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Human Mucus Protease Inhibitor in Airway Fluids Is a Potential Defensive Compound against Infection with Influenza A and Sendai Viruses[1]

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Tryptase Clara, a trypsin-like protease localized exclusively in and secreted from Clara cells of the bronchial epithelium of rat, proteolytically activates the infectivity of influenza A virus [H. Kido, Y. Yokogoshi, K. Sakai, M. Tashiro, Y. Kishino, A. Fukutomi, and N. Katunuma (1992) *J. Biol. Chem.* 267, 13573-13579]. We report here that human mucus protease inhibitor (MPI), a major inhibitor of granulocyte elastase in the lining fluids of the human respiratory tract, significantly inhibited proteolytic activation of the infectivity of influenza A and Sendai viruses by tryptase Clara *in vitro* and multi-cycles of mouse-adapted influenza A virus replication in rat lungs *in vivo*. Recombinant MPI and the C- but not the N-terminal domain of MPI inhibited both the proteolytic activity of tryptase Clara and the activation of virus infection. The 50% inhibitory concentrations of recombinant MPI and the C-terminal domain for tryptase Clara with Sendai virus envelope glycoprotein as substrate were 7.4 and 61.6 nM, respectively. These results indicate that MPI is a defensive compound against virus infection. Since there is evidence suggesting that concentrations of MPI in respiratory fluids are insufficient for prevention of virus infection, administration of MPI in the airway may be useful for treatment of these virus infections.

Key words: influenza virus, mucus protease inhibitor, Sendai virus, typtase Clara, virus infection.

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Influenza and Sendai viruses are exclusively pneumotropic and the target of infection is restricted to the airway epithelial cells of human and rodent species, although the virus receptor sialic acid is widely distributed among various cell types in the lungs and other organs (*1-5*). It has been postulated that the pneumotropism of these viruses is determined by a specific protease(s) present in the respiratory tract, which cleaves precursors of envelope fusion glycoprotein of the progeny viruses to induce fusion activity, thereby enabling the viruses to undergo multiple cycles of replication (*2-6*). The cleavage sites of the envelope glycoproteins, hemagglutinin (HA) of mammalian influenza viruses and fusion glycoprotein (F) of Sendai virus, have a single arginine in the consensus cleavage motif, Gln (Glu)-X-Arg (*2, 7-9*), and thus an arginine-specific serine protease(s) or a trypsin-like protease(s) in the respiratory tract may be a processing enzyme for viral envelope glycoproteins. A limited amount of pancreatic trypsin (*1, 4*) and blood clotting factor Xa in the allantoic fluid of chick embryos (*10*) activate these viruses experimentally *in vitro*. However, there is no evidence that these proteases participate in the proteolytic activation of pneumotropic viruses in the mammalian respiratory tract.

We found a novel trypsin-like serine protease, designated tryptase Clara, from the rat lung and the bronchial lavage fluid (*11*). This protease is localized exclusively in and secreted from Clara cells of the bronchial epithelium of rats and is enzymatically and immunologically distinct from pancreatic trypsin and lung mast cell tryptase. Tryptase Clara in the airway extracellularly activates the precursor F_0 protein of Sendai virus and the HA of human influenza A virus (*11, 12*). Similar protease was partially purified from the mucus epithelium of the human nasal cavity and human bronchial lavage fluid (H. Kido, unpublished data). These results suggest that an inhibitor of tryptase Clara prevents multi-cycles of influenza virus and Sendai virus replication.

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Abbreviations: HA, hemagglutinin; F, fusion glycoprotein; MPI, mucus protease inhibitor; rMPI, recombinant mucus protease inhibitor; Boc, *N*-*tert*-butoxycarbonyl; MCA, 4-methyl-coumaryl-7-amide; Suc(OMe), *N*-methoxysuccinyl; GST, gluthathione S-transferase; PBS$^-$, calcium and magnesium-free phosphate buffered saline; PAGE, polyacrylamide gel electrophoresis; HN, hemagglutinin neuraminidase; PFU, plaque forming unit; IC_{50}, 50% inhibitory concentration.

To find the inhibitor of tryptase Clara, we examined human bronchial lavage fluid and showed that pulmonary surfactant is a specific inhibitor of tryptase Clara, although relatively large amounts are needed for inhibition (*13*). By further investigation of the lavage, we isolated a potent protease inhibitor of the enzyme with a molecular mass of 14 kDa. Sequence analysis of the inhibitor revealed that it was identical to human mucus protease inhibitor (MPI) (*14*).

MPI, also named antileukoprotease and secretory leukoprotease inhibitor, is a nonglycosylated serine protease inhibitor of 107 amino acids, organized in two homologous domains (*14-18*). MPI is produced by nonciliated secretory airway epithelial cells, such as Clara and goblet cells (*19, 20*), and is found in bronchial lavage fluid, nasal and salivary secretions (*21*) as well as alveolar walls (*22*). MPI accounts for 70-90% of the protease inhibitory capacity of normal bronchial secretions and has been extensively studied as an inhibitor of granulocyte elastase to provide protection against the parenchymal destruction induced by granulocyte elastase in pulmonary emphysema and cystic fibrosis (*23-25*).

Here, we found the inhibitory effects of MPI purified from human bronchial lavage fluid and recombinant MPI (rMPI) on tryptase Clara. MPI inhibited the proteolytic activation by tryptase Clara of Sendai and influenza viruses *in vitro* and *in vivo*. Furthermore, we determined that the inhibitory domain of MPI for tryptase Clara is localized in the C-terminal. We also discuss the role of MPI as a defensive compound against infection by pneumotropic viruses and the potential use of MPI as a new drug for treatment of influenza A virus infection.

MATERIALS AND METHODS

Materials—Tryptase Clara was purified from rat lungs by the method of Kido *et al.* (*11*). MPI from human bronchia lavage collected from volunteers was isolated as described by Ohlsson *et al.* (*26*) and further purified by reversed-phase HPLC on a Cosmosil $_5C_4$-300 column (4.6 × 150 mm). MPI was eluted at 1 ml/min with a linear gradient of acetonitrile in 0.05% trifluoroacetic acid. Porcine pancreatic trypsin and human granulocyte elastase were obtained from Sigma (St. Louis, MO), elafin was from Peptide Institute (Osaka). All other reagents were commercial products of the highest grade available.

Virus and Cells—Sendai virus (Z strain) and both influenza A/Aichi/2/68 (H3N2) and mouse-adapted influenza A/Asia/1/57 (H2N2) viruses were propagated in LLC-MK_2 cells and MDCK cells, respectively (*11, 12*). Both cell lines were cultured in minimum essential medium supplemented with 10% fetal calf serum. Inactive viruses were propagated in the cell lines in the absence of trypsin as described previously (*11, 12*).

Enzyme and Inhibitor Assays—Inhibitory effects of MPI, rMPI, and rMPI fragments on various serine proteases were examined as follows. Human granulocyte elastase (0.05 nM) and trypsin (0.1 nM) were incubated with inhibitors in a total volume of 1.495 ml of 0.2 M triethanolamine-HCl buffer, pH 8.0, containing 1.0 M NaCl/0.1% Brij 35 and 0.1 M Tris-HCl buffer, pH 8.0, respectively. After incubation for 5 min at 25°C, 7.5 μl of fluorogenic substrates (20 mM) Suc(OMe)-Ala-Ala-Pro-Val-MCA for granulocyte elastase or Boc-Phe-Ser-Arg-MCA for trypsin was added to the reaction mixtures. The residual activity of each protease was measured in a quartz cuvette controlled thermostatically at 25°C. The amount of 7-amino-4-methylcoumarin liberated from the substrate was determined fluorimetrically at excitation and emission wavelengths of 375 and 460 nm, respectively. One unit of enzyme activity was defined as the amount required to degrade 1 μmol of substrate/min. The inhibition constant (K_i) for trypsin (0.1 nM) or human granulocyte elastase (0.05 nM) was determined from a Dixon plot.

Expression of MPI—The expression vector, pGEX-4T-1, containing a nucleotide sequence encoding human MPI (Ser^1-Ala^{107}) was constructed. The cDNA for human MPI was generated by polymerase chain reaction using two synthetic oligonucleotide primers with a λgt11 human submaxillary gland 5′-stretch cDNA library (Clontech), as the template. Primer 1 (5′-GGGAATTCTCTGGAAAGTC-CTTCAAAGCTG-3′) contained additional nucleotides to provide an *Eco*RI site, while primer 2 (5′-GGCTCGAGTC-AGCTTTCACAGGGGAAACG-3′) contained an *Xho*I restriction site. The polymerase chain reaction was performed for 30 cycles (94°C, 1 min; 55°C, 2 min; 72°C, 3 min). The generated 333 bp cDNA fragment was subcloned into plasmid vector pUC 19(X) at the *Eco*RI/*Xho*I sites and the nucleotide sequence was confirmed to be identical to the reported human MPI sequence. The MPI plasmid was then inserted into pGEX-4T-1 expression vector at the *Eco*RI/*Xho*I sites and transformed into *Escherichia coli* strain DH5a. Cells were grown at 37°C in LB medium containing 20 μg/ml ampicillin with constant shaking. When the A_{590} of the culture reached 1.0, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After 2 h of incubation, cells were pelleted by centrifugation and stored at −80°C.

Purification of rMPI—Cell pellets from 1 liter of culture were resuspended in 4 ml of lysis buffer containing 0.5% NP-40 and 50 mM $MgCl_2$ and sonicated, after which the supernatant was collected by centrifugation. Solubilized GST-MPI fusion protein in the extract was affinity-purified using gluthathione-Sepharose 4B beads (Pharmacia Biotech). The beads were washed with 20 mM Tris-HCl buffer, pH 7.5/2 mM $MgCl_2$, then GST-MPI was eluted with 5 mM reduced gluthathione in 50 mM Tris-HCl buffer, pH 8.0. The eluate was dialyzed against H_2O overnight, and the insoluble precipitate was collected by centrifugation. The precipitate was dissolved with a small volume of 20 mM Tris-HCl buffer, pH 8.0/250 mM NaCl/0.5% NP40/1 mM EDTA/10 mM DTT and digested with 9.25 U of thrombin (Sigma) at 37°C overnight. After the reaction, GST in the digest was adsorbed onto gluthathione-Sepharose beads. Unadsorbed supernatant was finally purified on a Sephadex 75HR 10/30 column (10 × 300 mm) equilibrated with PBS^-, and the MPI eluate was concentrated by lyophilization. Purified MPI appeared to be homogeneous according to SDS-PAGE, which showed a major sharp protein band with faint degradation products (Fig. 1).

Decomposition of rMPI by Formic Acid and Separation of N- and C-Terminal Domain MPI Peptides—Recombinant MPI (7.6 mg) was incubated with 1.6 ml of 70% formic acid for 24 h at 37°C to produce the N(Ser^1-Asp^{49})- and the C(Pro^{50}-Ala^{107})-terminal domain peptide of MPI as described by Van-Seuningen and Davril (*27*). After incuba-

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tion, cleaved peptides in the reaction mixture were separated by HPLC on a TSK-gel Sp-5PW column (7.5×75 mm) with a 60 min linear gradient of 0-2 M NaCl in 50 mM acetate buffer, pH 5.0, at a flow rate of 1 ml/min. Each peptide was finally purified by gel permeation FPLC on a Superdex Peptide HR 10/30 column (10×300 mm) (Pharmacia Biotec) with 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The total amounts of purified N- and C-terminal domain peptides were 540 and 1,596 μg, respectively. Each purified peptide migrated as a single protein band with a molecular mass of about 6.5 kDa on SDS-PAGE. The complete amino acid sequence of each domain peptide (100-200 pmol) was determined by use of a protein sequenator (Applied Biosystems model 470-A) and a 120A peptide analyzer.

Analysis of Cleavage of Viral Envelope Glycoproteins by Tryptase Clara and Its Inhibition by MPI—Sendai and influenza A viruses were labeled with [^{3}H]glucosamine in LLC-MK_2 and MDCK cells, respectively (*11*). To determine the inhibitory effect of MPI, rMPI, and its fragments on cleavage, tryptase Clara was incubated with these peptides for 5 min at 37°C, then [^{3}H]glucosamine labeled viruses were added, and the reaction mixture was incubated for another 30 min at 37°C. The reaction was stopped by cooling at 0°C, and the resulting proteolytic products were separated by SDS-PAGE (10-20% gradient) under reducing conditions followed by fluorography (*11*). The bands of F_0, F_1, and F_2 were scanned with a densitometer (Shimazu CS-9000 model). Percentage cleavage was calculated by using the formula: $100\times[(\text{intensities of } F_1+F_2 \text{ produced})-(\text{intensities of } F_1+F_2 \text{ contaminating the original virus})]/[(\text{intensity of uncleaved } F_0 \text{ remaining})+(\text{intensities of } F_1+F_2 \text{ produced})-(\text{intensities of } F_1+F_2 \text{ contaminating the original virus})]$. The amounts of F_1+F_2 contaminating the original virus did not exceed 3%.

Inhibitory Effect of MPI on Proteolytic Activation of Infectivity of Sendai and Influenza A Viruses In Vitro—Non-activated (non-infectious) Sendai and influenza A/Aichi/2/68(H3N2) viruses were propagated in LLC-MK_2 and MDCK cells, respectively (*11, 12*). Briefly, monolayer cultures of LLC-MK_2 and MDCK cells were inoculated with egg-grown wild-type seed viruses at a multiplicity of infection of 10 PFU/cell. After adsorption for 60 min at 37°C, the cells were washed and fed again with Eagle minimum essential medium without serum and protease. After incubation for 10 and 15 h for influenza virus and Sendai virus, respectively, progeny non-infectious virus in culture fluid was collected. To quantify activated and non-activated viruses differentially, both activated and non-activated viruses were counted by the plaque assay without or with 4 μg/ml of trypsin in the agar overlay (*5*). Aliquots of the progeny viruses were suspended in PBS−, pH 7.2, and incubated with various concentrations of tryptase Clara for 10-30 min at 37°C. To determine the effect of MPI and its fragments on the viral activation by tryptase Clara, the enzyme was incubated with MPI and MPI fragments for 5 min at 37°C, then activation of infectivity was assayed by immunofluorescent cell-counting method (*5*) using a polyclonal antibody against the F_2 subunit of Sendai virus fusion glycoprotein, F2C12 (*28*), or with monoclonal antibody against influenza A (Chemicon International).

Inhibitory Effect of MPI on Infection of Rat Lungs with a Mouse-Adapted Influenza A Virus—Specific-pathogen-free, 3-week-old male rats of the CD (SD) strain were obtained from Charles River Japan and housed under bio-clean conditions at 23°C and 55% humidity. Under ether anesthesia, the rats were infected intranasally with 1×10^4 PFU of activated mouse-adapted influenza A/Asia/1/57 (H2N2) virus. One group of 24 animals received intranasal administration of 5 μg of rMPI in 50 μl of PBS− 15 times at 8-h intervals, and a second group of 24 animals received 50 μl of PBS− alone as a control. The rats were weighed and clinical signs, pathological changes, and virus replication in the lungs were examined daily (*12*). Lung lesions were scored from 1 to 4 according to the extent of macroscopic consolidation of the lung surface (*5*). Lungs were homogenized and assayed differentially for total virus yield and activated viruses (*5*).

Protein Measurement—Protein concentration was measured with the bicinchoninic acid protein assay reagent (Pierce Chemical) according to Smith *et al.* (*29*).

RESULTS

Characterization of MPI, rMPI, and Fragments of rMPI—Purity of MPI purified from human bronchial lavage, rMPI and N- and C-terminal domain fragments of rMPI was analyzed by SDS-PAGE under reducing conditions (Fig. 1). Purified MPI and rMPI migrated as major bands with a molecular mass of 14±1.5 kDa with very faint minor bands under reducing (Fig. 1, lanes 1 and 2, respectively) and non-reducing conditions (data not shown). These minor contaminants were degradation products of MPI, since they were immunoreactive with a monoclonal antibody against MPI (data not shown). The N- and C-terminal domain peptides had molecular masses of 6.5±0.5 kDa as shown in Fig. 1, lanes 3 and 4, respectively. Amino acid sequences (data not shown) and the molecular

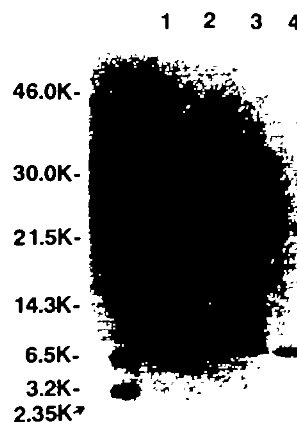

Fig. 1. **Electrophoretic analyses of MPI, rMPI, and rMPI fragments.** Purity of MPI purified from human bronchial lavage (2 μg) (lane 1), rMPI (2 μg) (lane 2), the N-terminal domain rMPI, Ser^1-Asn^{49}, (2 μg) (lane 3) and the C-terminal domain rMPI, Pro^{50}-Ala^{107}, (1 μg) (lane 4) was analyzed by SDS-PAGE (15-25% gradient) under denaturing and reducing conditions. Gels were calibrated with SDS-PAGE low range Rainbow™ colored protein molecular weight markers (Amersham): ovalbumin (46 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa), insulin B chain (3.4 kDa), and insulin A chain (2.35 kDa).

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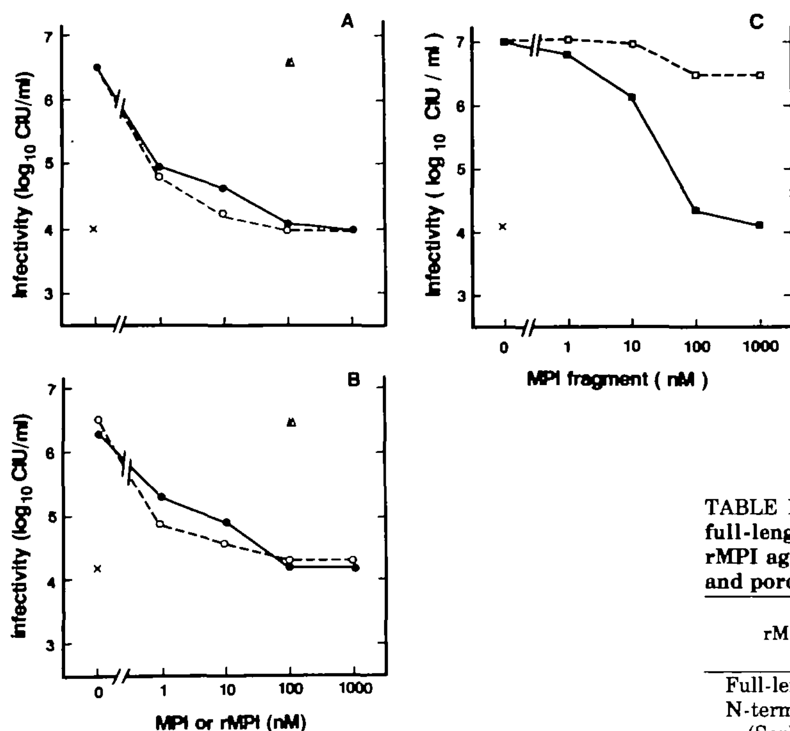

Fig. 2. **Inhibitory effect of MPI, rMPI, and rMPI fragments on the activation of Sendai and influenza A/Aichi/2/68 (H3N2) viruses by tryptase Clara *in vitro*.** Tryptase Clara (1.2 mg/ml) was incubated with various concentrations of MPI purified from bronchial lavage (○), rMPI (●), and the C- (■) and the N-terminal (□) domain fragments of rMPI, at 37°C for 5 min. Mixtures were then incubated with inactive Sendai virus (×) grown in LLC-MK_2 cells (A and C) or inactive influenza A virus (×) grown in MDCK cells (B) for 20 min. The infectivity of the viruses was assayed by immunofluorescent cell-counting. The effects of MPI (△) and rMPI (▲) on the infectivity of active Sendai (A) and influenza A (B) viruses which had been proteolytically activated and grown in the allantoic cavity of chick embryos (*5, 6*) were also measured. CIU, cell infecting unit (*5*).

TABLE I. **Fifty per cent inhibitory concentrations (IC_{50}) of full-length rMPI as well as the N- and C-terminal domains of rMPI against rat tryptase Clara, human granulocyte elastase, and porcine pancreatic trypsin.**

rMPI	IC_{50} values (nM)		
	Granulocyte elastase	Pancreatic trypsin	Tryptase Clara
Full-length rMPI	1.2	15.8	7.4
N-terminal domain (Ser^1-Asp^{49})	N.I.	N.I.	N.I.
C-terminal domain (Pro^{50}-Ala^{107})	3.8	208.9	61.6

N.I.: no inhibition at 10^{-6} M of the inhibitors.

masses of domain peptides revealed that the N-terminal domain peptide was comprised of Ser^1-Asp^{49} and the C-terminal domain, Pro^{50}-Ala^{107}.

The 50% inhibitory concentrations (IC_{50}) of rMPI and its fragments against rat tryptase Clara was analyzed with Sendai virus envelope glycoprotein as substrate (Table I). The IC_{50} values against human granulocyte elastase and porcine pancreatic trypsin were also analyzed with their synthetic peptide substrates. Full-length rMPI inhibited tryptase Clara efficiently, with a IC_{50} value of 7.4 nM. It also inhibited human granulocyte elastase and porcine pancreatic trypsin, with IC_{50} values of 1.2 and 15.8 nM, respectively. The inhibition constants (K_i values) of the rMPI against elastase and trypsin with their synthetic substrates were 4.5×10^{-10} and 2.5×10^{-8} M, respectively. The K_i values of MPI are largely compatible with the values reported (*30*).

X-ray crystallography has shown that MPI is comprised of two separate domains with an architecture that is similar to other serine proteinase protein inhibitors and interconnected by four disulfide bridges (*14*). The C-terminal domain has a reactive site at Leu^{72} to Met^{73} with elastase inhibitory activity, while the function of the N-terminal domain remains unknown (*14*). To elucidate which domain of MPI interacts with tryptase Clara, we isolated the N (Ser^1-Asp^{49})- and C (Pro^{50}-Ala^{107})-terminal domains of MPI after decomposing rMPI with formic acid and measured the IC_{50} value of each fragment. In comparison with the IC_{50} value of full-length rMPI, the value of the C-terminal domain fragment against tryptase Clara increased by about one order of magnitude. The IC_{50} value against pancreatic trypsin also increased in a similar manner to that against tryptase Clara, although the value against human granulocyte elastase increased only about threefold. On the other hand, the N-terminal domain fragment of MPI inhibited neither the activity of tryptase Clara nor those of granulocyte elastase and trypsin. To confirm the effect of the N-terminal domain fragment of MPI on tryptase Clara, the recombinant N-terminal domain peptide of MPI (Ser^1-Pro^{54}) was expressed and purified. In addition, the N-terminal domain peptide (Ser^1-Asn^{78}) was prepared by limited hydrolysis of MPI by asparaginyl-endopeptidase in 50 mM sodium acetate buffer, pH 5.0, containing 10 mM DTT and 1 mM EDTA. Both of the N-terminal domain peptides had no inhibitory effect on the proteolytic activation by tryptase Clara of [^{3}H]glucosamine-labeled Sendai virus (data not shown). These results indicate that the inhibitory domain of MPI for tryptase Clara is localized in the C-terminal. The previous reports on the function of the recombinant N- and C-terminal domains of MPI against human granulocyte elastase and pancreatic trypsin (*30-32*) support our findings.

Effects of MPI and rMPI Fragments on the Activation of Sendai and Influenza A Viruses In Vitro—We showed that tryptase Clara from rat lungs activates Sendai and influenza A/Aichi/2/68 (H3N2) viruses *in vitro* (*11, 12*) and that anti-tryptase Clara antibody almost completely inhibits the multiple cycles of Sendai virus replication in cell culture and in rat lungs (*12*). These results suggest that this enzyme in rats or the tryptase Clara-like protease in the mucus epithelium of the human airway is one of the major determinants of the pneumo-pathogenicity of these viruses.

To determine whether MPI inhibits the proteolytic activation of Sendai and influenza A/Aichi/2/68 (H3N2) viruses by tryptase Clara, we examined the infectivity of these viruses after *in vitro* activation by tryptase Clara that

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previously had been exposed to various concentrations of MPI for 5 min at 37°C. Infectivity was measured by immunofluorescent cell-counting (*5*), in which only the activated viruses are counted. As shown in Fig. 2, tryptase Clara increased the infectivity of Sendai (A) and influenza A (B) viruses 200-500-fold. Native MPI inhibited the activation of Sendai and influenza A viruses by this enzyme in a dose-dependent manner. It almost completely inhibited viral activation at concentrations from 100 nM to 1 μM. The inhibitory effect of full-length rMPI expressed in *E. coli* was similar to that of native MPI (Fig. 2, A and B).

The inhibitory effect of the C- and N-terminal fragments of rMPI on the proteolytic activation of Sendai virus by tryptase Clara was tested. As shown in Fig. 2C, the C-terminal domain of rMPI inhibited Sendai virus activation but the N-terminal domain had little effect, indicating that the former is the reactive domain of the enzyme. However, the efficiency of inhibition by the C-terminal domain at concentrations below 100 nM was reduced in comparison with full-length rMPI. Ying *et al.* have reported that the N-terminal domain stabilizes the conformation of the C-terminal domain of MPI and the protease-MPI complex (*31*). This may explain the reduced inhibitory efficiency of the C-terminal domain at low concentrations. In addition, MPI and rMPI by themselves did not affect the infectivity of these active viruses which were previously proteolytically activated and grown in the allantoic cavity of chick embryos (Fig. 2, A and B). These results indicate that MPI and the C-terminal domain MPI specifically inhibit the process of proteolytic activation during viral infection but not membrane fusion induced by activated viruses and viral replication in cells.

To confirm our interpretation of the effect of MPI on virus activation, we examined whether MPI and rMPI fragments inhibit proteolytic cleavage of the F_0 of Sendai virus and the HA of influenza A/Aichi/2/68 (H3N2) virus by tryptase Clara. Tryptase Clara was preincubated with various concentrations of rMPI or rMPI fragments for 5 min at 37°C, and the proteolytic cleavage by the enzyme of [^{3}H]glucosamine-labeled inactive Sendai and influenza A viruses grown in LLC-MK_2 and MDCK cells, respectively, was tested. Recombinant MPI inhibited the conversion of F_0 and HA into its subunits F_1 and F_2 and HA_1 and HA_2, respectively, in a dose-dependent manner (Fig. 3, A and B). At concentrations of 100 nM to 1 μM, rMPI almost completely inhibited the proteolytic conversion of these viral glycoproteins. Native MPI was inhibitory in a similar dose-dependent manner (data not shown). The C(Pro^{50}-Ala^{107})- but not the N(Ser^1-Asp^{49})-terminal domain fragment of rMPI inhibited cleavage by tryptase Clara (Fig. 4).

Inhibitory Effects of MPI on Infection with Influenza A/Asia/1/57 (H2N2) Virus In Vivo—To evaluate the effect of administration of MPI for treatment of influenza virus infection *in vivo*, rats that were infected with mouse-adapted influenza A/Asia/1/57 (H2N2) virus were treated with MPI intranasally. No pulmonary toxicity was reported in male golden hamsters which were given four intratracheal instillations of up to 3 mg of MPI (*24*).

Before starting the *in vivo* experiments, we evaluated the proteolytic activation of mouse-adapted influenza A/Asia/1/57(H2N2) virus by tryptase Clara as described in "MATERIALS AND METHODS." Tryptase Clara activated the infectivity of the virus in a similar dose-dependent manner to that reported on the activation of influenza A/Aichi/2/68(H3N2) virus (*11*) (data not shown).

Three-week-old rats that were infected with 1×10^4 PFU of mouse-adapted influenza A/Asia/1/57 (H2N2) virus were given 6 μg of rMPI intranasally 15 times at 8-h intervals. Viral replication and pathological changes in the lungs were examined. Without treatment of MPI, virus in the lungs was produced in the activated form and underwent multi-cycles of replication until days 3 to 5, when termination began, presumably by host immunological

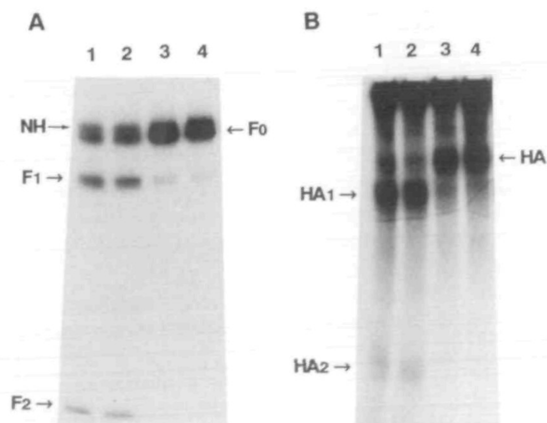

Fig. 3. Effects of rMPI on the proteolytic cleavage of F_0 of Sendai and HA of influenza A/Aichi/2/68 (H3N2) viruses by tryptase Clara. Tryptase Clara (2.4 μg/ml) was incubated without rMPI (A and B, lane 1) or with 10 nM (A and B, lane 2), 100 nM (A and B, lane 3), 1 μM (A and B, lane 4) of rMPI in 20 μl of 100 mM Tris-HCl buffer, pH 7.2, at 37°C for 5 min. Thereafter, [^{3}H]glucosamine-labeled inactive Sendai virus (A) or the labeled inactive influenza virus (B) was incubated with the reaction mixture at 37°C for 30 min. Proteolytic products were separated by SDS-PAGE under reducing conditions followed by fluorography. HN, hemagglutinin neuraminidase.

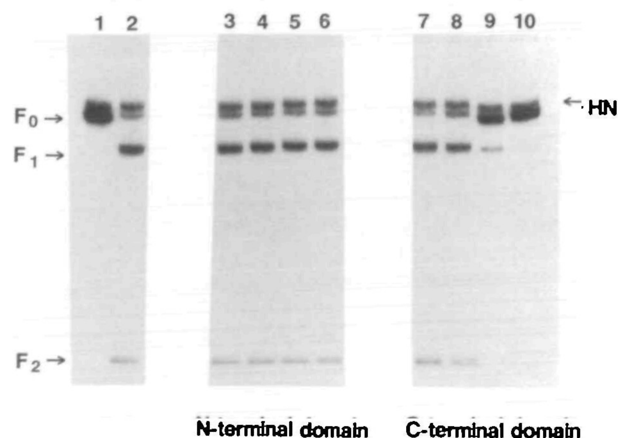

Fig. 4. Effects of the N- and C-terminal domains of rMPI on proteolytic cleavage of F_0 of Sendai virus by tryptase Clara. Tryptase Clara (2.4 μg/ml) was incubated without inhibitor (lane 2), with the N-terminal domain fragment of rMPI (lanes 3-6) or with the C-terminal domain fragment of rMPI (lanes 7-10) at concentrations of 1 nM (lanes 3 and 7), 10 nM (lanes 4 and 8), 100 nM (lanes 5 and 9), and 1 μM (lanes 6 and 10) at 37°C for 5 min. Thereafter, [^{3}H]-glucosamine-labeled inactive Sendai virus (lane 1) was incubated with the reaction mixture at 37°C for 30 min. Viral polypetides were separated as described in the legend to Fig. 3.

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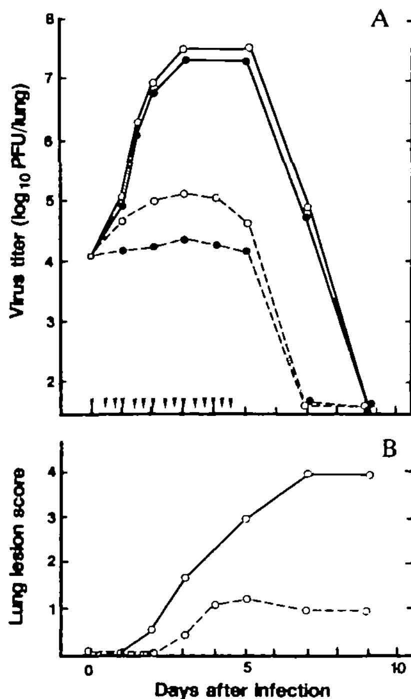

Fig. 5. **Effects of rMPI on viral replication in the lung and pulmonary pathology of rats infected with mouse-adapted influenza A/Asia/1/57 (H2N2) virus.** Three-week-old male rats of the CD (SD) strain were infected intranasally with 1×10^4 PFU of influenza A Asia/1/57 (H2N2) virus (□). One group was administered 15 times with 6 μg of rMPI in 50 μl of PBS− intranasally every 8 h (arrowheads) and the other group received 50 μl of PBS− as a control. A: Viral replication and activation in the lungs in the absence (solid lines) or presence (broken lines) of rMPI. Lung homogenates were assayed for total yield (○) and activated viruses (●). Each plot represents the mean value of three animals which were sacrificed on the indicated days. B: Lung pathology of infected rats without (solid lines) or with (broken lines) the administration of rMPI. Lung lesions were scored from 1 to 4 according to the extent of macroscopic consolidation of the lung surface as described (*5, 6*). Each plot represents the mean value of the three animals described above.

response (Fig. 5A). Severe pathological changes were also manifested in the lungs after 2 days (Fig. 5B). On the other hand, when rMPI was administered, activation of the progeny virus and viral replication in the lungs were markedly reduced to less than 10%, and over 90% of the virus was inactivated. Lung lesions were induced but only slightly compared with untreated animals. These results suggest that administration of MPI prevents proteolytic activation and inhibits multi-cycles of the viral replication *in vivo*. Furthermore, the results may also support our previous findings that tryptase Clara is a major activating protease in rat lungs. Taken together, our findings suggest that MPI may be a novel candidate for treating infections with these viruses.

DISCUSSION

We previously found that pulmonary surfactant in the bronchial fluid is a specific inhibitor of tryptase Clara. It inhibits the proteolytic activation of Sendai and influenza A viruses by tryptase Clara but not by trypsin *in vitro* and in organ cultures of the rat lung, although fairly high concentrations at 0.5-5 mg/ml are needed for complete inhibition (*13, 33*). The mechanism of the inhibition remains to be elucidated, but our preliminary experiments suggest that the pulmonary surfactant adsorbs tryptase Clara, resulting in its inactivation. By further investigation of human bronchial lavage, we found that MPI is another inhibitor of tryptase Clara. Although the physiological role of MPI has yet to be fully elucidated, the principal role is thought to be protection of the epithelial surface against human granulocyte elastase, an enzyme that can cause epithelial damage (*34*), and against mast cell chymase (*35*). Our results indicated a new function of MPI: the prevention of infection with pneumotropic influenza A and Sendai viruses. Furthermore, MPI may also inhibit bacterial serine proteases released from bacteria, which facilitate virus infections through proteolytic activation of viral glycoproteins (*23, 36-38*).

Major serine protease inhibitors in human bronchial lavage are MPI and α1-antitrypsin (*39*), but α1-antitrypsin was shown to have no inhibitory effect on tryptase Clara (*11*). MPI levels in human bronchial and nasal fluids average 11.0 ± 1.2 (*39*) and 208.9 ± 44.5 nM (*40*), respectively, suggesting that MPI contributes to protection against proteolytic activation of pneumotropic viruses by tryptase Clara or tryptase Clara-like protease in the airway. However, concentrations of MPI constitutively secreted may be insufficient to effect complete inhibition, because the activity of tryptase Clara in rat bronchial lavage (*13*) and proteolytic activation of influenza virus in human nasal fluid were observed (*9*). Furthermore, Sendai virus infection stimulates secretion of tryptase Clara and may induce an imbalance between the amount of tryptase Clara and that of the inhibitors (*13, 33*). *In vivo* experiments revealed that administration of MPI in airways efficiently inhibited multi-cycles of influenza A/Asia/1/57(H2N2) virus replication in rat lungs. This result suggests that concentrations of the free form of MPI at physiological conditions in rat airway fluids is insufficient for complete suppression of the inoculated influenza virus activation by tryptase Clara. Although methods by enzyme-immunoassay for determination of concentrations of free form of MPI and tryptase Clara, tryptase Clara-MPI complex and tryptase Clara-pulmonary surfactant complex in rat and human respiratory fluids have not yet been established, the proportion of the free form of tryptase Clara to the free form of the inhibitors may be a principal determinant host factor for these pneumotropic viral infections.

It was initially suggested that the N-terminal domain of MPI contains the anti-tryptic active site located at the Arg^{20}-Tyr^{21} bond, whereas the C-terminal domain contains the active site (Leu^{72}-Met^{73}) against α-chymotrypsin and human granulocyte elastase (*14*). However, investigations by recombinant means or by mild acid hydrolysis of MPI have demonstrated that the inhibitory activity against human granulocyte elastase, cathepsin G, trypsin, and α-chymotrypsin is located in the C-terminal domain (*27, 30, 39-41*). Although a very weak inhibitory activity against trypsin has been reported in the N-terminal domain by recombinant means (*40, 42*), we found that the inhibitory activity of MPI against tryptase Clara and against infections with Sendai and influenza A viruses *in vitro* was located in the C-terminal domain.

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Elafin, an elastase-specific inhibitor, which is also found in airway mucous secretions, is composed of a single peptide chain of 57 amino acid residues, the sequence of which is 38% identical with the C-terminal domain of MPI, but which lacks the domain corresponding to the N-terminal domain of MPI (*43*). Furthermore, elafin and the C-terminal domain of MPI conserve the same four disulfide connections, and the same alignment of five proline residues, suggesting that their tertiary structures are similar. We therefore studied the effects of human elafin on proteolytic activation by tryptase Clara of [^{3}H]glucosamine-labeled Sendai virus. Elafin, however, at concentrations up to 10 μM had no inhibitory effect on proteolytic activation by tryptase Clara (data not shown).

In conclusion, MPI, a major protease inhibitor in fluids lining the human respiratory tract, inhibited tryptase Clara and infection with pneumotropic Sendai and influenza A viruses by inhibiting proteolytic activation of viruses. Moreover, we showed that the inhibitory activity was located in the C-terminal domain. To our knowledge, this is the first report that MPI plays a defensive role against infection with these viruses. Administration of MPI or the C-terminal domain peptide may be useful for the prevention and treatment of infections with pneumotropic viruses.

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