Changes in specific cleavability of the Sendai virus fusion protein: implications for pathogenicity in mice


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Sendai virus mutants, KDe-21 and KDe-62, which had undergone multiple cycles of replication in Madin Darby canine kidney (MDCK) cells in the absence of exogenous proteases were isolated. The fusion (F) protein of the mutants regained proteolytic cleavability in MDCK cells and chick embryos, but the F protein remained non-cleavable in other cell lines. Unlike the F protein of wild-type (wt) virus, the mutant F was resistant to trypsin but was sensitive to elastase and, to a lesser extent, to chymotrypsin. Sequence analyses of the F gene and the F protein revealed an amino acid substitution at the cleavage site, Arg(116) to Ile, which conferred trypsin resistance and enhanced cleavability at Ile(116) by elastase and host proteases present in MDCK cells and in chicken embryos. In contrast to the pneumopathogenicity in mice of wt Sendai virus, the KDe mutants were non-pathogenic; cleavage activation of the F protein did not occur in the lungs and thereby infection was terminated after an initial cycle of replication.

Sendai virus, the paramyxovirus prototype, is exclusively pneumotropic in mice. Post-translational proteolytic cleavage of the envelope fusion (F) glycoprotein into subunits F₁ and F₂ is a prerequisite for the virus to express infectivity (Homma & Ohuchi, 1973; Scheid & Choppin, 1974). The cleavage site at amino acid position 116 is composed of a single arginine residue, which is not cleaved by cellular proteases of most cell types, but is cleaved by trypsin (EC 3.4.21.4), a trypsin-like endoprotease present in embryonated chicken eggs (Gotoh et al., 1990; Muramatsu & Homma, 1980; Suzuki et al., 1991) and by host proteases in primary cell cultures (Seto et al., 1980; Shibuta et al., 1971; Silver et al., 1978) and in the lungs of mice (Tashiro & Homma, 1983a, b; Tashiro et al., 1990).

It has been demonstrated previously that cleavage activation of the F protein in the lungs of mice is a primary determinant for the pneumopathogenicity of Sendai virus (Itoh et al., 1990; Mochizuki et al., 1988; Tashiro & Homma, 1983a, b; Tashiro et al., 1990, 1991). Pantomotropic properties of a Sendai virus mutant, F1-R, correlate with enhanced cleavability of the F protein in various organs of mice in addition to the lungs, a property which is based on an amino acid exchange in the vicinity of the cleavage site (Tashiro et al., 1990, 1991). On the other hand, protease (elastase) activation mutants, TR-2, TR-5 and pa-c1, have F proteins which are cleavable by chymotrypsin (EC 3.4.21.1) and elastase (EC 3.4.21.36), but are resistant to trypsin and proteases present in various cell types and in chick embryos (Itoh et al., 1987; Hsu et al., 1987; Scheid & Choppin, 1976; Tashiro & Homma, 1983a, b). Infection by the mutants is restricted to a single cycle of replication in mouse lungs, since progeny virus remains non-infectious at this site (Itoh et al., 1990; Hsu et al., 1989; Mochizuki et al., 1988; Tashiro & Homma, 1983a, 1985; Tashiro et al., 1988a). These results indicate that the enzyme(s) in the lungs that cleaves wild-type (wt) F is similar, though not identical, to trypsin rather than to elastase and chymotrypsin, and that the appropriate proteases that activate the mutant F are absent in mouse lungs (Itoh et al., 1990; Tashiro & Homma, 1983a, b). Such protease activation mutants have been proposed as a model for live vaccines (Hsu et al., 1989; Tashiro & Homma, 1985; Tashiro et al., 1988a).

By serial passaging of Sendai virus (Z strain) in Madin Darby canine kidney (MDCK) cells in the absence of exogenous proteases in the culture medium, we have submitted the GenBank/EMBL/DDBJ Data Libraries and assigned the accession numbers M30202, M76994 and M77002.

The nucleotide sequence data reported in this paper have been submitted to the GenBank/EMBL/DDBJ Data Libraries and assigned the accession numbers M30202, M76994 and M77002.
isolated several mutants with pathogenic properties different to those of wt virus (Tashiro et al., 1992). Two of the mutants, designated KDe-21 and KDe-62, are described in detail herein. The KDe mutants were shown to undergo cleavage activation of the F protein in MDCK cells and were not activated in other cell types, such as LLC-MK2, Madin Derby bovine kidney (MDBK), Vero and L cells (Table 1). In contrast to wt virus, non-active progeny of the KDe mutants produced by a non-permissive cell type, such as LLC-MK2 cells, was not activated by trypsin in vitro but had an increased sensitivity to elastase and, less efficiently, to chymotrypsin, similarly to the elastase activation mutants TR-2, TR-5, pa-c1 and pa-e2 described previously (Itoh et al., 1987; Scheid & Choppin, 1976; Tashiro & Homma, 1983a). This was shown by multicycle replication experiments in the absence or presence of the different proteases in the culture medium (Fig. 1) and verified by SDS–PAGE analysis (data not shown). The F protein was found to be readily cleaved into F1 and F2 by elastase, but approximately 70% of the precursor F0 remained uncleaved after treatment with chymotrypsin (data not shown). In contrast to the other elastase mutants, however, the KDe mutants replicated in the chick embryo as efficiently as did the wt virus and underwent multiple cycles of replication in MDCK cells. Thus, the KDe mutants were found to have a host range different from that of wt virus and other elastase activation mutants described previously (Table 1).

Since the pathogenicity of Sendai virus and its mutants is primarily determined by the cleavability of the F protein, the pathogenic properties of the KDe mutants were investigated. Three-week-old male mice (ICR; CD-1) were infected intranasally with virus. After various time intervals lung homogenates were assayed by the differential plaque assay to quantify activated and non-activated viruses (Tashiro et al., 1988b). Although the KDe mutants infected the lungs of mice, the progeny virus, in contrast to that of wt virus, remained non-activated, and thereby infection was terminated after an initial cycle of replication resulting in marginal lung lesions (Fig. 2). These results suggest a lack of mutant F protein cleavage activation protease in mouse lungs. The non-pathogenic property of the KDe mutants, in contrast to that of wt virus, remained non-activated, and thereby infection was terminated after an initial cycle of replication resulting in marginal lung lesions (Fig. 2). These results suggest a lack of mutant F protein cleavage activation protease in mouse lungs. The non-pathogenic property of the KDe mutants was therefore similar to that found for the elastase activation mutants TR-2, TR-5 and pa-c1 (Hsu et al., 1989; Mochizuki et al., 1988; Tashiro & Homma, 1983a, 1985).

To investigate the genetic basis for the altered cleavability of the F protein of the KDe mutants, the nucleotide sequence of the F gene was determined by the dideoxynucleotide chain termination method using viral RNA as template (Middleton et al., 1990; Tashiro et al., 1988b). The deduced amino acid substitutions presented in Table 2 illustrate that the F proteins of the KDe mutants were shown to undergo cleavage activation of the F protein in MDCK cells and were not activated in other cell types, such as LLC-MK2, Madin Derby bovine kidney (MDBK), Vero and L cells (Table 1). In contrast to wt virus, non-active progeny of the KDe mutants produced by a non-permissive cell type, such as LLC-MK2 cells, was not activated by trypsin in vitro but had an increased sensitivity to elastase and, less efficiently, to chymotrypsin, similarly to the elastase activation mutants TR-2, TR-5, pa-c1 and pa-e2 described previously (Itoh et al., 1987; Scheid & Choppin, 1976; Tashiro & Homma, 1983a). This was shown by multicycle replication experiments in the absence or presence of the different proteases in the culture medium (Fig. 1) and verified by SDS–PAGE analysis (data not shown). The F protein was found to be readily cleaved into F1 and F2 by elastase, but approximately 70% of the precursor F0 remained uncleaved after treatment with chymotrypsin (data not shown). In contrast to the other elastase mutants, however, the KDe mutants replicated in the chick embryo as efficiently as did the wt virus and underwent multiple cycles of replication in MDCK cells. Thus, the KDe mutants were found to have a host range different from that of wt virus and other elastase activation mutants described previously (Table 1).

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**Table 1. Activation of Sendai virus in vivo and in vitro**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Egg</th>
<th>MDCK</th>
<th>MDBK</th>
<th>LLC-MK2</th>
<th>Vero</th>
<th>L</th>
<th>T</th>
<th>C</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KDe-21,62</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TR-2,5</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pa-c1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pa-e2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

* Virus was injected into the allantoic cavity of 10-day-old chicken eggs at 10^3 p.f.u. or added to tissue culture cells at an m.o.i. of 10^-3 p.f.u. per cell. After incubation for 4 days at 37°C, haemagglutination tests were done; +, ≥ 32 HA units/ml; ±, 4 to 16 HA units/ml; -, < 4 HA units/ml.

† LLC-MK2 cells or MDBK cells were infected at 10^-3 p.f.u. per cell and incubated for 4 days at 37°C in the presence of various concentrations of trypsin (O), chymotrypsin (●) and elastase (●) in the culture medium. After 96 h, the culture medium was assayed for HA activity.

‡ Data are from Scheid & Choppin (1976).
Fig. 2. Virus replication in the lung and lung lesions of mice infected with wt Sendai virus (a) and KDe-62 (b). Three-week-old mice (ICR strain) were infected intranasally with 10⁵ p.f.u. of virus (□). At the indicated time intervals, the virus titre in lung homogenates was assayed by the differential plaque method for total virus (○) and activated virus (●). Lung lesions are denoted by consolidation score according to the extent of macroscopic pathology (▲).

mutants have amino acid exchanges that differentiate them from the elastase mutants reported by others. The exchange of Arg(116) for Ile resulted in the loss of the basic cleavage site for trypsin, identical to that reported for TR-2, TR-5, pa-c1 and pa-c2 (Hsu et al., 1987; Itoh et al., 1987). Additional amino acid exchange, Ile(47) to Val and Lys(79) to Asn, occurred for KDe-21 and KDe-62, respectively. Whether these exchanges are a prerequisite for the enhanced cleavability by host proteases has yet to be determined.

Direct amino acid sequencing of the F₁ subunit of the KDe mutants grown in MDCK cells and in chick embryos revealed residues Phe-Phe-Gly-Ala-Val at the N terminus. The same findings were obtained after treatment of LLC-MK₂ cell-grown KDe mutants with elastase. This indicated that Ile(116) was a target of cleavage activation by elastase and by cellular proteases present in MDCK cells and in the allantoic membrane/fluid of chick embryos (Table 2). The lack of any changes in the F₁ subunit N terminus by cleavage of the F protein by these proteases is consistent with the observation that the KDe mutants had cell–cell fusion activity similar to that of wt virus with cleaved F protein (Fig. 3). Therefore, it should follow that the KDe mutants possess the same cleavage site as the pa-e2 mutant, although they have a different host range and the chymotrypsin cleavability of the F proteins differs (Hsu et al., 1987; Scheid & Choppin, 1976). On the other hand, the chymotrypsin cleavage site in TR-2, TR-5 and pa-c1 mutants has been revealed to be Gln(114) (Hsu et al., 1987; Itoh et al., 1987). This was also confirmed by amino acid sequence analysis of the F₁ subunit N terminus of the KDe mutants. These results suggest that the host proteases in MDCK cells and in chick embryos that activate the F protein of the KDe mutants are similar, although not identical, to elastase. They are

Table 2. Amino acid differences and activation cleavage sites of the F protein of Sendai viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>47</th>
<th>79</th>
<th>110</th>
<th>Amino acid residue of F protein</th>
<th>115</th>
<th>120</th>
<th>172</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt (Z)*</td>
<td>I</td>
<td>K</td>
<td>Q</td>
<td>N</td>
<td>A</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>KDe-21</td>
<td>V</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KDe-62</td>
<td>-</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wt (Fushima, RU)†</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TR-2‡</td>
<td>-</td>
<td>-</td>
<td>D</td>
<td>-</td>
<td>V</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TR-5§</td>
<td>-</td>
<td>-</td>
<td>D</td>
<td>-</td>
<td>V</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pa-c1‡</td>
<td>-</td>
<td>-</td>
<td>D</td>
<td>-</td>
<td>V</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pa-e2‡</td>
<td>-</td>
<td>-</td>
<td>D</td>
<td>-</td>
<td>V</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Amino acid residues different from wt virus (Z strain) are shown.
† Cleavage sites in vivo in chicken eggs (▼) and MDCK cells (▼), and in vitro by trypsin (○), chymotrypsin (●) and elastase (■).
‡ Data for wt (Fushima strain), TR-2 and TR-5 are from Itoh et al. (1987) and those for wt (RU strain), pa-c1 and pa-c2 are from Hsu et al. (1987).
§ The elastase cleavage site for TR-5 was not determined.
different, however, from trypsin, chymotrypsin and the clotting factor Xa present in the allantoic fluid of chick embryos which cleaves the wt virus F protein at Arg(116) (Gotoh et al., 1990; Muramatsu & Homma, 1980; Suzuki et al., 1991).

The results described above confirm the previous view that cleavability of the F protein by a trypsin-like serine protease(s) in mouse lungs is required for multiple cycles of wt virus replication and thus is responsible for pneumopathogenicity (Itoh et al., 1990; Mochizuki et al., 1988; Tashiro & Homma, 1983a, b). The lung protease(s) efficiently cleaves not only the single basic residue Arg(116) of wt F, but also the proline-directed monobasic cleavage site, Lys(116) of the pantropic mutant F1-R (Tashiro & Homma, 1983b; Tashiro et al., 1988b, 1990). In addition, the single basic residue with a decreased positive charge, Lys(116) of the pneumotropic revertants derived from F1-R, was also cleaved but less efficiently (Tashiro et al., 1991). On the other hand, the chymotrypsin cleavage site, Gln(114), and the elastase cleavage site, Ile(116), of the F proteins of TR-2, TR-5 and pa-cl including the KDe mutants are resistant to lung protease(s) (Hsu et al., 1989; Mochizuki et al., 1988, 1985). Elastase, which is present in neutrophils and is secreted by alveolar macrophages, would not cleave the F protein of the mutants in mouse lungs. The results provide evidence that elastase-like proteases are present in MDCK cells and in chick embryos and that the lung protease(s) for activation of wt F is different from chymotrypsin and elastase.

Although an identical exchange of Arg(116) to Ile, which confers trypsin resistance, occurred at the cleavage site of the KDe mutants and the other elastase activation mutants, the F protein of the KDe mutants was cleavable in MDCK cells and in chick embryos, in contrast to the resistance shown by other elastase mutants (Itoh & Homma, 1988; Itoh et al., 1987; Scheid & Choppin, 1976; Tashiro & Homma, 1983a, 1985). The KDe mutant F was also less sensitive to chymotrypsin than the F protein of TR-2, TR-5 and pa-cl. The differential cleavability of the F protein should therefore be influenced by the remaining amino acid differences (Table 2). The substitution in the vicinity of the cleavage site, Asn(109) to Asp, has been postulated to be involved in the cleavability of the pa-cl F protein by chymotrypsin (Hsu et al., 1987). Since no such exchange was found with the KDe mutants, it might be that the relatively high concentration of chymotrypsin needed for cleavage is required for activation of these mutants. It should be noted that higher concentrations of chymotrypsin can also activate wt virus (Fig. 1).

The exchange at the cleavage site, Arg(116) to Ile, has been suggested to confer both trypsin resistance and chymotrypsin sensitivity to TR-5 (Itoh & Homma, 1988). Amino acid differences at residue 112, Ala in the KDe mutants and Val in TR-2, TR-5, pa-cl and pa-e2, might influence the susceptibility to cellular proteases in MDCK cells and in chick embryos. The substitutions of
Val(47) and Asn(79), which occurred in KDe-21 and KDe-62, respectively, could also be responsible for enhanced cleavability by host proteases. It has been shown that amino acid exchanges removed from the cleavage site of Sendai virus F protein (Tashiro et al., 1992) and influenza virus haemagglutinin (HA) (for references see Orlich et al., 1990) could alter cleavability by host cell proteases, plasmin or bacterial proteases. Investigation with other mutants or by site-directed mutagenesis should clarify these questions. It would also be of interest to study whether elastase-like endoprotease(s) is present in host cells other than those described. If this were so, the host range of the elastase activation mutants could be extended, and they obviously could be obtained at relatively high frequency.

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References


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