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Mini-plasmin found in the epithelial cells of bronchioles triggers infection by broad-spectrum influenza A viruses and Sendai virus

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Extracellular cleavage of virus envelope fusion glycoproteins by host cellular proteases is a prerequisite for the infectivity of mammalian and nonpathogenic avian influenza viruses, and Sendai virus. Here we report a protease present in the airway that, like tryptase Clara, can process influenza A virus haemagglutinin and Sendai virus envelope fusion glycoprotein. This protease was extracted from the membrane fraction of rat lungs, purified and then identified as a mini-plasmin. Mini-plasmin was distributed predominantly in the epithelial cells of the upward divisions of bronchioles and potentiated the replication of broad-spectrum influenza A viruses and Sendai virus, even that of the plasmin-insensitive influenza A virus strain. In comparison with plasmin, its increased hydrophobicity, leading to its higher local concentrations on membranes, and decreased molecular mass may enable mini-plasmin to gain ready access to the cleavage sites of various haemagglutinins and fusion glycoproteins after expression of these viral proteins on the cell surface. These findings suggest that mini-plasmin in the airway may play a pivotal role in the spread of viruses and their pathogenicity.

Keywords: influenza A virus; mini-plasmin; plasmin; rat lung; Sendai virus.

Post-translational proteolytic cleavage of precursors of the envelope fusion glycoproteins of RNA viruses is indispensable for virus entry into host cells. It is widely known that the pathogenicity of mammalian and nonpathogenic avian influenza viruses, and Sendai virus is determined primarily by host cellular processing proteases in the respiratory tract, which proteolytically induce fusion of the viral envelope glycoproteins, influenza virus haemagglutinin (HA) and Sendai virus F_0, with the plasma membrane of target cells, allowing the viral genome to enter the cytoplasm [1–4]. The cleavage of these viral fusion glycoproteins occurs extracellularly and is restricted to the membranes of airway epithelial cells and/or the airway lumen after expression of these viral proteins on the cell surface [1,5,6]. Proteolytic activation of viral envelope glycoproteins involves cleavage at a specific cleavage site, the carboxyl moiety of an arginine residue, by trypsin-like endoproteases. Some of the biologically relevant cleavage enzymes have recently been identified by us and other groups in the rat respiratory tract [5,6], pig lung [7], and chicken embryos [8]. Plural trypsin-like arginine endoproteases may be involved in the spread of viral infection, host range and pathogenicity in the respiratory tract, because differences in susceptibility to processing enzymes of HA of various influenza A virus strains and Sendai virus F_0 were evident [9].

Previously we found a trypsin-like protease, tryptase Clara, that processes human influenza A virus HA and Sendai virus F_0, in the soluble fraction of rat lung and bronchial lavage fluid. We extracted it with low-salt buffer, and then purified it [5]. The enzyme is distributed predominantly in the secretory granules of Clara cells in the downward divisions of bronchioles, terminal and respiratory bronchioles [5,10]. In the present study, another processing enzyme with different biochemical properties from those of tryptase Clara was found specifically in the insoluble membrane fraction of rat lungs, and was purified. Studies on its amino-acid sequence and enzymatic properties revealed that this processing enzyme is mini-plasmin. Mini-plasmin is formed from plasmin *in vitro* by elastases from granulocytes and the pancreas, or from plasminogen through sequential processing by a plasminogen activator and these elastases [11]. The presence and localization of mini-plasmin *in vivo* had not been reported previously; we now report purification of this enzyme from rat lung tissue its distribution in the epithelial cells of the upward divisions of bronchioles. Like pancreatic trypsin, mini-plasmin potentiated the infectivity of a wide variety of human influenza A virus strains, even that of the plasmin-insensitive influenza A virus. We also discuss the role of mini-plasmin in the progression of virus infection in lungs.

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Abbreviations: HA, hemagglutinin; F_0, fusion glycoprotein precursor; MCA, 4-methyl-coumaryl-7-amide; Boc, N-*tert*-butyloxycarbonyl; DFP, diisopropylfluorophosphate; Bz, benzoyl; Suc, succinyl; MPI, mucus protease inhibitor; HN, hemagglutinin neuraminidase; CIU, cell infecting units.

Enzymes: plasmin (EC 3.4.21.7); neuraminidase (EC 3.2.1.18); elastase (EC 3.4.21.11).

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MATERIALS AND METHODS

Materials

Male Wistar strain rats weighing 200–250 g were used. 4-Methyl-coumaryl-7-amide (MCA) peptide substrates were from the Peptide Institute (Osaka, Japan). Chromatography media, S-Sepharose, benzamidine-Sepharose 6B, EAH-Sepharose 4B, and Superdex 200 PC 3.2/30 were from Pharmacia Biotech. D-[^{3}H]Glucosamine hydrochloride was from Muromachi Chemicals Ltd. (Fukuoka, Japan). Kunitz-type soybean trypsin inhibitor was supplied by K. Takamatsu (Fuji Oil Co., Ltd, Tokyo, Japan). All other reagents were commercial products of the highest grade available. Sendai (Z strain) virus and various influenza virus strains were supplied by M. Tashiro (National Institute of Infectious Diseases, Tokyo, Japan) and H. Kida (Hokkaido University, Japan), respectively.

Purification of a virus envelope glycoprotein-processing protease

All procedures were carried out at 4 °C unless stated otherwise. Minced rat lungs (250 g) were washed with NaCl/P_i and then homogenized in 4 vol. 50 mM sodium acetate buffer pH 5.5, with a Polytron (Kinematica GmbH), for 10 min. After centrifugation at 12 000 *g* for 15 min the supernatant was discarded. The precipitate was further extracted twice with an equal volume of 50 mM sodium acetate buffer pH 5.0 containing 0.5 M NaCl and then centrifuged at 12 000 ***g*** for 10 min. The supernatant was adjusted to pH 4.5 by drop-wise addition of 1 M acetic acid with stirring. The precipitated protein was removed by centrifugation as described above, and the supernatant was treated with ammonium sulfate. The material precipitated on 0–80% saturation with ammonium sulfate was resuspended in 50 mM sodium acetate buffer, pH 4.5 (buffer A). The suspension was dialysed overnight against buffer A and insoluble material was removed by centrifugation. The soluble fraction was applied to an S-Sepharose column (4.1 × 21 cm) previously equilibrated with buffer A. The column was washed with the same buffer until the absorbance of the eluate at 280 nm had returned to the baseline value, and then the enzyme was eluted stepwise with buffer A containing 0.5 M, 0.8 M, and 0.9 M NaCl, respectively. The enzyme eluted in the 0.5 M NaCl/buffer A fraction was concentrated by ultrafiltration on a YM-10 membrane (Amicon), and then dialysed against buffer A containing 0.3 M NaCl overnight. Insoluble material was removed by centrifugation, and the resulting soluble material was rechromatographed on an S-Sepharose column (2.8 × 10 cm) equilibrated with buffer A containing 0.3 M NaCl. After washing the column with equilibration buffer, the enzyme was eluted with a linear gradient of 0.3–0.8 M NaCl in buffer A. The active fractions eluted with 0.5–0.6 M NaCl were pooled, concentrated by ultrafiltration on a YM-10 membrane, and then dialysed against 50 mM Tris/HCl buffer pH 7.5 containing 0.5 M NaCl (buffer B) overnight. Insoluble material was removed by centrifugation, and the soluble material was applied to a benzamidine-Sepharose 6B column (2 × 5 cm), which was previously equilibrated with buffer B. The column was washed with 50 mM sodium acetate buffer pH 5.0 containing 0.5 M NaCl until the absorbance of the eluate at 280 nm had returned to baseline. The enzyme was then eluted with 50 mM benzamidine hydrochloride hydrate in the washing buffer and concentrated with a YM-10 membrane. After exhaustive dialysis against 50 mM Tris/HCl buffer pH 7.5 containing 0.1 M NaCl to remove the benzamidine, the sample was stored at −20 °C in small aliquots. Rat plasminogen from serum was purified as described previously [12].

Preparation of human mini-plasmin and microplasmin

Human plasmin (5 mg) in 2 mL Tris/HCl buffer pH 8.0, was incubated with 15 μg porcine pancreatic elastase or human granulocyte elastase at room temperature for 195 min with stirring. The reaction product was then applied to a soybean trypsin inhibitor-conjugated EAH-Sepharose 4B column (0.6 × 3 cm), which was previously equilibrated with 50 mM Tris/HCl buffer pH 8.0 containing 0.5 M NaCl. The column was washed with the same buffer, eluted with 50 mM glycine/HCl buffer pH 2.8 containing 0.5 M NaCl, and then concentrated with a YM-10 membrane. Finally, the material was applied to a Superdex 200 PC 3.2/30 gel permeation HPLC column, which had been equilibrated with 50 mM potassium phosphate buffer pH 8.0 containing 0.2 M NaCl. Human mini-plasmin was eluted in the fraction corresponding to a 38-kDa protein. Human recombinant microplasmin was prepared as described previously [13].

Protein measurement

Protein concentrations were measured with bicinchoninic acid protein assay reagent (Pierce) [14] using BSA as a standard.

Enzyme and inhibitor assays

Protease activity was determined with a Hitachi fluorescence spectrophotometer, Model 650-10 MS, by measuring the amount of 7-amino-4-methylcoumarin released upon hydrolysis of various MCA peptide substrates in a quartz cuvette controlled thermostatically at 37 °C as described [5]. The reaction was initiated by adding the enzyme sample to 0.1 mM substrate in 100 mM Tris/HCl buffer pH 8.0; the assay mixture volume was 0.5 mL. The synthetic peptide substrate Boc-Gln-Ala-Arg-MCA, which resembles the consensus cleavage site motif of Sendai and various human influenza A viruses, was used unless otherwise noted. One unit of enzyme activity was defined as the amount cleaving 1 μmol substrate per min. For inhibition studies, the enzyme was preincubated with various inhibitors for 5 min at 37 °C before activity measurement.

Electrophoresis

SDS/PAGE was carried out according to Laemmli [15] in a 10–20% gradient gel. The gel was stained for protein with a Silver Stain Kit (Wako Pure Chemical Industries, Ltd, Tokyo, Japan). The SDS/PAGE low range standards (Apro Science, Inc., Tokushima, Japan) used as molecular mass markers were rabbit muscle phosphorylase *b* (97.2 kDa), BSA (66.4 kDa), ovalbumin (45.0 kDa), carbonic anhydrase

(29.0 kDa), soybean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa).

DFP labelling of proteins and gel electrophoresis

The purified protease (4 μg) was treated with [^{3}H]diisopropylfluorophosphate (DFP; 74 GBq·mmol^{-1}) at 37 °C for 10 h. The reaction was stopped by the addition of unlabelled DFP (10 mM) and proteins were precipitated with cold acetone, with 50 μg of BSA as a carrier. The resulting precipitate was washed twice with 90% cold acetone by centrifugation, dried *in vacuo*, and then subjected to SDS/PAGE (10–20% gradient). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250, destained, treated with Amplify TM (Amersham), dried and then fluorographed with Fuji RX film.

N-terminal amino-acid sequence determination

Purified enzyme (4.8 μg) was transferred electrophoretically to a ProBlott^{TM} membrane (Applied Biosystems), and the N-terminal amino-acid sequences of the protein band materials were determined with an Applied Biosystems model 492 automatic protein sequencer according to the manufacturer's instructions.

Preparation of antibodies

An immunogen peptide corresponding to the five N-terminal residues, SAIVP, of the 12-kDa subunit, i.e. the kringle 5 domain of plasminogen, and with cysteine at the C-terminus to allow conjugation of this peptide to a carrier protein and the mouse plasminogen light-chain immunogen peptide, NRVEYLNNRVKSTELC(732–747), were synthesized by the solid phase method with an automated peptide synthesizer (Model 430 A; Applied Biosystems) according to the manufacturer's instructions. Each hapten peptide (2 μmol) was conjugated to 2 mg of maleimide-activated keyhole limpet haemocyanin (Pierce) by incubation at room temperature for 2 h. The conjugate was emulsified with an equal volume of complete Freund's adjuvant for the primary injection and with the incomplete form for booster injections, and injected into a Japan white rabbit (1 mg each time) several times at 3-week intervals. Each antiserum with a satisfactory titre was applied to an affinity column of immunogen peptide-coupled epoxy-activated Sepharose 6B (20 mg peptide per 2 g epoxy-activated Sepharose 6B), and antigen-specific antibodies were obtained by elution with 0.2 M glycine/HCl buffer pH 2.8. The eluate was immediately adjusted to neutral pH, concentrated and then dialysed against NaCl/P_i.

Immunohistochemical staining

Immunohistochemical staining of rat lungs was performed after perfusion of the lungs with heparinized NaCl/P_i and fixing with 10% buffered formalin pH 7.2, to eliminate artefacts due to autolysis and contamination by serum factors [10]. They were then excised, fixed in the same fixtative for 1 day at 4 °C and embedded in paraffin. Lung sections of thickness 4 μm were cut and stained immunohistochemically by the avidin-biotin-peroxidase complex method [5,10]. After being deparaffinized, the sections were treated with 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to block endogenous peroxidase activity. After hydration and rinsing in NaCl/P_i, the sections were treated with 10% normal goat serum for 20 min at room temperature to reduce the nonspecific background staining, and then with antigen-specific antibodies (3 μg·mL^{-1}) at room temperature for 1 h. After immunohistochemical staining, cell nuclei were counterstained for 10 min with 1% methyl green, and the lung sections were then dehydrated and mounted.

Processing activity toward viral envelope fusion glycoprotein precursors

[^{3}H]Glucosamine-labelled nonactivated (noninfectious) Sendai and influenza A/Aichi/2/68 (H3N2) viruses, prepared in LLC-MK2 cells and MDCK cells, respectively [16] were incubated with the purified protease in 200 mM Tris/HCl buffer pH 7.5 at 37 °C. The proteolytic product was subjected to SDS/PAGE, followed by fluorography.

Activation of the infectivity of influenza A viruses

Nonactiviated influenza A/Aichi/2/68 (H3N2), A/WSN/33 (H1N1) (WSN) and A/seal/Massachusetts/1/80 (H7N7) viruses, which exhibit different susceptibilities to host-cellular processing proteases [9,17], were prepared in MDCK cells [18]. These viruses were incubated with various concentrations of plasmin, mini-plasmin, microplasmin and trypsin at 37 °C for 30 min, and then inoculated into CV-1 cell cultures to analyse infectivities. At 15 h postinfection, infected cells were detected by haemadsorption with guinea pig erythrocytes. Haemadsorption-positive cells were counted under an inverted microscope at 100-fold magnification in 30 microscopic fields (area of each field, ≈ 2.4 mm^2) [19]. Infectivity was expressed as cell infecting units (CIU)·mL^{-1}.

RESULTS

Purification of a processing protease for the viral envelope fusion glycoproteins from the membrane fraction of rat lung

Processing activity for the envelope fusion glycoprotein precursors of human influenza A and Sendai viruses was found in the membrane fraction of rat lungs. Unlike tryptase Clara, a trypsin-like processing protease found in Clara cells of rat lungs and bronchial lavage fluid and extracted readily with low-salt buffer [5], the processing enzyme was extractable from the membrane fraction with > 0.5 M NaCl. The processing enzyme was purified by measuring the hydrolytic activities on the synthetic substrate Boc-Gln-Ala-Arg-MCA, resembling to the cleavage motif Gln(or Glu)-X-Arg of HA and F_0, and [^{3}H]glucosamine-labelled nonactivated Sendai virus as substrates. After homogenization in low-salt buffer and centrifugation to remove a large amount of soluble proteins including tryptase Clara, the insoluble membrane fraction was extracted with 0.5 M NaCl in 50 mM sodium acetate buffer pH 5.0, and the processing enzyme was then purified by a series of standard chromatographic procedures and affinity chromatography on a benzamidine–Sepharose column as the final purification

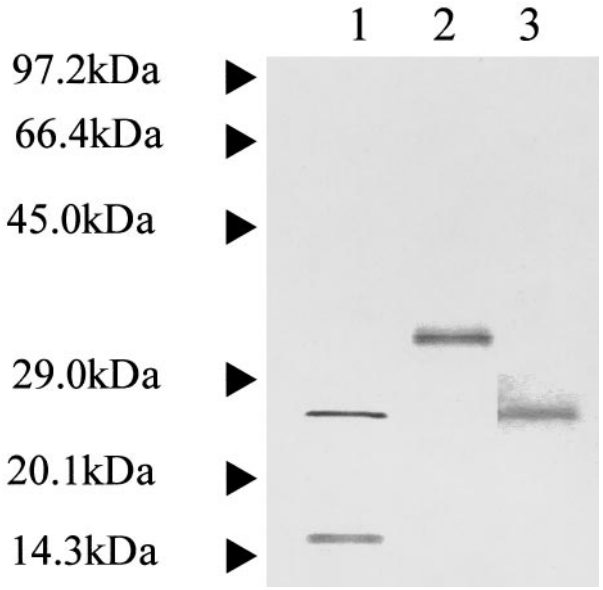

Fig. 1. Electrophoretic analyses of the purified enzyme. The purified enzyme (1 μg) was subjected to SDS/PAGE (10–20% gradient) under reducing (lane 1) and nonreducing (lane 2) conditions, followed by silver staining as described in Materials and methods. The protein band material (4 μg) with a molecular mass of 28 kDa under reducing conditions was labelled with [^{3}H]DFP (lane 3). The positions of SDS/PAGE low range standards are indicated on the left.

step. Although several hydrolysing activities on the synthetic substrate were observed in the membrane fraction and the fractions on sequential chromatography steps, only one peak with hydrolysing activity on the synthetic substrate coincided with the processing activity of [^{3}H]glucosamine-labelled nonactivated Sendai virus on each purification step. Starting from 2 kg of rat lungs, 9.6 μg of the purified enzyme was obtained. The specific activity of the purified enzyme was 1.51 U·mg^{-1} protein with Boc-Gln-Ala-Arg-MCA as substrate, the value being almost identical with that of the purified human mini-plasmin described below. The purified enzyme gave a protein band corresponding to a molecular mass of 38 kDa under nonreducing conditions, and two bands corresponding to molecular masses of 28 kDa and 12 kDa under reducing conditions on SDS/PAGE (Fig. 1). This indicates that the purified 38-kDa protein has an intermolecular disulfide bond(s) between the 28-kDa subunit and the 12-kDa subunit. As the processing activity of the purified enzyme was inhibited by DFP, as described below, it was treated with [^{3}H]DFP before electrophoresis under reducing conditions. A fluorogram of the gel revealed a labelled 28-kDa band and an unlabelled 12-kDa band, indicating that the 28-kDa protein is a catalytic subunit having an active site serine residue.

N-terminal amino-acid sequence

The N-terminal 49 amino acid residues of the 28-kDa band material and the 30 residues of the 12-kDa band material were determined after Western blotting of the purified enzyme under reducing conditions (Fig. 2). Sequence alignment of the 12-kDa band material revealed 100% identity with the N-terminal kringle 5 domain sequence of the heavy chain of rat plasminogen [20]. Although sequence information on rat plasminogen is limited and none on the catalytic subunit is available so far [20], the N-terminal amino-acid sequence of the 45 residues except four unidentified residues of the 28-kDa band material exhibited 93.3% identity with that of the catalytic subunit of mouse plasminogen. These sequence data and the enzymatic properties of the purified enzyme described below show that the processing enzyme is rat mini-plasmin, which consists of the kringle 5 domain and the catalytic light chain of plasminogen. Because of the absence of the N-terminal hydrophilic kringle 1–4 domains, i.e. the 'angiostatin' domain, mini-plasmin exhibited an increased hydrophobic moment [24]: the hydrophobic moment is −146.09 for human plasminogen, and −48.66 for human mini-plasmin. The subcellular fractionation of mini-plasmin in the insoluble membrane fraction is consistent with its increased hydrophobicity.

Substrate and inhibitor specificity

The purified enzyme showed maximum activity in the pH range 7.5–8.0. The activity of the enzyme toward various synthetic peptide substrates is shown in Table 1. The

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bovine plasminogen   421 ENYPNAGLTMNYCRNPDADK-SPWCYTTDPRVRWEFCNLKKCSETPEQVPAAPQAPGVEN
human plasminogen    414 ENYPNAGLTMNYCRNPDADK-GPWCFTTDPSVRWEYCNLKKCSGTEASVVAPPPVVLLPD
mouse plasminogen    414 ENFPDAGLEMNYCRNPDGDK-GPWCYTTDPSVRWEYCNLKRCSETGGSVVELPTVSQEPS
rat plasminogen       -a ANFPDDSGLEMNYCRNPDNDQRGPWCFTTDPSVRWEYCNLKRCSETGGGVAESAIVPQVPS
purified enzyme (12kDa)                                                SAIVPQVPS

bovine plasminogen   480 PDEA---DCMIGDGKSYRGKKATTVAGVPCQEWAAQEPHRHSIFTPETNPQSGLERNYCR
human plasminogen    473 VETPSEEDCMFGNGKGYRGKRATTVTGTPCQDWAAQEPHRHSIFTPETNPRAGLEKNYCR
mouse plasminogen    473 GPSDSETDCMYGNGKDYRGKTAVTAAGTPCQGWAAQEPHRHSIFTPQTNPRADLEKNYCR
rat plasminogen       -  APGTSETDCMYGNGKEYRGKTAVTAAGTPCQEWAAQEPH
purified enzyme (12kDa) APGTSETDCMYGNGKEYRGKT

bovine plasminogen   537 NPDGDVNGPWCYTMNPRKPFDYCDVPQCES--SFDCGKPKVEPKKCSGRIVGGCVSKPHS
human plasminogen    533 NPDGDVGGPWCYTTNPRKLYDYCDVPQCAAP-SFDCGKPQVEPKKCPGRVVGGCVAHPHS
mouse plasminogen    533 NPDGDVNGPWCYTTNPRKLYDYCDIPLCASASSFECGKPQVEPKKCPGRVVGGCVANPHS
rat plasminogen       -
purified enzyme (28kDa)                                                VVGGCVANPHS

bovine plasminogen   595 WPWQVSLRRSSR--HFCGGTLISPKWVLTAAHCLDNILALSFYKVILGAHNEKVREQSVQ
human plasminogen    592 WPWQVSLRTRFGM-HFCGGTLISPEWVLTAAHCLEKSPRPSSYKVILGAHQEVNLEPHVQ
mouse plasminogen    593 WPWQISLRTRFTGQHFCGGTLIAPEWVLTAAHCLEKSSRPEFYKVILGAHEEYIRGLDVQ
rat plasminogen       -
purified enzyme (28kDa) WPWQISLRTRFXGQHFCGGTLIXNRWVLTAAXCLHKSS
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Fig. 2. Alignment of the amino-acid sequences of the N-terminal 49 and 30 residues of the 28- and 12-kDa protein band materials, respectively, from the purified enzyme, and plasminogens from various species. The reported sequences of bovine plasminogen [21], human plasminogen [22], and mouse plasminogen [23] were deduced from their cDNAs and the partial sequence of rat plasminogen was reported by Kannalas and Makker [20]. Residues identical with those among them are boxed. Residue numbers, from the translation-initiation site, are shown on the left. [a]Residue number from the translation-initiation site has not been determined.

Table 1. Substrate specificity. Relative activities (32.3 mU·mL^{-1}) of the purified enzyme are expressed as percentages of the activity toward Boc-Gln-Ala-Arg-MCA.

Substrate	Relative activity (%)
Boc-Gln-Ala-Arg-MCA	100
Boc-Leu-Thr-Arg-MCA	10.6
Boc-Phe-Ser-Arg-MCA	15.4
Boc-Val-Pro-Arg-MCA	15.4
Boc-Gly-Gly-Arg-MCA	25.0
Boc-Ala-Gly-Pro-Arg-MCA	7.7
Boc-Ile-Gln-Gly-Arg-MCA	4.8
Pro-Phe-Arg-MCA	15.4
Bz-Arg-MCA	0.0
Boc-Gln-Arg-Arg-MCA	50.0
Boc-Gly-Lys-Arg-MCA	9.6
Boc-Leu-Arg-Arg-MCA	9.6
Boc-Val-Leu-Lys-MCA	54.8
Boc-Glu-Lys-Lys-MCA	155.8
Suc-Leu-Leu-Val-Thy-MCA	0.0
Suc-Ala-Ala-Pro-Phe-MCA	0.0

enzyme recognized not only Arg but also Lys at the P1 position. Of the compounds examined, Boc-Glu-Lys-Lys-MCA was the best substrate; this is known to be the best synthetic peptide substrate for plasmin. Boc-Gln-Ala-Arg-MCA, which is homologous to the consensus cleavage site sequence of human influenza A virus, Gln (or Glu)-X-Arg [2,25–27], was the second best substrate. Furthermore, Boc-Gln-Arg-Arg-MCA, which is identical to the cleavage site sequence of Newcastle disease virus Miyadera [1], was also a fairly good substrate for this enzyme among substrates with a dibasic sequence. Unlike trypsin, the enzyme scarcely hydrolysed substrates with a single basic amino acid residue, such as Bz-Arg-MCA, suggesting that it requires more than a single amino acid residue for its hydrolytic activity. This substrate specificity was almost identical to those of plasmin and mini-plasmin [28,29].

The effects of various protease inhibitors on the activity of the purified enzyme are summarized in Table 2. Phenylmethylsulfonyl fluoride and DFP at the concentration of 10 mM, and benzamidine at the concentration of 1 mM effectively inhibited the activity, indicating that the enzyme is a member of the serine protease family. The activity was also inhibited markedly by inhibitors of plasmin, such as aprotinin, Kunitz-type soybean trypsin inhibitor and Bowman–Burk soybean trypsin inhibitor. Mucus protease inhibitor (MPI) [16] and α_1-anti-trypsin had no inhibitory effect on the enzyme. Inhibitors of chymotrypsin-type serine, metallo-, carboxyl-and thiol-proteases exhibited weak or no inhibitory effect on the activity.

Table 2. Inhibitor specificity. The purified enzyme was preincubated with an effector at the concentration indicated for 5 min at 37 °C and then the residual activity was determined as described in Materials and methods. Enzyme activity in the absence of an effector was taken as 100%.

Effector	Final concentration (mM)	Relative activity (%)
None		100
Phenylmethylsulfonyl fluoride	1	95.1
	10	29.5
DFP	1	65.6
	10	3.3
Aprotinin	0.01	0.0
MPI	0.01	97.4
Leupeptin	0.01	18.6
Elastatinal	0.01	69.2
Benzamidine	0.01	67.6
	1	22.9
Kunitz-type soybean trypsin inhibitor	0.01	0.0
Chymostatin	0.01	100
Bowman–Birk soybean trypsin inhibitor	0.01	3.7
α_1-Anti-trypsin	0.01	100
E-64	0.01	61.5
Pepstatin A	0.01	64.0
Phosphoramidon	0.01	100

Immunohistochemical staining

On Western immunoblotting analyses (Fig. 3), antibodies against the mouse light chain of plasminogen reacted with both rat mini-plasmin and plasminogen, but antibodies against the rat N-terminal five residues of the kringle 5 domain (anti-rat NT kringle 5) reacted specifically with rat mini-plasmin but not with plasminogen. A light micrograph showed that the folded epithelial cells in the relatively thick upward divisions of bronchioles were stained predominantly with anti-rat NT kringle 5 antibodies (Fig. 4A and C) and anti-plasminogen light chain antibodies (Fig. 4B). However, terminal and respiratory bronchioles were little

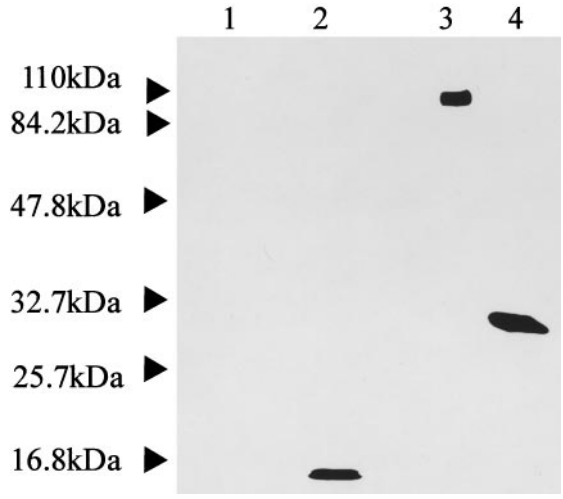

Fig. 3. Immunochemical analysis of the specificity of anti-rat NT kringle 5 and anti-light chain of plasminogen antibodies. Rat plasminogen purified from serum (50 ng, lanes 1 and 3) and mini-plasmin from lungs (50 ng, lanes 2 and 4) were subjected to SDS/PAGE (10–20% gradient) under reducing conditions and then transferred electrophoretically to an immobilon transfer membrane. Immunoreactivity of anti-rat NT kringle 5 antibodies (lanes 1 and 2) and anti-light chain of mouse plasminogen antibodies (lanes 3 and 4) was then analysed.

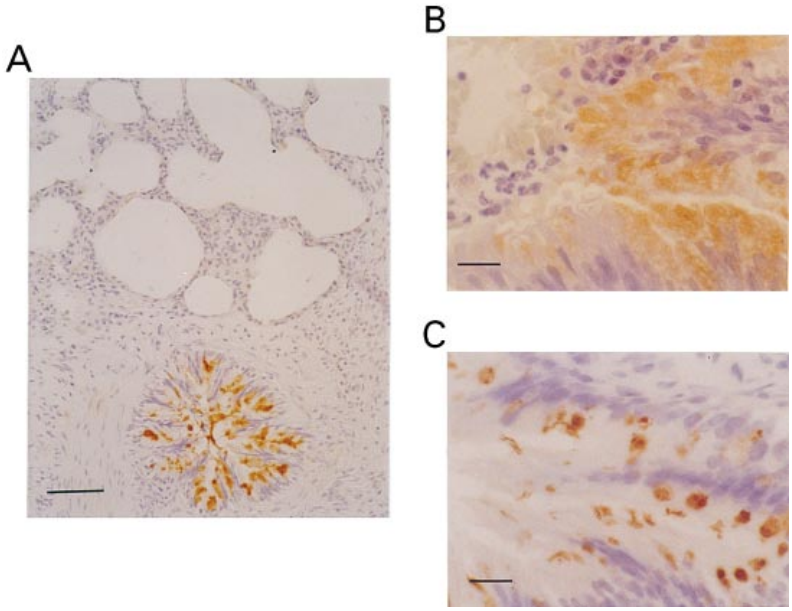

Fig. 4. Immunohistochemical localization of mini-plasmin in rat lungs. Serial sections of a bronchiole of rat lung were immunostained with anti-rat NT kringle 5 (A, × 140, bar = 80 μm; and C, × 420, bar = 20 μm) and anti-light chain of mouse plasminogen (B, × 420, bar = 20 μm) antibodies as described in Materials and methods. Mini-plasmin, which reacts with both antibodies, was distributed in a granular manner, and plasminogen, which reacts with the anti-light chain of plasminogen but not the anti-rat NT kringle 5 antibodies, was distributed diffusely in the cytoplasm, but not in nuclei. Nuclei were counterstained with methyl green.

stained with these antibodies, and alveolar ducts, alveoli, veins, arteries and circular connective tissues were not stained at all. The cytoplasm of the immunoreactive epithelial cells was stained diffusely with antibodies against the light chain of plasminogen (Fig. 4B). However, positive staining with anti-rat NT kringle 5 antibodies was seen in restricted and granular patterns (Fig. 4C) and deposits of the reaction products overlapped parts of those reacted with anti-plasminogen light chain antibodies. Control sections of lungs incubated in the presence of nonimmune rabbit IgG at the same concentration were not labelled at all.

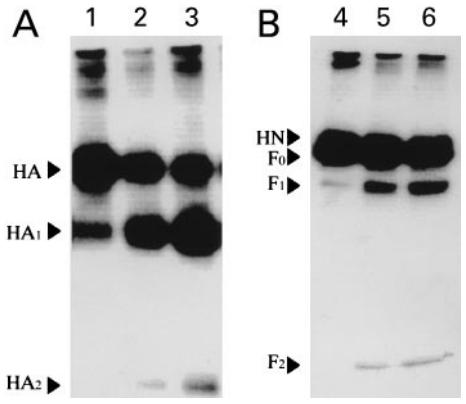

Fig. 5. Processing activity toward HA of influenza A/Aichi/2/68 (H3N2) virus and of Sendai (Z strain) virus F_0. (A) The [^{3}H]glucosamine-labelled and nonactived influenza A virus grown in MDCK cells (lane 1) was treated with plasmin (6.2 mU, lane 2) and mini-plasmin (3.1 mU, lane 3) for 10 min at 37 °C. (B) The [^{3}H]glucosamine-labelled and nonactivated Sendai virus grown in LLC-MK2 cells (lane 4) was treated with 1 mU of plasmin (lane 5) and mini-plasmin (lane 6) for 60 min at 37 °C. The proteolytic products were separated by SDS/PAGE under reducing conditions and visualized by fluorography.

Processing by mini-plasmin of virus envelope fusion glycoproteins

Besides the amidolytic activity toward peptide substrates, the purified mini-plasmin efficiently converted the [^{3}H]glucosamine-labelled envelope glycoprotein precursor of HA into HA_1 and HA_2, and F_0 into F_1 and F_2 as shown in Fig. 5. The N-terminal amino-acid sequences of F_1 and HA_2 revealed that the enzyme recognized the R116–F117 bond of F_0 and the R325–G326 bond of HA, respectively, and cleaved at the arginine residue (data not shown). When plasmin and mini-plasmin were incubated with human influenza A/Aichi/2/68 (H3N2) virus, mini-plasmin converted HA into HA_1 and HA_2 more efficiently than plasmin (Fig. 5A). Plasmin and mini-plasmin, however, processed Sendai virus F_0 with similar efficiency (Fig. 5B). We then analysed the effects of various inhibitors of serine proteases and metallo-proteases on the processing by mini-plasmin of Sendai virus F_0 (Fig. 6). Low molecular mass inhibitors of trypsin-type serine proteases, such as DFP at 1 mM and benzamidine at 10 μM, and proteinaceous inhibitors at 10 μM, such as Kunitz-type soybean trypsin inhibitor and Bowman–Birk soybean trypsin inhibitor, significantly inhibited the processing of F_0 by mini-plasmin. MPI, which is a potent inhibitor of tryptase Clara and is found in bronchial and nasal fluid [16], did not inhibit the processing by mini-plasmin at all. Inhibitors of chymotrypsin-type serine and metallo proteases and an inhibitor of elastase had no effect on the activity.

Potentiation by human mini-plasmin of the infectivity of various strains of influenza A viruses

Plasminogen concentration in normal human plasma is as high as ≈2 μM (158 μg·mL^{-1}) [13] and mini-plasmin was localized in the upward divisions of bronchioles. We analysed the efficiency of the proteolytic potentiation of various strains of influenza A viruses by human plasmin, mini-plasmin and microplasmin, together with that by

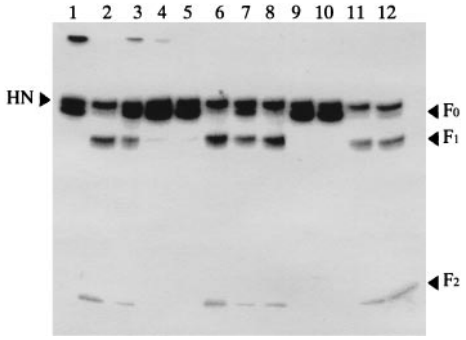

Fig. 6. Effects of protease inhibitors on F_0 processing by mini-plasmin. Rat mini-plasmin (0.24 μg) was incubated with various protease inhibitors in 50 mM Tris/HCl buffer pH 7.5 for 30 min on ice and then [^{3}H]glucosamine-labelled nonactivated Sendai virus was added to the reaction mixture. After incubation for 30 min at 37 °C, SDS/PAGE was performed under reducing conditions, followed by fluorography. Lane 1, nonactivated virus; lane 2, virus treated with mini-plasmin. Lanes 3–12, virus treated with mini-plasmin in the presence of 1 mM phenylmethylsulfonyl fluoride (lane 3), 1 mM DFP (lane 4), 10 μM aprotinin (lane 5), 10 μM MPI (lane 6), 10 μM leupeptin (lane 7), 10 μM elastatinal (lane 8), 10 μM benzamidine (lane 9), 10 μM soybean trypsin inhibitor (lane 10), 10 μM o-phenanthlorin (lane 11), and 10 μM chymostatin (lane 12).

porcine pancreatic trypsin (Fig. 7). Plasmin has been reported to process HA, resulting in potentiation of the infectivity of some specific influenza virus strains, i.e. not all strains [9,17]. In these experiments, we selected three typical influenza A virus strains: WSN strain, which exhibits the highest susceptibility to plasmin and can replicate in cultured cells in the presence of serum containing plasminogen [17]; influenza A/seal/Massachusetts/1/81 (H7N7) strain, the infectivity of which is not potentiated by plasmin [9]; and influenza A/Aichi/2/68 (H3N2), which is activated by tryptase Clara [5], trypsin and plasmin.

In the case of strain WSN, plasminogen binds to the C-terminal lysine of the WSN neuraminidase through the lysine binding region in its heavy-chain subunit, leading to enhanced HA cleavage and greater virulence of this pantropic influenza A virus [30]. Mini-plasmin, which has no lysine binding region in its molecule, however, also potentiated the infectivity of WSN at concentrations > 3 μg·mL^{-1}, with a plateau at 10 μg·mL^{-1}, although it was less efficient than plasmin and trypsin, as shown in Fig. 7A. On the other hand, the infectivity of the plasmin-insensitive virus, influenza A/seal/Massachusetts/1/81 (H7N7), was potentiated by mini-plasmin, but not by plasmin, at concentrations > 10–30 μg·mL^{-1} (Fig. 7B). For influenza A/Aichi/2/68 (H3N2) virus, trypsin exhibited the highest potentiating effect among the proteases examined. Mini-plasmin showed ≈ 3-fold higher efficiency of proteolytic activation of the virus infectivity than plasmin (Fig. 7C); the result was consistent with that in Fig. 5A. Micro-plasmin, i.e. the light chain subunit of plasminogen, exhibited proteolytic potentiation of all influenza A viruses examined similar to that of mini-plasmin. The results indicate that the kringle 5 domain of mini-plasmin has no effect on the proteolytic potentiation of influenza A viruses.

DISCUSSION

In the present study, we isolated, from the membrane fraction of rat lungs, a virus envelope glycoprotein-processing protease that potentiates the infectivity of broad-spectrum influenza A viruses and Sendai virus, as does pancreatic trypsin. Studies on the amino-acid sequence and enzymatic properties, such as the specific activity, the substrate specificity and the inhibitor sensitivity of the purified enzyme, indicated that the HA- and F_0- processing enzyme is mini-plasmin. Although *in vitro* formation of mini-plasminogen or mini-plasmin on treatment of plasminogen with neutrophil or pancreatic elastase and/or urokinase has been reported [11,29,31], the localization and function *in vivo* of mini-plasmin have been unclear. Immunohistochemical studies in Fig. 4 have indicated that mini-plasmin is distributed predominantly in the epithelial cells of the upward divisions of bronchioles, but not in arteries or veins, alveoli or circular connective tissues, and little in terminal and respiratory bronchioles. In contrast with mini-plasmin, tryptase Clara, another virus

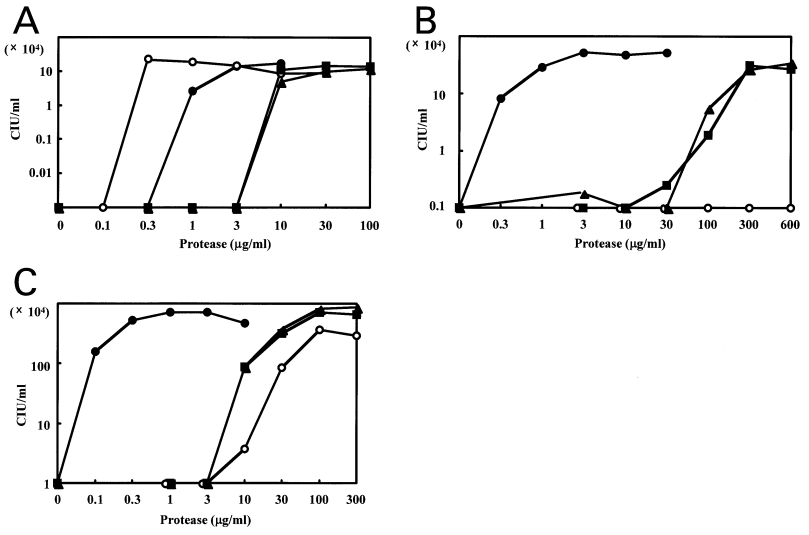

Fig. 7. Activation by human plasmin, mini-plasmin, microplasmin and bovine pancreatic trypsin of WSN (A), influenza A/seal/Massachusetts/1/81 (H7N7) (B), and influenza A/Aichi/2/68 (H3N2) (C). MDCK cell-grown nonactivated influenza A viruses were treated with trypsin (●), plasmin (○), mini-plasmin (▲), or microplasmin (■) at the concentrations indicated in NaCl/P_i for 30 min at 37 °C as described in Materials and methods. Infectivity was assayed by the haemadsorption cell-counting method [19], which can detect only the active virus.

envelope-processing protease in lungs, is distributed mainly in the secretory epithelial cells (Clara cells) of the downward divisions of bronchioles, such as terminal and respiratory bronchioles, but not alveoli. Deposits of the immunoreaction products with anti-plasminogen light chain antibodies were distributed diffusely in the cytoplasm, but not the nuclei, of rat bronchiole epithelial cells (Fig. 4B); this staining pattern is in fair agreement with that obtained in rat liver, the major *de novo* biosynthesis organ of plasminogen. In liver, plasminogen is distributed in the endoplasmic reticulum and/or the Golgi apparatus and is secreted into the blood. Deposits in the airway epithelial cells of the reaction products with the anti-rat NT kringle 5 were distributed in restricted and granular patterns, suggesting that mini-plasmin is probably distributed in secretion vesicles in the epithelial cells, although further studies on the subcellular localization of mini-plasmin and plasminogen in the airway epithelial cells by immunoelectron micrograph are needed. Deposits of the reaction products with the anti-rat NT kringle 5 antibodies were not, however, detected in liver or kidney.

The enzymatic properties of mini-plasmin have been studied extensively *in vitro*, because of the striking functional difference from those of plasmin: mini-plasmin is relatively free from the control of α_2-anti-plasmin, which is generally considered to be a primary physiological inhibitor of plasmin [29,32]. A similar property of mini-plasmin was observed in the inhibition by α_2-anti-plasmin on the processing of Sendai virus F_0 (data not shown). Because of its increased hydrophobicity and escaping rapid inactivation by α_2-anti-plasmin, mini-plasmin could easily bind to the membrane, leading to increased local concentrations, and thus potentiate the infectivity of influenza A viruses and Sendai virus effectively for a long time in the airway epithelial cells.

Like pancreatic trypsin, mini-plasmin potentiated the infectivity of broad-spectrum influenza A virus strains and Sendai virus, i.e. not only the plasmin-sensitive strains but also the plasmin-insentitive strains, although it was less efficient than trypsin (Fig. 7). These data suggest that 38-kDa mini-plasmin without lysine binding domains can gain easier access to the cleavage sites of HA of various influenza A virus strains than 94-kDa plasmin. As the plasminogen concentration in human plasma is relatively high, mini-plasmin may also be produced at inflammatory loci [31] or during clot formation [33] in lungs by granulocyte elastase. Therefore, the studies on the pathological roles of mini-plasmin at inflammatory loci in the progression and the infiltration on influenza virus infection in the lungs are now under investigation.

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REFERENCES

1. Klenk, H.-D. & Garten, W. (1994) Host cell proteases controlling virus pathogenicity. *Trends Microbiol.* **2**, 39–43.
2. Klenk, H.-D. & Rott, R. (1988) The molecular biology of influenza virus pathogenicity. *Adv. Virus Res.* **34**, 247–281.
3. Homma, M. & Ohuchi, M. (1973) Trypsin action on the growth of Sendai virus in tissue culture cell. *J. Virol.* **12**, 1457–1465.
4. Scheid, A. & Choppin, P.W. (1974) Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity by proteolytic cleavage of an inactive precursor protein of Sendai virus. *Virology* **57**, 475–490.
5. Kido, H., Yokogoshi, Y., Sakai, K., Tashiro, N., Kishino, Y., Fukutomi, A. & Katunuma, N. (1992) Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. Possible activator of the viral fusion glycoprotein. *J. Biol. Chem.* **267**, 13573–13579.
6. Tashiro, M., Yokogoshi, Y., Tobita, K., Seto, J.T., Rott, R. & Kido, H. (1992) Tryptase Clara, an activating protease for Sendai virus in rat lungs, is involved in pneumopathogenicity. *J. Virol.* **66**, 7211–7216.
7. Chen. Y., Shiota, M., Ohuchi, M., Towatari, T., Tashiro, J., Murakami, M., Yano, M., Yang, B. & Kido, H. (2000) Mast cell tryptase from pig lungs triggers infection by pneumotropic Sendai virus and influenza A viruses. *Eur. J. Biochem.* **267**, 3189–3197.
8. Gotoh, B., Ogasawara, T., Toyoda, T., Inocencio, N., Hamaguchi, M. & Nagai, Y. (1990) An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo. *EMBO J.* **9**, 4189–4195.
9. Scheiblauer, H., Reinacher, M., Tashiro, M. & Rott, R. (1992) Interactions between bacteria and influenza A virus in the development of influenza pneumonia. *J. Infect. Dis.* **166**, 783–791.
10. Sakai, K., Kawaguchi, Y., Kishino, Y. & Kido, H. (1993) Electron immunohistochemical localization in rat bronchiolar epithelial cells of tryptase Clara, which determines the pneumotropism and pathogenicity of Sendai virus and influenza virus. *J. Histochem. Cytochem.* **41**, 89–93.
11. Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T.E. & Magnusson, S. (1978) The primary structure of human plasminogen: Isolation of two lysine-binding fragments and one 'mini-' plasminogen (MW, 38,000) by elastase-catalyzed-specific limited proteolysis. *Prog. Chem. Fibrinol. Thrombol.* **3**, 191–209.
12. Thorsen, S., Clemmensen, I., Sottrup-Jensen, L. & Magnusson, S. (1981) Adsorption to fibrin of native fragments of known primary structure from human plasminogen. *Biochim. Biophys. Acta* **668**, 377–387.
13. Parry, M.A.A., Fernandez-Catalan, C., Bergner, A., Huber, R., Hopfner, K.-P., Schlott, B., Gührs, K.-H. & Bode, W. (1998) The ternary microplasmin-staphylokinase-microplasmin complex is a proteinase-cofacter-substrate complex in action. *Nature Struct. Biol.* **5**, 917–923.
14. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85.
15. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
16. Beppu, Y., Imamura, Y., Tashiro, M., Towatari, T., Ariga, H. & Kido, H. (1997) Human mucus protease inhibitor in airway fluid is a potential defensive compound against infection with influenza A and Sendai virus. *J. Biochem.* **121**, 309–316.
17. Lazarowitz, S.G., Goldberg, A.R. & Choppin, P.W. (1973) Proteolytic cleavage by plasmin of a HA polypeptide of influenza virus: host cell activation of serum plasminogen. *Virology* **56**, 172–180.
18. Tashiro, M., Ciborowski, P., Reinacher, M., Pulverer, G., Klenk, H.-D. & Rott, R. (1987) Synergistic role of staphylococcal proteases in the induction of influenza virus pathogenicity. *Virology* **157**, 421–430.
19. Ohuchi, M., Ohuchi, R., Feldmann, A. & Klenk, H.-K. (1997) Regulation of receptor binding affinity of influenza virus hemmagglutinin by its carbohydrate moiety. *J. Virol.* **71**, 8377–8384.
20. Kannalas, J.J. & Makker, S.P. (1991) Identification of the rat

Heymann nephritis autoantigen (GP330) as a receptor site for plasminogen. *J. Biol. Chem.* **266**, 10825–10829.
21. Schaller, J., Moser, P.W., Dannegger-Muller, G.A., Rosselet, G.A., Kampfer, U. & Rickli, E.E. (1985) Complete amino-acid sequence of bovine plasminogen. Comparison with human plasminogen. *Eur. J. Biochem.* **149**, 267–278.
22. Petersen, T.E., Martzen, M.R., Ichinose, A. & Davie, E.W. (1990) Characterization of the gene for human plasminogen, a key proenzyme in the fibrinolytic system. *J. Biol. Chem.* **265**, 6104–6111.
23. Degen, S.J., Bell, S.M., Schaefer, L.A. & Elliott, R.W. (1990) Characterization of the cDNA coding for mouse plasminogen and localization of the gene to mouse chromosome 17. *Genomics* **1**, 49–61.
24. Eisenberg, D., Wilcox, W. & McLachlan, A.D. (1986) Hydrophobicity and amphiphilicity in protein structure. *J. Cell Biochem.* **31**, 11–17.
25. Barber-Morel, C.L., Oeltmann, T.N., Edwards, K.M. & Wright, P.F. (1987) Role of respiratory tract proteases in infectivity of influenza A virus. *J. Infect. Dis.* **155**, 667–672.
26. Blumberg, B.M., Giorgi, C., Rose, K. & Kolakofsky, D. (1985) Sequence determination of the Sendai virus fusion gene. *J. Gen. Virol.* **66**, 317–331.
27. Gething, M.-J., Bye, J., Skehel, J. & Waterfield, M. (1980) Cloning and DNA sequences of double-stranded copies of hemaggulutinin genes from H2 and H3 strains elucidates antigenic shift and drift in human influenza virus. *Nature* **287**, 301–306.
28. Robbins, K.C. & Summaria, L. (1970) Human plasminogen and plasmin. *Methods Enzymol.* **19**, 184–199.
29. Christensen, U., Sotterup-Jensen, L., Magnusson, S., Petersen, T.E. & Clemmensen, I. (1979) Enzymic proterties of the neoplasmin-Val-442 (mini-plasmin). *Biochem. Biophys. Acta* **567**, 472–481.
30. Goto, H. & Kawaoka, Y. (1998) A novel mechanism for the acquisition of virulence by a human influenza A virus. *Proc. Natl Acad. Sci. USA* **95**, 10224–10228.
31. Moroz, L.A. (1981) Mini-plasminogen: a mechanism for leukocyte modulation of plasminogen activation by urokinase. *Blood* **58**, 97–104.
32. Pertersen, L.C. & Clemmensen, I. (1981) Kinetics of plasmin inhibition in the presence of a synthetic tripeptide substrate. *Biochem. J.* **199**, 121–127.
33. Plow, E.E. & Plescia, J. (1988) Neutrophil secretion during blood coagulation: evidence for a prekallikrein independent pathway. *Thromb. Haemost.* **59** 360–363.