSP in bronchoalveolar lavage fluid. Bronchoalveolar lavage (BAL) fluid (BALF) was obtained from healthy nonsmoking volunteers ($n = 4$) by methods previously described (34). BAL was performed with 150 ml saline and the recovered BALF was filtered through sterile gauze. The cell-free supernatant of BALF was obtained by centrifugation for 10 min at 300 *g* and frozen immediately at −70°C. MSP concentrations in BALF supernatants were measured by sandwich-type ELISA. Briefly, a microtiter plate was coated with a mouse monoclonal anti–human MSP antibody for overnight at 4°C. After blocking with 3% BSA in PBS, the plate was incubated with diluted standards or samples. After washing, the plate was incubated with a rabbit polyclonal anti–human MSP antibody, followed by HRP-conjugated anti–rabbit IgG. Specific binding was detected by incubating the plate with 0.05 M phosphate-citrate buffer containing 0.4 mg/ml *o*-phenylenediamine dihydrochloride and 0.4 mg/ml urea hydrogen peroxide. The absorbance at 450 nm was measured in an automated microplate reader (Molecular Devices Corp., Sunnyvale, CA). MSP concentrations were determined by interpolation of their absorbance from a standard curve.

Measurement of ciliary beat frequency (CBF) of human nasal cilia in vitro. Human airway ciliated epithelium was obtained from the nasal mucosa of healthy volunteers ($n = 4$) by a brushing technique (35). Strips of ciliated epithelium were dispersed by agitation of the cytology brush in Medium 199 (Gibco Laboratories, Grand Island, NY). To exclude endogenous MSP, cells were incubated overnight at 4°C, then centrifuged at 200 *g* for 5 min. The epithelial pellet was resuspended in Medium 199. The suspension (450 μl) of the epithelial strips was poured into each well of Falcon 3047 multiwell plate (Becton Dickinson Labware, Lincoln Park, NJ), placed on an electronically controlled warm-stage (Microtec) at 37°C, and allowed to equilibrate for 15 min. The epithelial strips were viewed directly through a Nikon Diaphot-TMD phase-contrast microscope at a magnification of 400. An intact and fully ciliated epithelial strip with a smooth surface was selected, and serial readings of CBF were taken from the same point of the epithelial strip for 50 min using a photometric technique (36, 37). After the determination of baseline CBF for 15 min, 50 μl of Medium 199 alone (control) or Medium 199 containing MSP (final concentration; 1, 10, 50, or 100 ng/ml) was gently added to the well, and CBF was continuously measured. The test samples (Medium 199 alone and Medium 199 containing MSP) were randomized at the beginning of each experiment so that the observer was unaware of the nature of the test samples. Baseline CBF was defined as mean CBF during the period of 15 min before adding the test samples and maximum CBF was defined as maximal value of CBF after the addition. The statistical significance of the differences between maximum and baseline CBF was determined using the two-tailed Student's *t* test.

Results

Expression of RON in human tissues. The expression of RON was examined in a number of human adult tissues by Northern blot analysis. As shown in Fig. 1, a 5.4-kb RON transcript was detected in the lung, pancreas, thymus, prostate, testis, small intestine, and colon. The densitometric ratio (RON signal/β-actin signal) was quantitated as follows; lung (0.38), pancreas (0.67), thymus (0.74), prostate (0.18), testis (1.09), small intestine (0.77), and colon (0.82).

Immunolocalization of RON on the ciliated epithelium in the lung and distinct expression of RON and HGF receptor in the bronchial ciliated epithelium. To identify specific cell types expressing RON in the lung, we performed immunohistochemical staining of normal human lung and bronchial tissue using an anti-RON antibody. In the lung specimens, the bronchiolar ciliated epithelia were clearly stained with anti-RON (Fig. 2 *A*). Notably, the positive staining was localized at the apical surface of the cells. The specific reactivity of anti-RON antibody with RON was confirmed by the finding that positive staining was completely blocked by an excess molar ratio of antigen peptide (Fig. 2 *B*). A similar staining pattern was observed also in the bronchial ciliated epithelia (Fig. 2 *C*). Negative staining of goblet cells in bronchial epithelia was confirmed by comparing immunohistochemical staining with anti-RON and PAS staining using serial sections (Fig. 3, *A* and *B*).

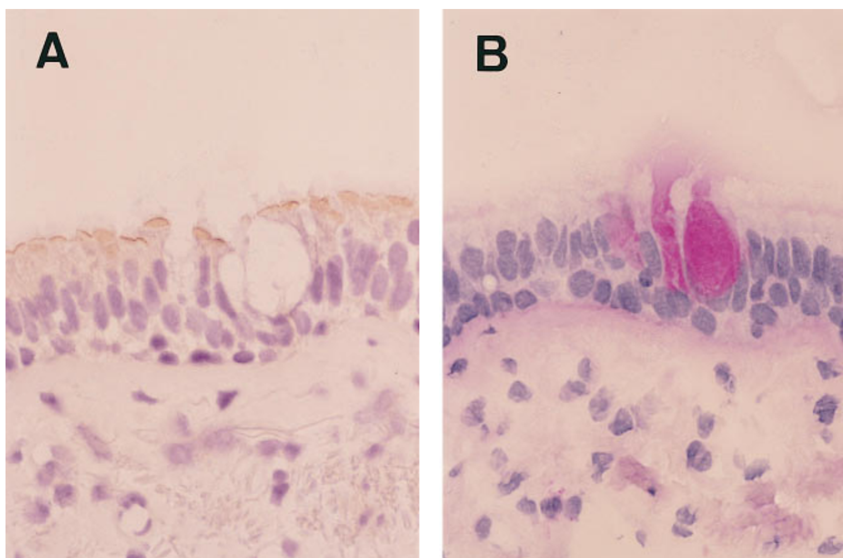

Figure 3. Examination of RON expression in goblet cells of bronchial epithelia. Comparison of immunohistochemical staining with anti-RON (*A*) and PAS staining (*B*) using serial sections showed that goblet cells are negative for RON (*A* and *B*, ×132).

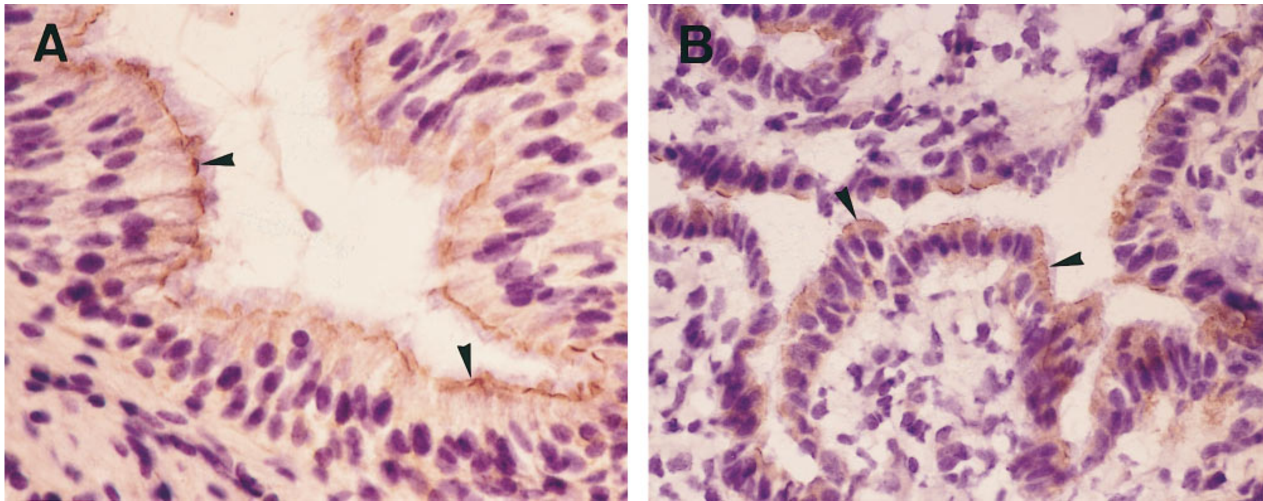

Figure 4. Immunolocalization of RON in the human nasal mucosa (*A*) and oviduct (*B*). The apical portion of the pseudostratified ciliated epithelium of nasal mucosa and of the ciliated epithelium of oviduct showed positive staining (*arrowheads*; *A*, ×160; *B*, ×132).

No specific staining was detected in other types of cells in the lung, including alveolar epithelial cells and alveolar macrophages (data not shown).

The HGF receptor is expressed in bronchial epithelial cells where it mediates a growth signal (38). Because of the structural similarities between RON and HGF receptor, we compared the expression of HGF receptor with RON in bronchial ciliated epithelium. As demonstrated in other polarized epithelial cells by Crepaldi et al. (39), a monoclonal antibody against the HGF receptor selectively stained the basolateral cell membrane of bronchial ciliated epithelia (Fig. 2 *D*). This staining pattern presents a striking contrast to the restricted expression of RON to the apical cell membrane (Fig. 2 *C*).

Ultrastructural localization of RON on the bronchial ciliated epithelium. To identify the precise localization of RON in the bronchial ciliated epithelium, we performed immunoelectron microscopic analysis using anti-RON antibody. As shown in Fig. 2, *E* and *F*, electro-dense reaction products were localized at the apical cell membrane beneath the cilia in the ciliated epithelium.

Expression of RON in the ciliated epithelium of nasal cavity and oviduct. Because the expression of RON was restricted to ciliated epithelium in the lung and bronchus, we focused on whether RON is expressed in the ciliated epithelium in other organs, such as nasal cavity and oviduct. Fig. 4, *A* and *B* show that RON was similarly expressed at the apical portion of the ciliated epithelia of nasal mucosa and oviduct. The specific staining for RON in these cells was also confirmed by antigen-peptide blocking (data not shown).

Expression of RON in NHBE cells, and activation of RON receptor in NHBE cells and bronchial epithelial cell lines by MSP. To examine the expression of RON on NHBE cells, we performed FACS analysis of NHBE cells using an anti-RON antibody. As shown in Fig. 5, FACS analysis shows that NHBE cells are uniformly positive for RON. The specific staining for RON was confirmed by the finding that the histogram of positive staining completely shifted to the basal level by peptide blocking. Specific staining for RON to BET-1A cells was also detected (data not shown).

To further confirm the expression of RON on NHBE cells, we performed the binding assay using ^{125}I-MSP. Fig. 6 *A* shows a typical saturation curve of ^{125}I-MSP binding to RON on cultured NHBE cells and a Scatchard plot. The specific binding to NHBE cells was saturated at $\sim$ 1 nM of ^{125}I-MSP. The K_d value and the average number of RON receptors on a cell were calculated from the Scatchard plot as 0.5 nM and 5,000 sites/cell, respectively. Specific binding of ^{125}I-MSP to BET-1A cells was also detected (K_d = 0.5 nM; 6,000 sites/cell).

To investigate the functional involvement of MSP-induced signals in bronchial epithelial cells, we examined whether MSP induces autophosphorylation of RON in NHBE cells and cell lines. MSP-stimulation induced autophosphorylation of the 150-kD RON β-chain in these cells (Fig. 6 *B*), suggesting that RON mediates the signals for MSP in bronchial epithelial cells.

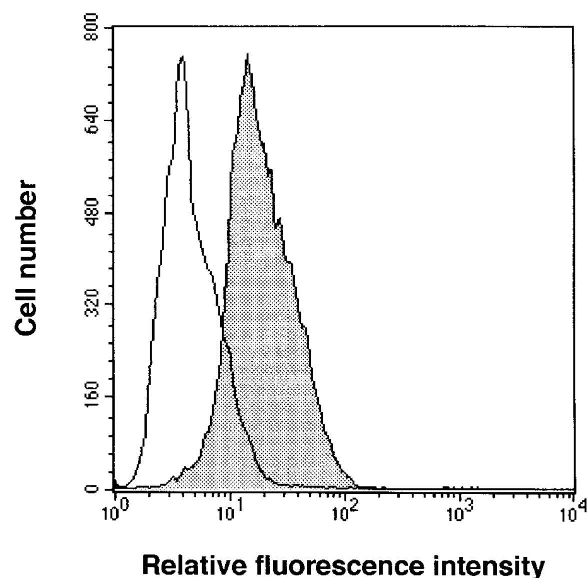

Figure 5. FACS analysis of RON expression on NHBE cells with anti-RON. Stippled histogram shows analysis of NHBE cells stained with anti-RON and open histogram shows antigen-peptide blocking.

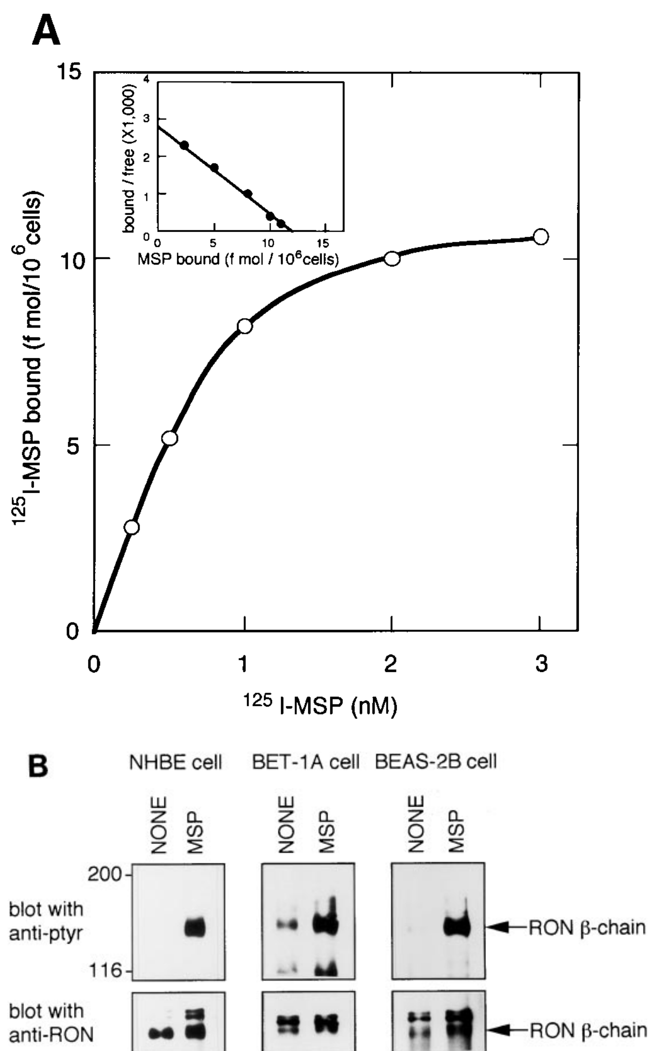

Figure 6. (*A*) Concentration-dependent binding of ^{125}I-MSP to its receptor on NHBE cells. Specific binding was obtained by subtracting nonspecific binding from total binding. The inset panel shows a Scatchard plot. (*B*) Induction of autophosphorylation of RON β-chain by MSP. NHBE, BET-1A, and BEAS-2B cells were stimulated with medium only (NONE) or 4 nM human MSP. Proteins in cell lysates were immunoprecipitated with anti-RON. Immunoprecipitates were Western blotted with antiphosphotyrosine (anti-ptyr; 4G10; *top*) and with anti-RON (*bottom*). The bands above the 150-kD protein in blots with anti-RON, which were not phosphorylated by MSP-stimulation, represent the cytoplasmic single-chain precursors as reported (13).

Detection of MSP in BALF from healthy volunteers. Because the expression of RON is localized at the apical cell membrane of bronchiolar and bronchial ciliated epithelia, we examined whether its ligand MSP is present in the bronchoalveolar space. MSP content was evaluated based upon the MSP content per milliliter of BALF. As shown in Fig. 7, MSP was detected in BALF obtained from healthy nonsmoking volunteers at concentrations of 1.3–5.8 ng/ml BALF.

Effects of MSP on CBF of human nasal cilia. The fact that RON is specifically expressed on the ciliated epithelia in the airways and oviduct, and that RON is localized at the apical cell membrane beneath the cilia encouraged us to focus on the involvement of MSP in ciliary function, particularly in ciliary motility. MSP significantly increased the CBF of human nasal cilia at concentrations of 1 to 50 ng/ml (Fig. 8 *A*). The maximum increase in CBF (20%) was observed at the concentration of 10 ng/ml. This dose response curve to MSP showed a characteristic bell-shaped profile. MSP is reported to induce chemotactic attraction of resident peritoneal macrophages (22) and stimulate [^{3}H]thymidine incorporation of neuroendocrine cell line PC12 (26). The dose-response curves to MSP in these experiments were similar to our finding. Fig. 8 *B* shows the time course of CBF changes in each experiment after addition of 10 ng/ml MSP. The maximum increase in CBF was observed during 10–30 min after the addition of MSP. Control preparations showed no significant change in CBF.

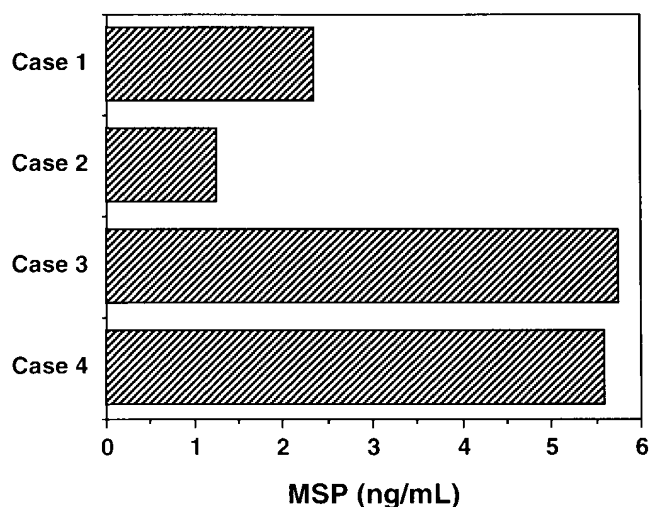

Figure 7. Concentration of MSP in BALF from healthy nonsmoking volunteers (n = 4). Results are presented as nanograms per milliliter BALF.

Discussion

Previous studies have shown that the MSP ligand and the RON receptor function primarily in mononuclear phagocytes such as peritoneal resident macrophages (12, 16, 21–23) and osteoclasts (24). In addition, RON is expressed in the nervous systems, epithelia of the gut, and the skin and lung (9, 11, 25, 26). However, the function of the MSP-RON signaling pathway in cells other than mononuclear phagocytes has not been elucidated.

By Northern blot analysis, we confirmed that human RON mRNA is abundantly expressed in the lung as previously described (9, 11). Immunohistochemical and immunoelectron microscopic analyses demonstrated that RON shows a unique localization to the apical cell membrane beneath the cilia in the bronchiolar and bronchial ciliated epithelia. On the other hand, the HGF receptor showed a distinct localization to the basolateral cell membrane. This difference in subcellular localization suggests that these highly related receptors may mediate distinct functions in the bronchial ciliated epithelium. Singh-Kaw et al. demonstrated that HGF stimulates cell growth of NHBE cells using the bromodeoxyuridine labeling technique (38). These findings show that HGF acts as a mitogenic factor in NHBE cells as well as other epithelial cells. On the contrary, MSP does not stimulate DNA synthesis in NHBE cells (data

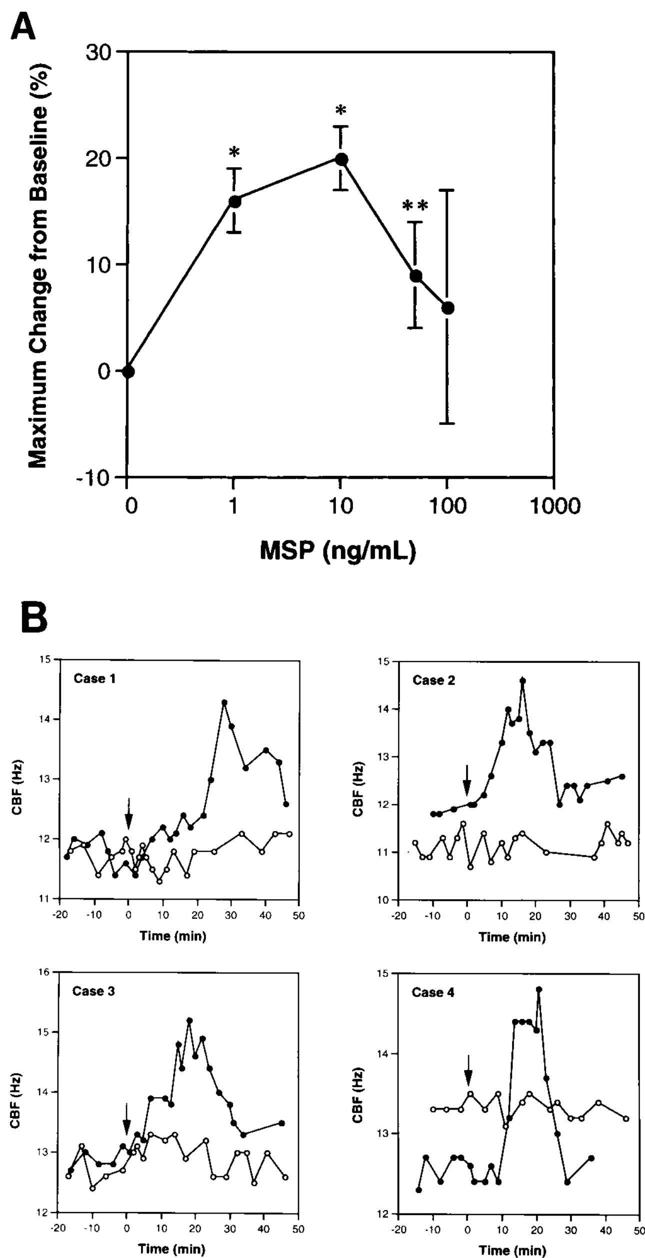

Figure 8. Effects of MSP on CBF of human nasal cilia in vitro. (*A*) Dose-response curve to MSP. MSP significantly increased CBF at concentrations of 1 to 50 ng/ml. The maximum increase in CBF (20%) was observed at the concentration of 10 ng/ml. Maximum change (%) = (maximum CBF − baseline CBF) ÷ baseline CBF × 100. Each value represents mean (n = 4)±SEM. *P = 0.001, **P = 0.05. (*B*) Time course of CBF changes in each experiment after addition of 10 ng/ml MSP (*closed circles*). The maximum increase in CBF was observed during 10–30 min after the addition of MSP. Control preparations showed no significant change in CBF (*open circles*). Arrows indicate the addition of Medium 199 containing MSP or Medium 199 alone. Ciliary dyskinesia and epithelial disruption were not observed in our experiments.

not shown). The apical versus basolateral localization of two related receptors may play a role in their distinct functions in polarized epithelial cells.

The bronchiolar and bronchial ciliated epithelia function as the mucociliary transport apparatus. Notably, in other mucociliary transport systems such as nasal cavity and oviduct, RON was also expressed at the apical portion of the ciliated epithelia.

To further analyze the expression of RON in bronchial epithelial cells, we used NHBE cells and two bronchial epithelial cell lines, BET-1A and BEAS-2B cells (29). These cell lines were derived from NHBE cells and have been shown to have similar biological properties to NHBE cells (40–42). In general, intermediate cells (presecretory, columnar cells) and/or basal cells are considered to be progenitors of bronchial epithelial cells and can differentiate further into ciliated cells in vivo (43), and previous studies indicate that the majority of NHBE cells used in our experiments consist of both the intermediate and the basal cells (44, 45). Expression of RON in NHBE cells and BET-1A cells by FACS analysis and receptor binding assay suggests that RON is expressed also on the progenitor cells. In addition, the RON protein was immunoprecipitated from NHBE cells and cell lines, and MSP induced autophosphorylation of RON β-chain in these cells. Our speculation is that both the intermediate and the basal cells in vivo may express RON at the detectable level by FACS analysis, and during the course of differentiation of the cells in vivo, expression of RON may upregulate or accumulate at the apical surface on the ciliated epithelium at the detectable level by immunohistochemistry. Thus, these findings suggest that RON can be activated by MSP and transduces signals in bronchial epithelial cells.

In the case of the HGF–HGF receptor system, the HGF receptor is expressed at the basolateral but not the apical cell membrane of epithelia lining the lumen in several organs, while HGF is expressed in the surrounding mesenchyme (27, 39), suggesting a paracrine mode of HGF action. On the other hand, MSP is mainly produced by hepatocytes (15, 17, 19). MSP is not expressed in the lung (data not shown), and is presumed to be derived from the circulation. We found that BALF from healthy volunteers contains MSP at concentrations of 1.3–5.8 ng/ml. Because we detected diluted MSP in BALF, the absolute concentration of MSP in the bronchoalveolar space should be higher. This finding suggests that MSP is supplied to the bronchoalveolar space from the circulation in concentrations high enough to be biologically significant and that MSP binds to and activates RON at the apical surface of ciliated epithelium in vivo.

The expression of RON on the apical cell membrane of ciliated epithelia and the possible interaction of MSP with RON on their apical cell surface in vivo suggest that RON is involved in the control of functions specific to ciliated epithelium, especially in ciliary functions. In fact, MSP significantly increased CBF of human nasal cilia in vitro. The stimulatory effect of MSP on ciliary motility was comparable to that of inflammatory mediators and neuropeptides previously reported, namely, leukotriene-C_4 (maximum increase in CBF, 33%), leukotriene-D_4 (16%), prostaglandin E_1 (13%), and neurokinin A (15%) (46). To date, however, no evidence of direct hormonal control of ciliary motility has been demonstrated (47). Thus, it seems reasonable to conclude that MSP is the first serum protein with an ability to stimulate ciliary motility. Although highly purified recombinant MSP, as judged from SDS–PAGE and HPLC analyses, was used in the present work, we cannot exclude the possibility that another factor may exist in our MSP solution that affects CBF because at present we can-

not obtain any antagonists of the MSP–RON signaling pathway to examine this possibility.

Cilia are a major component of the mucociliary transport system. The primary function of cilia is to move fluid over the surface of epithelial cells or to propel single cells through a fluid. On the epithelial cells lining the human respiratory tract, huge numbers of cilia sweep layers of mucus. Cilia also help to sweep eggs along the oviduct, and a closely related structure, the flagellum, propels sperm. Ciliary dysfunction causes several conditions such as chronic respiratory tract infections and sterility in individuals with primary ciliary dyskinesia (48–50). Therefore, ciliary motility plays an essential role in both host defense and fertilization.

The structural components of cilia and flagella have been well characterized and are conserved among ciliated epithelia and the sperm tail, and intracellular Ca^{2+} concentration or cAMP-stimulated phosphorylation has been implicated in the regulation of ciliary motility (51–53). However, no intrinsic factor that regulates ciliary motility in vivo has been elucidated so far (47). Our findings establish that the MSP–RON signaling pathway functions as one regulatory system of ciliary motility. Furthermore, we have found in rats that MSP mRNA is expressed in the epithelium of epididymis and RON mRNA is expressed in sperm, suggesting that locally produced MSP may be involved in sperm motility (54). Dey et al. showed that activation of *Ciona* sperm motility are inhibited by a tyrosine kinase inhibitor (55). Taken together, these findings suggest that tyrosine kinase may play an important role in sperm motility. How activation of RON by MSP stimulates motility of cilia as well as peritoneal macrophages and osteoclasts is currently under investigation.

In conclusion, we showed that the MSP–RON signaling pathway is a novel regulatory system of ciliary motility. This pathway may function as a universal regulator of mucociliary transport.

Acknowledgments

We are grateful to Drs. Y. Suenaga and M. Araki for providing surgical samples and Drs. K. Iyonaga, N. Hirata, and T. Inada for technical support.

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

References

1. Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell.* 61:203–212.

2. Schlessinger, J., and A. Ullrich. 1992. Growth factor signaling by receptor tyrosine kinases. *Neuron.* 9:383–391.

3. Fantl, W.J., D.E. Johnson, and L.T. Williams. 1993. Signaling by receptor tyrosine kinases. *Annu. Rev. Biochem.* 62:453–481.

4. Hanks, S.K., A.M. Quinn, and T. Hunter. 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science (Wash. DC).* 241:42–52.

5. Yarden, Y., and A. Ullrich. 1988. Growth factor receptor tyrosine kinases. *Annu. Rev. Biochem.* 57:443–478.

6. Cohen, T., R. Ren, and D. Baltimore. 1995. Modular binding domains in signal transduction proteins. *Cell.* 80:237–248.

7. Pawson, T. 1995. Protein modules and signaling networks. *Nature (Lond.).* 373:573–580.

8. Huff, J.L., M.A. Jelinek, C.A. Borgman, T.J. Lansing, and J.T. Parsons. 1993. The protooncogene c-*sea* encodes a transmembrane protein-tyrosine kinase related to the Met/hepatocyte growth factor/scatter factor receptor. *Proc. Natl. Acad. Sci. USA.* 90:6140–6144.

9. Ronsin, C., F. Muscatelli, M.-G. Mattei, and R. Breathnach. 1993. A novel putative receptor protein tyrosine kinase of the met family. *Oncogene.* 8: 1195–1202.

10. Iwama, A., K. Okano, T. Sudo, Y. Matsuda, and T. Suda. 1994. Molecular cloning of a novel receptor tyrosine kinase gene, *STK*, derived from enriched hematopoietic stem cells. *Blood.* 83:3160–3169.

11. Gaudino, G., A. Follenzi, L. Naldini, C. Collesi, M. Santoro, K.A. Gallo, P.J. Godowski, and P.M. Comoglio. 1994. Ron is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3524–3532.

12. Iwama, A., M.-H. Wang, N. Yamaguchi, N. Ohno, K. Okano, T. Sudo, M. Takeya, F. Gervais, C. Morissette, E.J. Leonard, et al. 1995. Terminal differentiation of murine resident peritoneal macrophages is characterized by expression of the STK protein tyrosine kinase, a receptor for macrophage-stimulating protein. *Blood.* 86:3394–3403.

13. Wang, M.-H., C. Ronsin, M.-C. Gesnel, L. Coupey, A. Skeel, E.J. Leonard, and R. Breathnach. 1994. Identification of the *ron* gene product as the receptor for the human macrophage stimulating protein. *Science (Wash. DC).* 266:117–119.

14. Wang, M.-H., A. Iwama, A. Skeel, T. Suda, and E.J. Leonard. 1995. The murine *stk* gene product, a transmembrane protein tyrosine kinase, is a receptor for macrophage-stimulating protein. *Proc. Natl. Acad. Sci. USA.* 92:3933–3937.

15. Han, S., L.A. Stuart, and S.J.F. Degen. 1991. Characterization of the DNF15S2 locus on human chromosome 3: identification of a gene coding for four kringle domains with homology to hepatocyte growth factor. *Biochemistry.* 30:9768–9780.

16. Skeel, A., T. Yoshimura, S.D. Showalter, S. Tanaka, E. Appella, and E.J. Leonard. 1991. Macrophage stimulating protein: purification, partial amino acid sequence, and cellular activity. *J. Exp. Med.* 173:1227–1234.

17. Yoshimura, T., N. Yuhki, M.-H. Wang, A. Skeel, and E.J. Leonard. 1993. Cloning, sequencing, and expression of human macrophage stimulating protein (MSP, MST1) confirms MSP as a member of the family of kringle proteins and locates the MSP gene on chromosome 3. *J. Biol. Chem.* 268:15461–15468.

18. Shimamoto, A., T. Kimura, K. Matsumoto, and T. Nakamura. 1993. Hepatocyte growth factor-like protein is identical to macrophage stimulating protein. *FEBS Lett.* 333:61–66.

19. Bezerra, J.A., D.P. Witte, B.J. Aronow, and S.J.F. Degen. 1993. Hepatocyte-specific expression of the mouse hepatocyte growth factor-like protein. *Hepatology.* 18:394–399.

20. Wang, M.-H., A. Skeel, T. Yoshimura, T.D. Copeland, K. Sakaguchi, and E.J. Leonard. 1993. Antibodies to macrophage stimulating protein (MSP): specificity, epitope interactions, and immunoassay of MSP in human serum. *J. Leukocyte Biol.* 54:289–295.

21. Leonard, E.J., and A. Skeel. 1976. A serum protein that stimulates macrophage movement, chemotaxis and spreading. *Exp. Cell Res.* 102:434–438.

22. Skeel, A., and E.J. Leonard. 1994. Action and target cell specificity of human macrophage-stimulating protein (MSP). *J. Immunol.* 152:4618–4623.

23. Wang, M.-H., G.W. Cox, T. Yoshimura, L.A. Sheffler, A. Skeel, and E.J. Leonard. 1994. Macrophage-stimulating protein inhibits induction of nitric oxide production by endotoxin- or cytokine-stimulated mouse macrophages. *J. Biol. Chem.* 269:14027–14031.

24. Kurihara, N., A. Iwama, J. Tatsumi, K. Ikeda, and T. Suda. 1996. Macrophage-stimulating protein activates STK receptor tyrosine kinase on osteoclasts and facilitates bone resorption by osteoclast-like cells. *Blood.* 87:3704–3710.

25. Quantin, B., B. Schuhbaur, M.-C. Gesnel, P. Dollé, and R. Breathnach. 1995. Restricted expression of the ron gene encoding the macrophage stimulating protein receptor during mouse development. *Dev. Dyn.* 204:383–390.

26. Gaudino, G., V. Avantaggiato, A. Follenzi, D. Acampora, A. Simeone, and P.M. Comoglio. 1995. The proto-oncogene *RON* is involved in development of epithelial, bone and neuro-endocrine tissues. *Oncogene.* 11:2627–2637.

27. Sonnenberg, E., D. Meyer, K.M. Weidner, and C. Birchmeier. 1993. Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *J. Cell Biol.* 123:223–235.

28. Comoglio, P.M. 1993. *In* Hepatocyte Growth Factor-Scatter Factor (HGF-SF) and the C-Met Receptor. I.D. Goldberg, and E.M. Rosen, editors. Birkäuser Verlag, Basel, Switzerland. 131–165.

29. Reddel, R.R., Y. Ke, B.I. Gerwin, M.G. McMenamin, J.F. Lechner, R.T. Su, D.E. Brash, J.-B. Park, J.S. Rhim, and C.C. Harris. 1988. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer Res.* 48:1904–1909.

30. Coleman, D.L., I.K. Tuet, and J.H. Widdicombe. 1984. Electrical properties of dog tracheal epithelial cells grown in monolayer culture. *Am. J. Physiol.* 246:C355–C359.

31. Isobe, Y., S.-T. Chen, P.K. Nakane, and W.R. Brown. 1977. Studies on translocation of immunoglobulins across intestinal epithelium. I. Improvements in the peroxidase-labeled antibody method for application to study of human intestinal mucosa. *Acta Histochem. Cytochem.* 10:161–171.

32. Lillie, R.D., and M.F. Harold. 1976. Histopathologic Technic and Practical Histochemistry. McGraw-Hill Book Company, New York. 612–626.

33. Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

34. The BAL Cooperative Group Steering Committee. 1990. Bronchoalveolar lavage constituents in healthy individuals, idiopathic pulmonary fibrosis, and selected comparison groups. *Am. Rev. Respir. Dis.* 141(Suppl.):169–202.

35. Rutland, J., and P.J. Cole. 1980. Noninvasive sampling of nasal cilia for measurement of beat frequency and study of ultrastructure. *Lancet.* ii:564–565.

36. Amitani, R., R. Wilson, A. Rutman, R. Read, C. Ward, D. Burnett, R.A. Stockley, and P.J. Cole. 1991. Effects of human neutrophil elastase and *Pseudomonas aeruginosa* proteinases on human respiratory epithelium. *Am. J. Respir. Cell Mol. Biol.* 4:26–32.

37. Wilson, R., T. Pitt, G. Taylor, D. Watson, J. MacDermot, D. Sykes, D. Roberts, and P. Cole. 1987. Pyocyanin and 1-hydroxyphenazine produced by *Pseudomonas aeruginosa* inhibit the beating of human respiratory cilia in vitro. *J. Clin. Invest.* 79:221–229.

38. Singh-Kaw, P., R. Zarnegar, and J.M. Siegfried. 1995. Stimulatory effects of hepatocyte growth factor on normal and neoplastic human bronchial epithelial cells. *Am. J. Physiol.* 268:L1012–L1020.

39. Crepaldi, T., A.L. Pollack, M. Prat, A. Zborek, K. Mostov, and P.M. Comoglio. 1994. Targeting of the SF/HGF receptor to the basolateral domain of polarized epithelial cells. *J. Cell Biol.* 125:313–320.

40. Cromwell, O., Q. Hamid, C.J. Corrigan, J. Barkans, Q. Meng, P.D. Collins, and A.B. Kay. 1992. Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1β and tumor necrosis factor-α. *Immunology.* 77: 330–337.

41. Levine, S.J., P. Larivée, C. Logun, C.W. Angus, and J.H. Shelhamer. 1993. Corticosteroids differentially regulate secretion of IL-6, IL-8, and G-CSF by a human bronchial epithelial cell line. *Am. J. Physiol.* 265:L360–L368.

42. Bloemen, P.G.M., M.C. van den Tweel, P.A.J. Henricks, F. Engels, S.S. Wagenaar, A.A.J.J.L. Rutten, and F.P. Nijkamp. 1993. Expression and modulation of adhesion molecules on human bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 9:586–593.

43. Jetten, A.M. 1992. Proliferation and differentiation in normal and neoplastic tracheobronchial epithelial cells. *In* Lung Cancer Differentiation. S.D. Bernal, and P.J. Hesketh, editors. Marcel Dekker Inc., New York. 3–43.

44. Widdicombe, J.H., D.L. Coleman, W.E. Finkbeiner, and I.K. Tuet. 1985. Electrical properties of monolayers cultured from cells of human tracheal mucosa. *J. Appl. Physiol.* 58:1729–1735.

45. Kauffman, S.L. 1980. Cell proliferation in the mammalian lung. *Int. Rev. Exp. Pathol.* 22:131–191.

46. Widdicombe, J.H. 1991. Physiology of airway epithelia. *In* The Airway Epithelium. S.G. Farmer, and D.W.P. Hay, editors. Marcel Dekker Inc., New York. 41–64.

47. Chediak, A.D., and A. Wanner. 1993. Mucociliary function. *In* Bronchial Asthma, 3rd ed. E.B. Weiss, and M. Stein, editors. Little, Brown and Company, Boston. 371–381.

48. Eliasson, R., B. Mossberg, P. Camner, and B.A. Afzelius. 1977. A congenital ciliary abnormality as an etiologic factor in chronic airway infections and male sterility. *New. Engl. J. Med.* 297:1–6.

49. Rossman, C.M., J.B. Forrest, R.M.K.W. Lee, and M.T. Newhouse. 1980. The dyskinetic cilia syndrome: ciliary motility in immotile cilia syndrome. *Chest.* 78:580–582.

50. Rossman, C.M., J.B. Forrest, R.M.K.W. Lee, A.F. Newhouse, and M.T. Newhouse. 1981. The dyskinetic cilia syndrome: abnormal ciliary motility in association with abnormal ciliary ultrastructure. *Chest.* 80:860–864.

51. Verdugo, P. 1980. Ca^{2+}-dependent hormonal stimulation of ciliary activity. *Nature (Lond.).* 283:764–765.

52. Sanderson, M.J., and E.R. Dirksen. 1986. Mechanosensitivity of cultured ciliated cells from the mammalian respiratory tract: implications for the regulation of mucociliary transport. *Proc. Natl. Acad. Sci. USA.* 83:7302–7306.

53. Hamasaki, T., K. Barkalow, J. Richmond, and P. Satir. 1991. cAMP-stimulated phosphorylation of an axonemal polypeptide that copurifies with the 22S dynein arm regulates microtubule translocation velocity and swimming speed in *Paramecium. Proc. Natl. Acad. Sci. USA.* 88:7918–7922.

54. Ohshiro, K., A. Iwama, K. Matsuno, T. Ezaki, O. Sakamoto, I. Hamaguchi, N. Takasu, and T. Suda. 1996. Molecular cloning of rat macrophage-stimulating protein and its involvement in the male reproductive system. *Biochem. Biophys. Res. Commun.* 227:273–280.

55. Dey, C.S., and C.J. Brokaw. 1991. Activation of *Ciona* sperm motility: phosphorylation of dynein polypeptides and effects of a tyrosine kinase inhibitor. *J. Cell Sci.* 100:815–824.