

Tryptase Clara, an Activating Protease for Sendai Virus in Rat Lungs, Is Involved in Pneumopathogenicity

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Tryptase Clara is an arginine-specific serine protease localized exclusively in and secreted from Clara cells of the bronchial epithelium of rats (H. Kido, Y. Yokogoshi, K. Sakai, M. Tashiro, Y. Kishino, A. Fukutomi, and N. Katunuma, *J. Biol. Chem.* 267:13573-13579, 1992). The purified protease was shown in vitro to behave similarly to trypsin, cleaving the precursor glycoprotein F of Sendai virus at residue Arg-116 and activating viral infectivity in a dose-dependent manner. Anti-tryptase Clara antibody inhibited viral activation by the protease in vitro in lung block cultures and in vivo in infected rats. When the enzyme-specific antibody was administered intranasally to rats that were also infected intranasally with Sendai virus, activation of progeny virus in the lungs was significantly inhibited. Thus, multiple cycles of viral replication were suppressed, resulting in a reduction in lung lesions and in the mortality rate. These findings indicate that tryptase Clara is an activating protease for Sendai virus in rat lungs and is therefore involved in pulmonary pathogenicity of the virus in rats.

Paramyxoviruses possess an envelope glycoprotein F which mediates entry of the viral genome into the host cells by fusing the viral envelope with the cell membranes. The F protein is synthesized in infected cells as a biologically nonactive precursor, F₀, and posttranslational proteolytic cleavage to two disulfide-linked subunits, F₁ and F₂, is a prerequisite for expression of the fusion activity and infectivity of the virus (9, 20, 25). If host cells possess a protease which proteolytically activates F, infectious progeny virus is produced, resulting in multiple cycles of viral replication. By contrast, infection is restricted to a single cycle of replication in cells lacking an appropriate protease for cleavage activation (8, 11, 20-22, 27, 33). Sendai virus, the paramyxovirus prototype, possesses an F protein with a cleavage site, residue 116, that consists of a single arginine (2, 10). The F protein is cleaved in vitro only by specific proteases such as trypsin (7, 25) and the clotting factor Xa, present in the allantoic fluid of chicken embryos (5, 19), and is not cleavable by a ubiquitous host protease(s), present in various cell types, that will cleave preferentially multibasic cleavage sites (18, 34).

Sendai virus is exclusively pneumotropic in rodent species (3, 11, 30, 35). The target of intranasal infections is restricted to the bronchial epithelial cells (3a, 27, 33), although various cell types in the lungs and other organs possess sialic acid-containing receptors for the virus (12) which are potentially permissive for productive virus replication (33). We have postulated that the pneumopathogenicity of Sendai virus for mice is primarily determined by the presence in the lungs of an arginine-specific serine protease(s) that cleaves the F protein and thereby enables the virus to undergo multiple cycles of replication in this organ, resulting in fatal lung lesions (27, 31, 33). On the other hand, we have also

postulated that appropriate proteases for cleavage activation of F are absent or do not function in other organs, and therefore viral infection in these organs, if it occurs at all, must be terminated after an initial cycle of replication (32, 33). Although trypsin, which activates Sendai virus in vitro (7, 25), should be present in the intestinal tract, and primary cultures of kidney cells were suggested to support viral activation (25a), respiratory infections in vivo do not spread to these tissues (30, 32). This may be explained by the apical budding phenotype of Sendai virus in the bronchial epithelial cells, the primary target of natural infections; spread of the progeny virus remains localized at the surface epithelia of the respiratory tract (32, 33). Such a protease(s) for activation of Sendai virus has repeatedly been suggested to be present in the lungs as a key cellular determinant of pulmonary pathogenicity (8, 14, 26, 30, 31). This putative protease should also have characteristics similar, although not identical, to those of trypsin rather than those of chymotrypsin or elastase (7, 13, 27, 28, 31). However, the precise nature and distribution of the enzyme(s) remain unclear.

Recently, we isolated a novel serine protease, designated tryptase Clara, from rat lungs (17). The protease is localized exclusively in the nonciliated secretory cells, the so-called Clara cells, of the bronchial and bronchiolar epithelia of rats and is also secreted into the airway lumen. Tryptase Clara cleaves specifically single arginine residues of several peptides at neutral pH, and its spectrum of protease inhibitors is compatible with that suggested for the Sendai virus-activating protease(s) in mouse lungs (6, 14, 27, 28, 31). In addition, we have demonstrated that tryptase Clara cleaves the hemagglutinin of an influenza A virus and activates viral infectivity in vitro (17). These results suggested that tryptase Clara is an activating protease for Sendai virus in rat lungs. In this report, we show that tryptase Clara activates Sendai virus in vitro and in vivo and is involved in pulmonary pathogenicity of the virus in rats.

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MATERIALS AND METHODS

Viruses and cells. Wild-type Sendai virus (Z strain) and protease activation mutants TR-2, T-9, and KDe-62 were propagated, purified, and assayed as described previously (27, 29, 31). Nonactive virus was propagated in LLC-MK₂ cells in the absence of protease in the culture medium as described previously (27). LLC-MK₂ cells were grown in Dulbecco's minimum essential medium (MEM) supplemented with 10% fetal calf serum in plaque dishes or on chamber slides (catalog no. 177402; Nunc, Inc., Naperville, Ill.) for immunofluorescence studies. Cells were maintained with MEM containing 0.1% bovine serum albumin.

Proteases. Tryptase Clara was purified from rat lungs as reported previously (17). Specific activity was 622 U/mg when assayed with Boc (*N*-*tert*-butoxycarbonyl)-Gln-Ala-Arg-MCA (4-methylcoumaryl-7-amide) as a substrate. One unit was defined as the amount degrading 1 μ mol of the substrate per min (17). Bovine pancreatic trypsin (type III), chymotrypsin (type VII), and porcine pancreatic elastase (type III) were from Sigma Chemical Co., St. Louis, Mo.

Antibodies. Rabbit immunoglobulins against Sendai virus and trypsin Clara were prepared as described previously (17, 27). Fluorescein isothiocyanate-labeled sheep immunoglobulin against rabbit immunoglobulins G, M, and A was obtained from Serotec, Ltd., Oxford, United Kingdom.

Infectivity assays. To quantify activated and nonactivated viruses differentially, the comparative assay method was used as described previously (27). Whole virus (both activated and nonactivated) was counted by plaque assay with 4 μ g of appropriate protease per ml in the agar overlay, while only activated virus was assayed by the immunofluorescent cell-counting method without protease (7, 17, 27).

In vitro activation of Sendai virus. Nonactive virus suspended in phosphate-buffered saline deficient in Ca²⁺ and Mg²⁺ (pH 7.2) (PBS) was incubated with proteases at various concentrations for 15 or 30 min at 37°C. The enzyme reaction was stopped by the addition of 100 μ g of aprotinin per ml. Infectivity was assayed by the immunofluorescent cell-counting method as described above. Hemolytic activity was assayed as described previously (19, 29).

Radioisotope labeling and SDS-PAGE. Virus was labeled with [³H]glucosamine or [³⁵S]methionine in LLC-MK₂ cells as described previously (29, 30). Purified virus suspended in PBS was treated with proteases as described above. Viral polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions followed by fluorography (30).

Amino acid sequencing. The amino acid sequence was determined by the microsequencing method as described previously (29, 31). Briefly, SDS-PAGE of Sendai virus was run, and viral polypeptides were electrophoretically transferred to ProBlot membrane (Applied Biosystems, Foster City, Calif.). The amino acid sequence of the NH₂ terminus of the F₁ subunit band was determined with a Protein Sequencer (Applied Biosystems).

Infection of rats. Specific-pathogen-free, 3-week-old male rats of the CD(SD) strain were obtained from Charles River Japan, Inc., and kept under bio-clean conditions at 23°C and 55% humidity. Under anesthesia with ether, rats were infected intranasally with 2 \times 10⁴ PFU of activated virus. A group of the animals received intranasal administration of 50 μ l of anti-tryptase Clara rabbit immunoglobulins every 4 to 8 h and also an intraperitoneal injection of 500 μ l of antibody every 2 to 3 days. Rats were examined daily for body weight, clinical signs, and pathological changes and for virus repli-

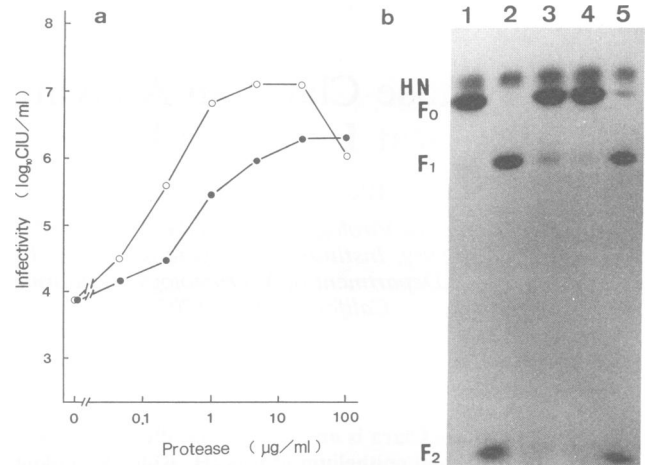


FIG. 1. Proteolytic activation of wild-type Sendai virus in vitro by tryptase Clara. (a) Activation of virus infectivity. LLC-MK₂ cell-grown virus suspended in PBS (pH 7.2) was treated with tryptase Clara for 15 min (●) or with trypsin for 10 min (○) at 37°C. Infectivity of activated virus was assayed by the immunofluorescent cell-counting method, using LLC-MK₂ cells in the absence of trypsin, and is shown as cell-infecting units (CIU). (b) SDS-PAGE of the viral glycoproteins. LLC-MK₂ cell-grown virus labeled with [³H]glucosamine (lane 1) was treated with trypsin at 5 μ g/ml for 10 min (lane 2) or with tryptase Clara at 50 (lane 3) and 10 (lane 4) μ g/ml for 15 min or at 50 μ g/ml for 30 min (lane 5). Viral glycoproteins were analyzed by SDS-PAGE under reducing conditions. HN, hemagglutinin-neuraminidase.

cation in the organs as described for mice (27). Lung lesions were scored from 1 to 4 according to the extent of macroscopic consolidation of the lung surface (27). Organs were homogenized and assayed for infectivity by the differential assay method as described above.

Organ block cultures of rat lungs. The lung block culture method described for mice (28) was applied to rats, with minor modifications. Rats were infected intranasally with 10⁴ PFU of virus. After 2 h, the animals were sacrificed and the lungs were removed and minced into blocks, which were then washed extensively with MEM. The organ blocks were incubated in MEM in the presence or absence of anti-tryptase Clara immunoglobulins at 34°C. At various time intervals, organ blocks were homogenized and assayed for infectivity as described above.

RESULTS

Proteolytic activation of Sendai virus in vitro by tryptase Clara. Nonactive Sendai viruses grown in LLC-MK₂ cells were treated with tryptase Clara as described in Materials and Methods. Activation of infectivity of wild-type virus is presented in Fig. 1a together with the results of trypsin treatment. Tryptase Clara was clearly shown to activate infectivity of wild-type Sendai virus in a dose-dependent manner. Although the activation of infectivity by the protease was less efficient than that induced by trypsin, it should be noted that in contrast to trypsin, tryptase Clara at higher concentrations did not decrease viral infectivity. The results suggest that tryptase Clara cleaves specifically at the activation cleavage site of the F protein and not at other residues, which may be cleaved by trypsin at a high concentration. Hemolytic activity of the virus was also enhanced by the treatment with tryptase Clara, but hemagglutinating activity

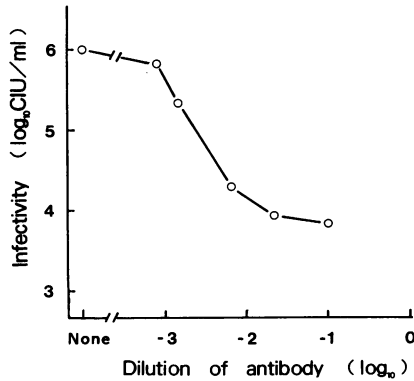


FIG. 2. Inhibitory effect of anti-tryptase Clara rabbit immunoglobulins on in vitro activation of Sendai virus by the protease. Tryptase Clara (50 μ g/ml) was preincubated with the antibody at the indicated dilutions for 60 min at 4°C and was tested for Sendai virus-activating capacity as described in the legend to Fig. 1. CIU, cell-infecting units.

was not affected (data not shown). On the other hand, the protease did not enhance the infectivity of the protease activation mutants with an amino acid exchange at the cleavage site of the F protein, Arg-116 to Ile for TR-2 (13) and KDe-62 (31) and Arg-116 to Lys for a revertant, T-9, derived from F1-R (29) (data not shown), also suggesting that tryptase Clara cleaves specifically at the arginine residue (residue 116) of wild-type F.

Results of SDS-PAGE analyses of [³H]glucosamine-labeled wild-type virus presented in Fig. 1b illustrate that tryptase Clara cleaved the precursor polypeptide F₀ into subunits F₁ and F₂, similar to trypsin. When [³⁵S]methionine-labeled virus was analyzed by SDS-PAGE, no change in the viral polypeptide pattern other than F protein was found after treatment with the protease (data not shown).

Direct amino acid sequencing of the NH₂ terminus of the F₁ subunit revealed the residues Phe-Phe-Gly-Ala-Val-Gly-, indicating that the cleavage site of wild-type F by tryptase Clara was between Arg-116 and Phe-117, identical to that for trypsin (4, 10, 13) and the clotting factor Xa in the allantoic fluid of chicken eggs (5).

Inhibition of in vitro viral activation by anti-tryptase Clara antibody. To confirm the role of tryptase Clara in the activation of Sendai virus in vitro and to establish optimal conditions for the experiments described below, 50 μ g of the protease per ml was preincubated with anti-tryptase Clara rabbit immunoglobulins at various dilutions for 60 min at 4°C, and the virus-activating capacity was assayed (Fig. 2). Activation of Sendai virus infectivity by tryptase Clara was suppressed to various degrees by the treatment, depending on the dilution of the antibody. At dilutions of greater than 1:40, activation was only at the background level. The antibody was devoid of any hemagglutination inhibiting and activated-virus neutralizing activity (data not shown). Immunoglobulins from normal rabbits did not inhibit viral activation by tryptase Clara (data not shown).

Inhibition by anti-tryptase Clara antibody of viral activation in organ block cultures of rat lungs. We have previously shown that progeny of wild-type Sendai virus is produced in the activated form in the lungs of mice (27). The same was also found in the lungs of rats, as described below. We therefore attempted to demonstrate that the cleavage activa-

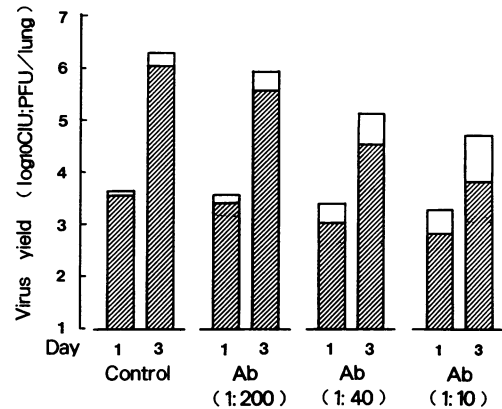


FIG. 3. Inhibitory effect of anti-tryptase Clara antibody on Sendai virus replication and activation in organ cultures of rat lungs. Lung block cultures prepared from rats that had been infected intranasally with 10⁴ PFU of Sendai virus for 2 h were incubated at 34°C with MEM in the absence or presence of anti-tryptase Clara rabbit immunoglobulins at the indicated dilutions. On days 1 and 3, organ blocks were homogenized and infectivity was assayed differentially for activated virus (hatched columns) and nonactivated virus (white columns). CIU, cell-infecting units; Ab, antibody.

tion of progeny virus in rat lungs was inhibited by anti-tryptase Clara antibody. Organ block cultures of rat lungs infected with Sendai virus were prepared and incubated with the antibody. Titers of activated and nonactivated viruses assayed on days 1 and 3 are presented in Fig. 3. In the lung culture system, Sendai virus was shown to be almost fully activated and to undergo multiple cycles of replication, similar to findings in the lungs of mice (27, 32). When anti-tryptase Clara immunoglobulins were present in the culture medium, activation of the progeny virus was inhibited significantly and multiple cycles of replication were suppressed. The inhibitory effect by the antibody was dose-dependent, and about 80% of the progeny virus remained nonactivated at an antibody dilution of 1:10. Normal rabbit serum at a 1:10 dilution had no effect on viral activation (data not shown). These results indicate that tryptase Clara was primarily, although not solely, responsible for activation of Sendai virus in organ cultures of rat lungs and that part of the viral activation by the protease occurred extracellularly.

Effects of anti-tryptase Clara antibody on intranasal infection of rats. Since the in vitro results presented above suggested that tryptase Clara plays a key role in the activation of Sendai virus in rat lungs in vivo and thus in pulmonary pathogenicity, rats that were infected intranasally with Sendai virus were treated with the immunoglobulins against the protease. They were examined for viral replication and pathological changes in the lungs (see Materials and Methods). Without the administration of antibody, progeny virus in the lungs was produced in the activated form and underwent multiple cycles of replication until days 5 to 6, when viral replication began to be terminated, presumably by the host immunological response (Fig. 4a). Severe pathological changes were manifest in the lungs (Fig. 4b), and more than 90% of infected rats died within 10 days after infection (Fig. 4c). Immunohistological examinations of the lung sections revealed that the target of the infection was restricted to the epithelial cells of the bronchi and bronchioles, i.e., not only Clara cells but also ciliated cells (data not shown). Virus was not recovered from any organs other than the lungs during

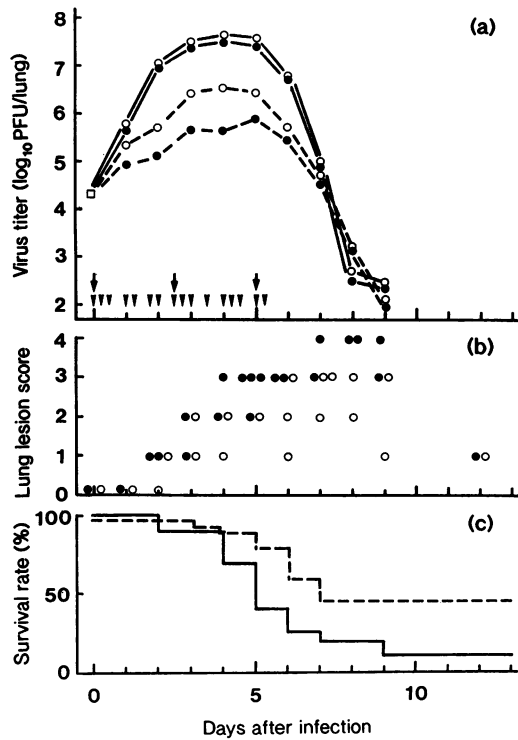


FIG. 4. Effects of anti-tryptase Clara antibody on viral replication in the lungs, pulmonary pathology, and mortality rates of rats infected with Sendai virus. Three-week-old rats were infected intranasally with 2×10^4 PFU of Sendai virus (\square). One group was administered 10-fold-diluted anti-tryptase Clara immunoglobulins intranasally (arrowheads) and intraperitoneally (arrows). (a) Viral replication and activation in the lungs in the absence (solid lines) or presence (broken lines) of the antibody. Lung homogenates were assayed differentially for total yield (\circ) and activated viruses (\bullet). There were 20 rats in the antibody-treated group and 21 in the untreated group. Each plot represents the mean value of two to four animals which had died or were sacrificed on the indicated days. (b) Lung pathology of infected rats without (\bullet) or with (\circ) administration of the antibody. Lung lesions were scored from 1 to 4 according to the extent of macroscopic consolidation of the lung surface. Each plot represents one of the rats described above. (c) Mortality rates of infected rats without (solid line) or with (broken line) administration of the antibody. Each group contained 22 rats.

the 2-week observation period. These findings were consistent with previous reports (3a, 35) and were essentially identical to those obtained for mice (27, 30, 33).

On the other hand, when anti-tryptase Clara antibody was administered intranasally and intraperitoneally to the infected rats, activation of the progeny virus in the lungs was reduced to 10 to 20% and viral replication was suppressed accordingly (Fig. 4a). Lung lesions were induced but only slightly compared with results for untreated animals (Fig. 4b). The mortality rate of infected rats was reduced, and half of the animals survived the normally fatal infection (Fig. 4c). When immunoglobulins from normal rabbits were administered, results identical to those described above for the control group were obtained (data not shown). These results indicate that tryptase Clara activates progeny virus in the lungs of rats and is involved in the pneumopathogenicity of Sendai virus in rats.

DISCUSSION

In this study, we have shown that tryptase Clara, present in rat lungs (17), is an activating protease for wild-type Sendai virus in the lungs. The protease was shown to cleave the F protein at the correct cleavage site, between residues Arg-116 and Phe-117, and to enhance infectivity of nonactive Sendai virus in vitro and in vivo. Therefore, with the aid of the protease, Sendai virus underwent multiple cycles of replication in the lungs and, as a result, produced extensive lung lesions as postulated previously (8, 14, 27, 29, 33). Since anti-tryptase Clara antibody was shown to suppress viral activation in the lungs to 10 to 20% of the activation in the control rats, it is concluded that tryptase Clara is a primary host protease that activates progeny virus in the lungs. The protease is therefore involved in the pulmonary pathogenicity of Sendai virus in rats.

Characterization of the protease has revealed that tryptase Clara is a novel serine-type endoprotease which cleaves preferentially arginine residues (17); its properties are similar to those of trypsin, which is an activating protease for Sendai virus in vitro (7, 9, 25). However, it differs from trypsin or trypsin-like proteases so far described with respect to molecular weight, subunit composition, serological reactivity, spectrum of protease inhibitors, and substrate specificity (17). Protease Clara requires several specific amino acid sequences around the arginine cleavage site, such as -Glu-Ser-Arg-, the sequence at the cleavage site of the Sendai virus F protein (2, 4, 10). The protease is inhibited by aprotinin and leupeptin, which have been shown to inhibit activation of Sendai virus and influenza virus in mouse lungs (6, 28, 37). The protease does not require Ca^{2+} ions for enzyme activity, which differentiates it from factor Xa, an activator of Sendai virus isolated from the allantoic and amniotic fluids of embryonated chicken eggs (5, 19). These characteristics of tryptase Clara are identical to the properties of an activating protease for Sendai virus that has been postulated to be present exclusively in the bronchial epithelium of mice (14, 27, 28, 31).

Immunohistological examinations have revealed that tryptase Clara is localized only in Clara cells of rat lungs (17) and is absent in other cell types in rat organs (23). These findings are consistent with the observation that if rat organ cultures other than lungs were infected with Sendai virus, progeny virus was produced mostly in the nonactivated form, similar to that found in mice (32, 33), suggesting the absence of Sendai virus-activating mechanisms in rat organs other than lungs (23). The distribution of tryptase Clara exclusively to the bronchial epithelium might therefore be responsible for the pneumotropism of Sendai virus in rats.

Tryptase Clara was also shown to be secreted into the bronchial lumen from Clara cells, and the secreted protein found in the bronchial lavage fluid was suggested to be a processed product (17). Extracellular administration of the antibody against the protease inhibited activation of the progeny virus in the lungs in vitro and in vivo to a large extent but not completely. These results taken collectively suggest that part of cleavage activation by tryptase Clara occurred extracellularly in the lumen of the respiratory tract. Since tryptase Clara was shown to be colocalized with the F protein in vesicles of infected Clara cells (23), the processing cleavage of F might also be expected to occur within the infected cells. The question remains, however, as to whether progeny virus grown in ciliated bronchial epithelial cells other than Clara cells that are also targets of Sendai virus infection (3a, 23, 27, 33) is produced in a nonactive form and

whether it is also activated extracellularly by trypsin Clara. An unidentified trypsin-like protease(s) may additionally be responsible for viral activation in the lungs. The latter possibility seems plausible from the finding that administration of anti-trypsin Clara antibody even at a high titer did not inhibit activation completely, although the antibody might not have reached the target regions efficiently. It is also supported by the previous observations that Sendai virus mutants, F1-R and T-9, with an amino acid exchange at the cleavage site, Arg-116 to Lys, are also activated, although less efficiently for the latter, in mouse lungs and undergo multiple cycles of replication (29, 30).

We have previously shown that when noninfectious virus with uncleaved F is inoculated intranasally into mice, the virus is not converted into an activated form in the lungs and infection will not ensue, suggesting the absence of a viral activating mechanism(s) inside the lumen of the respiratory tract (27). This finding is in contrast to the present observation of the extracellular activation of Sendai virus progeny by trypsin Clara. Our preliminary experiments suggest the presence of a potential inhibitor of trypsin Clara in the respiratory tract of rats (15). We propose that in the lungs of intact animals, trypsin Clara may be suppressed by the inhibitory substance and unable to activate incoming nonactive viruses, whereas once infection occurs, the inhibition might be released. Although the physiological role of trypsin Clara remains to be clarified, the antibody against the rat protease was shown to be cross-reactive with corresponding polypeptides in the lungs of mice and humans (16), suggesting that trypsin Clara is present in many animals and plays an essential role in the lungs. Like most proteolytic enzymes, trypsin Clara may also be regulated by inhibitors and activators. Such a regulation mechanism of the protease might be involved in the activation of Sendai virus in the lungs and thus modulate pulmonary pathogenicity.

Host protease-mediated viral activation and pathogenicity are also well documented for infections with avian influenza viruses (reviewed in references 18, 22, and 36). Bacterial proteases have also been shown to be involved in the pathogenesis of experimental influenza virus pneumonia in mice (1, 24, 26), suggesting insufficient viral activation in the lungs without exogenous proteases. However, the role of host proteases in the pathogenicity of mammalian influenza viruses awaits further investigation. Aprotinin, an inhibitor of trypsin Clara (17), has been shown to inhibit viral activation in mouse lungs (37). We have also demonstrated in vitro that trypsin Clara activates infectivity of a human influenza A virus by cleaving the hemagglutinin (17). Although the rat is not a natural host of influenza virus, this finding suggests that an activating protease like trypsin Clara must play an important role in determining the pathogenicity of influenza virus in humans.

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