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Pneumopathogenicity of a Sendai Virus Protease-Activation Mutant, TCs, Which Is Sensitive to Trypsin and Chymotrypsin

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A protease-activation mutant of Sendai virus, TCs, was isolated from a trypsin-resistant mutant, TR-5. TCs was activated in vitro by both trypsin and chymotrypsin. TCs was, however, less sensitive to trypsin and chymotrypsin than were the wild-type virus and TR-5, respectively. F protein of TCs had a single amino acid substitution at residue 114 from glutamine to arginine, resulting in the appearance of the new cleavage site for trypsin and the shift of the cleavage site for chymotrypsin. Activation of TCs in the lungs of mice occurred less efficiently than that of the wild type, and TCs caused a less severe pneumopathogenicity than did the wild-type virus, which supports our previous view that the in vitro trypsin sensitivity of Sendai virus can be a good indication of pneumopathogenicity in mice.

Sendai virus penetrates host cells through adsorption of HANA protein to the cellular receptors (19, 28) and fusion of the viral envelope with the plasma membrane which is mediated by F protein (5, 19). Since envelope fusion activity of F protein is acquired by posttranslational cleavage by certain proteases inherent to host cells (3, 14, 15, 20–23), the structure of F protein around the cleavage site is thought to be important for the virus to express infectivity and to determine host range and organ tropism. This view was verified by the fact that the wild-type virus whose F protein accepted the cleavage by trypsin next to arginine at residue 116 could multiply in the lungs of mice (24) in which trypsin like protease was present (23), while trypsin-resistant mutants TR-2 and TR-5 could not multiply because the arginine residue mutated to isoleucine and were not activated by the proteases in the lungs of mice (24, 25). The following report by Tashiro et al. (26) also supports the above-described view: a variant (F1-R) of Sendai virus which exhibited susceptibility to the activation by ubiquitous proteases as a result of the amino acid substitutions around the cleavage site and/or at the glycosylation site of the F_2 subunit caused a systemic infection in mice.

Recently, we isolated a variant of Sendai virus named TCs from the parent TR-5. As TCs was susceptible to both trypsin and chymotrypsin, we studied the mechanism of the activation of TCs by trypsin and the pneumopathogenicity of TCs in mice. The results obtained are presented in this report. The overall results will support our previous view that the in vitro trypsin sensitivity of Sendai virus correlates with pneumopathogenicity in mice.

TCs was isolated by plaquing the trypsin-resistant mutant TR-5 in the presence of 3 μg of trypsin per ml instead of chymotrypsin for TR-5 in the agar overlay. The sensitivity of TCs to activation by trypsin and chymotrypsin was compared with those of TR-5, the wild-type virus of the Fushimi strain of Sendai virus, and trypsin-sensitive revertant TSrev (isolated from TR-5, also the parent of TCs, by passaging the virus in the presence of 1 μg of trypsin per ml of the medium [8]). TCs formed plaques in the presence of both chymotrypsin and trypsin, unlike the wild-type virus and TSrev, which formed plaques only in the presence of trypsin (data not shown). To obtain more detailed information, multiple-cycle replication in the presence of trypsin and chymotrypsin (as well as elastase and plasmin at different concentrations) was studied (Fig. 1). The wild-type virus could be activated, as reported previously (7), by chymotrypsin in addition to trypsin (although a concentration as high as 2.5 μg/ml was required) but not by either elastase or plasmin. The protease sensitivity of TSrev-58 was exactly the same as that of the wild-type virus (data not shown). TR-5 was activated almost equally by chymotrypsin and elastase. Unlike the above-mentioned viruses, TCs could be activated by all of the four proteases. However, the minimum concentration of trypsin required for the activation of TCs was higher than those required for the wild-type virus and TSrev-58. The chymotrypsin susceptibility of TCs was also decreased compared with that of the parent TR-5.

In previous papers, we demonstrated that the recovery of the trypsin sensitivity and the decrease in the chymotrypsin sensitivity of TSrev was caused by a single amino acid reversion from isoleucine to arginine at the cleavage site (7, 8). The characteristic protease sensitivity of TCs prompted us to analyze its amino acid sequence. Cloning of cDNA and the identification of the clones containing sequences corresponding to the F gene were carried out according to the methods of Okayama and Berg (16) and of Sanger et al. (18), respectively, as described in a previous paper (8). A single mutation of G from A at position 394 was found in the F gene of TCs when compared with that of the parent TR-5 and was accompanied by an amino acid substitution from glutamine to arginine at residue 114 (Fig. 2). To determine the cleavage sites, the N-terminal ends of the F_1 subunit cleaved by trypsin and by chymotrypsin were sequenced as described previously (7). When TCs was activated by trypsin, an amino acid sequence of N-Ser-Ile-Phe-Phe-Gly-Ala-Val-Ile-Gly appeared at the N terminus of the F_1 subunit, indicating that the Arg substituted for Gln at residue 114 made a new target site of trypsin. From our results with the wild-type virus, TR, TSrev, and TCs, together with other results with trypsin-resistant variants of pa-c1 and pa-e2 which lacked Arg at residue 116 (6), the presence of Arg seems to be a prerequisite factor in determining the sensitivity of the F

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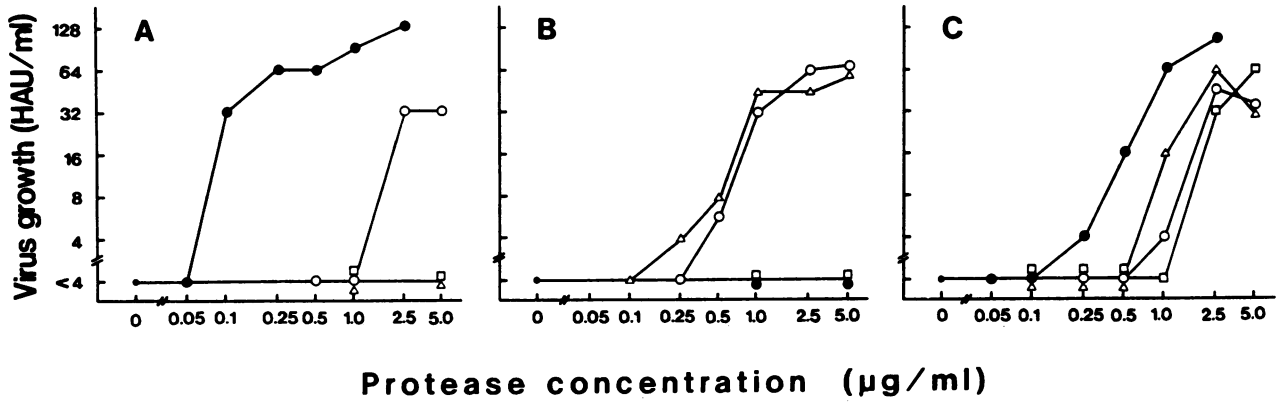

FIG. 1. Multiple-cycle replication of the wild-type (A), TR-5 (B), and TCs (C) viruses in the presence of trypsin (●), chymotrypsin (○), elastase (△), and plasmin (□). LLC-MK_2 cells were infected with each virus at a multiplicity of infection of 0.01 CIU per cell and incubated at 36°C in the presence of every one of the proteases, and the virus growth was monitored on the fifth day by measuring the hemagglutinin titer (8). HAU, Hemagglutinating units.

protein of Sendai virus to trypsin. On the other hand, when TCs was activated by chymotrypsin, a sequence starting with N-Gly-Ala-Val-Ile-Gly appeared at the N terminus of the F_1 subunit. Since the cleavage site of the F protein of TR-5 by chymotrypsin was the C-terminal side of Gln at residue 114 (7), the substitution of Arg for Gln at residue 114 was responsible for transition of the cleavage site for chymotrypsin from residue 114 to 118, which resulted in a slight decrease of the chymotrypsin sensitivity of TCs compared with that of TR-5. That fact shows that Gln is not necessarily the cleavage site of chymotrypsin and that the structure around the cleavage site may be important. As chymotrypsin is generally considered to cleave next to the aromatic amino acid, Phe at residue 117 or 118 would be a preferable cleavage site to Gln at residue 114. Therefore, some contortion in the structure of F protein is suggested to occur by the presence of Gln at residue 114 with the wild-type, TR-5, and TSrev-58 viruses other than TCs, so that it prevents chymotrypsin from cleaving next to Phe and affects the sensitivity of F protein to chymotrypsin. Although plasmin is generally considered to cleave after basic residues, Hsu et al. showed that an amino acid next to the basic residue had some influences on the plasmin activity for Sendai virus F protein because only pa-cp2 carrying an Arg-Ser sequence around the cleavage site permitted susceptibility to plasmin (6). Since TCs has the same Arg-Ser sequence at residues 114 to 115, plasmin may cleave the C-terminal side of this Arg. This possibility will be determined in the near future.

TSrev-58 as well as the wild-type Sendai virus underwent multiple-cycle replication in the lungs of mice and caused pneumonia (13). Since the amino acid structure of TCs around the cleavage site was different from those of the above-mentioned viruses and the spectrum of the sensitivity of TCs to various proteases was characteristic among the viruses studied, it was of great interest to examine whether TCs can undergo multiple-cycle replication in the lungs of mice and cause pneumonia. Both TCs and the wild-type virus were diluted serially in a fivefold manner and inoculated intranasally into mice. The animals were observed for symptoms and weighed daily. The rate of the body weight

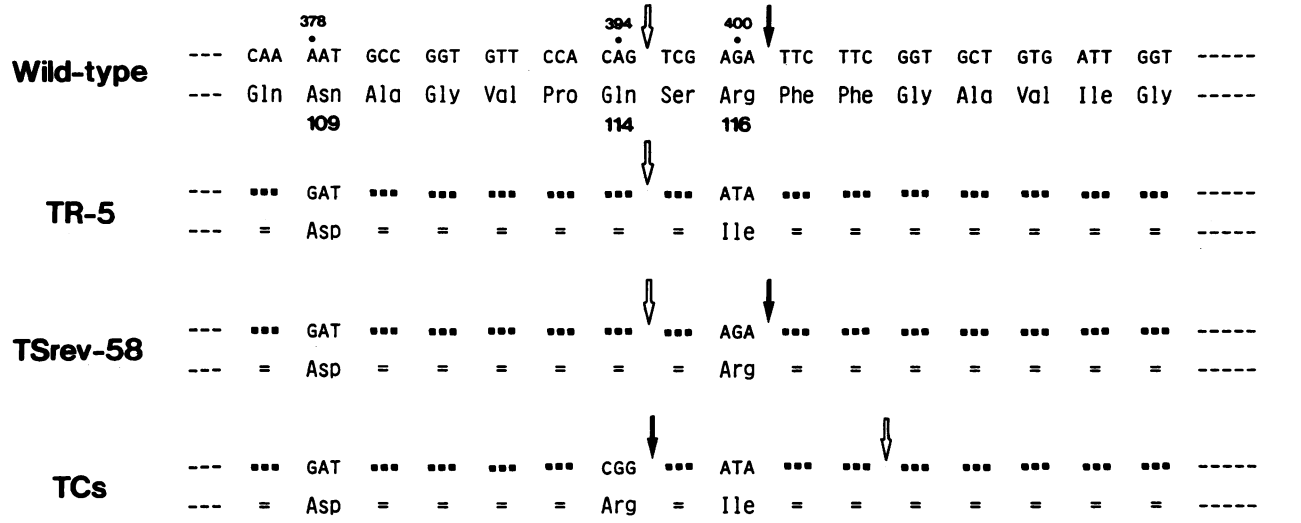

FIG. 2. Amino acid sequence around the cleavage site of F protein and the cleavage site by trypsin (↓) and chymotrypsin (⇩) of TCs in comparison with those of the wild-type virus, TR-5, and TSrev-58 (13, 14). Symbols (■ and =) indicate, respectively, the nucleotides and amino acid residues identical to those found in the wild-type virus.

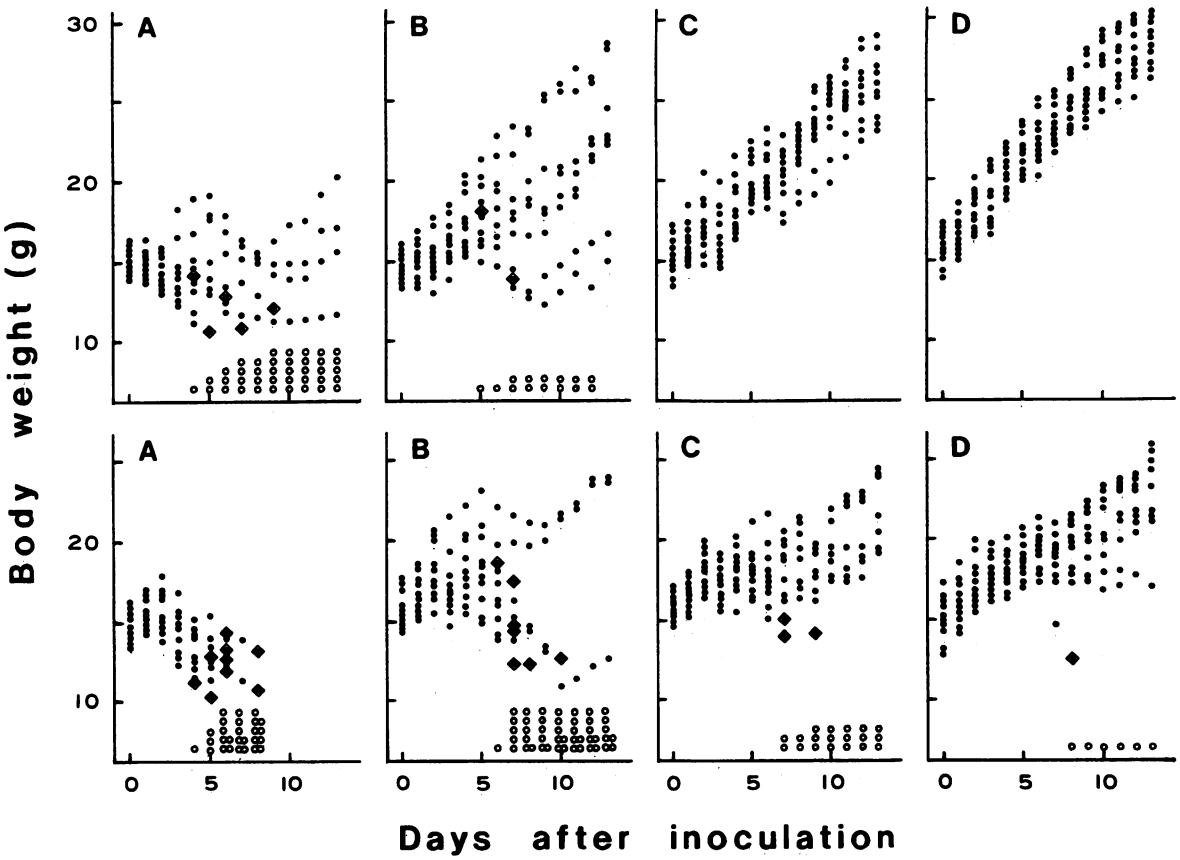

FIG. 3. Time course of the body weight of mice after inoculation with TCs (top) and the wild-type (bottom) of Sendai virus. Each virus was diluted fivefold serially, and samples (0.025 ml) were inoculated intranasally into mice. The inocula were 6.25×10^6 (A), 1.25×10^6 (B), 2.5×10^5 (C), and 5.00×10^4 (D) CIU per mouse. Symbols indicate the newly dead mice (◆) and accumulation of the dead mice (○).

decrease was lower in the mice inoculated with every dilution of TCs than in the animals inoculated with comparable dose of the wild-type virus (Fig. 3). All of the mice infected with 6.25×10^6 cell-infecting units (CIU) (9) of the wild-type virus died of pneumonia within 8 days. On the other hand, when infected with the same amount of TCs, four of nine mice survived. These results showed that TCs was less pathogenic than the wild-type virus. Since pneumopathogenicity of the wild-type Sendai virus in mice was shown to be directly connected with the virus replication in the lung (24), the growth of TCs in the lung was investigated. The mice inoculated with 3×10^6 CIU were sacrificed at certain intervals, and the infectivity of the supernatant of the lung homogenates was assayed by the following two different ways of modified fluorescent cell-counting method (9). The viral samples were adsorbed to the monolayer culture of MDCK cells (an established cell line of a canine kidney) at 4°C for 1 h. The cells were then fed with Eagle minimum essential medium with trypsin at 2 μg/ml for titration of both the active and inactive viruses (the whole virus) or without trypsin for titration of only the active virus and incubated for 30 min at 36°C. After washing, the cells were then refed with fresh Eagle minimum essential medium without trypsin, incubated for another 24 h at 36°C, fixed with 95% ethanol, and stained by the fluorescent antibody technique. The results are shown in Fig. 4. In the case of the wild-type virus, the increase of the infectivity was evident on day 5 after infection and the progeny viruses were found mostly in activated form. On the other hand, in the case of TCs, a gradual decrease of the infectivity was observed from the day after infection and the virus became undetectable after day 7. Moreover, only 5 to 30% of the progeny viruses were found to be in the activated form, suggesting that the activation proteases in the lungs of mice were less effective for the activation of the progeny virus of TCs than for activation of the progeny virus of the wild-type virus and that the inactive progeny virus would prevent the successive multiple-cycle replication which resulted in a gradual decrease of the infectivity in the lung.

The relationship between viral pathogenicity and cleavability of the viral glycoproteins by cleavage-activation proteases has been extensively studied with Newcastle disease and avian influenza viruses on the basis of the structural variations around the cleavage site. Generally, apathogenic strains had a single arginine around the cleavage site and were activated by proteases present only in limited host cells. On the contrary, pathogenic strains which caused systemic infections had a chain of basic residues around the cleavage site and could be activated by ubiquitous proteases (2, 4, 11, 27). By using a site-directed mutagenesis technique with a cDNA clone encoding the glycoprotein of influenza virus or simian virus 5, the number of basic amino acids around the cleavage site was shown to affect the cleavage efficiency by ubiquitous proteases in mammalian cells (10, 17). As for Sendai virus, a pantropic variant named F1-R has been isolated by Tashiro et al. and the cleavability of F protein by ubiquitous proteases was shown to be responsible for the pantropism (26). On the other hand, a series of our

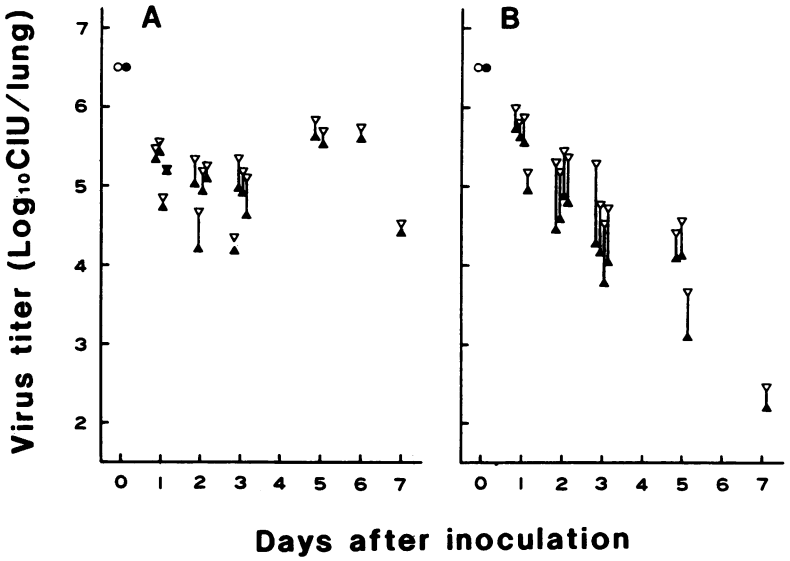

FIG. 4. Time course of the virus replication in the lungs of mice after infection with wild-type (A) and TCs (B) Sendai virus. The mice received each an inoculation of 0.025 ml of the virus containing 3×10^6 CIU. They were sacrificed on the indicated days, and 10% lung homogenates were prepared (24) for determination of the virus titer as described in the text. The whole virus (▽) and the activated virus (▲) recovered from each mouse are linked. Symbols indicate the levels of whole virus (○) and activated virus (●) in the inocula.

previous (13, 24) and present studies have clearly shown that the susceptibility of F protein to the cleavage-activation proteases in the lung is important in determining the pneumopathogenicity of Sendai virus in mice.

In the case of influenza virus, some strains were activated by plasmin in vitro (12, 29), and Akaike et al. indicated that the pathogenicity of influenza virus in mice was enhanced in parallel with the increase of the level of plasmin as a result of infection (1). Among a series of mutants of the Fushimi strain, only TCs was activated by plasmin in vitro, and we expected a potent pneumopathogenicity with TCs. However, this was not observed. TCs exhibited less sensitivity to in vitro activation by trypsin (Fig. 1) and less pneumopathogenicity (Fig. 3) than the wild-type virus. This fact reveals the importance of the in vitro trypsin sensitivity rather than plasmin sensitivity for pneumopathogenicity of Sendai virus in mice, which is in full accord with our previous view (13). Since a single nucleotide mutation of the F gene can alter the susceptibility to trypsin (8), a slight difference in the structure of F protein may generate variations in the susceptibility to the cleavage-activation proteases present in the lungs of mice and then affect the pneumopathogenicity. The fact that protease-activation mutants like TSrev and TCs were recovered from the same parent TR-5 by the use of different screening conditions suggests that such variants can easily emerge during passages in different host cells containing different proteases. Studies along this line with recent isolates of Sendai virus from moribund mice are in progress in our laboratory.

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