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Mild proteolysis induces a ready-to-fuse state on Sendai virus envelope

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Abstract The Sendai virus fuses with host cell membranes in a pH-independent manner through an unknown mechanism. Here we report that mild trypsin pre-treatments of Sendai virions, for example 15 min at 4°C, give Sendai virions the ability to fuse at a rate up to 10-fold higher than control. By using human erythrocytes as host cell membranes, viral fusion was assessed by hemolysis as well as fluorescence dequenching of octadecyl rhodamine B chloride. The mild protease treatment strikingly shortens the lag time taken by the virus to start the fusion process. Similar data were obtained on reconstituted Sendai virus envelope. Among proteases, tested as fusion enhancer, trypsin is more effective than either endoproteinase Lys-C, chymotrypsin, or endoproteinase Arg-C. After removal of trypsin from treated virions the fusion rate enhancement remains for hours at room temperature. The lack of protease specificity, together with the impossibility to detect any new N-terminal products, suggests that only a small percentage of viral envelope components are cleaved, still a large enough number to set the envelope in a ready-to-fuse state.

Key words: Sendai virus; Enveloped virus; Membrane fusion; Protease; Fluorescence dequenching

1. Introduction

The early stage of infection of enveloped viruses starts by binding to cell-surface receptors and terminates by fusion of the viral envelope with cell membranes [1]. In the influenza virus the fusion is triggered by a well documented pH-induced conformational change of the HA glycoprotein [2]. The structural rearrangement occurs at pH 5.5 in the lumen of endosomes where after binding the virion is taken up. By lowering the pH a specific HA hydrophobic peptide passes from a shielded conformation to an exposed one, then it becomes inserted into the host-cell membrane [2]. In contrast to the HA-fusion mechanism, no molecular event triggering fusion has been described for those enveloped viruses such as Sendai virus which fuse directly with the host membrane, in a pH-independent manner [1,3–5].

The viral envelope of Sendai virus (SV), belonging to the paramyxoviridae family, bears two transmembrane glycoproteins, the hemagglutinin-neuraminidase (HN) and the fusion (F) glycoprotein [3]. The envelope is similar in structure and lipid composition to the cell membrane which the virion buds from. The HN glycoprotein binds the virion to the external cell surface via sialic acid recognition, then the F glycoprotein induces fusion between the viral envelope and the host-cell membrane [3–5]. F is produced as a unique inactive peptide F_0 which, once cleaved at Arg-116, becomes the active F_1-F_2 form [6]. As a consequence of the cleavage, a 26 amino acids long hydrophobic peptide is located at the F_1 N-terminus [3]. Many data indicate that this peptide plays a crucial role in the viral fusion process [3–5]. A previous study on the F topology with respect to the viral envelope rules out an insertion of this hydrophobic peptide in the viral envelope [7]. Therefore the fusion peptide, in analogy with the influenza virus, seems to be buried in a portion of the F glycoprotein which protrudes out of the viral envelope. What, if anything, determines the insertion in the host cell membrane is completely unknown. The hypothesis that a proteolytic activity may cause fusion was proposed long ago [8]. Recent data, showing that a protease of host origin seems associated with F glycoprotein [9], give new strength to this hypothesis. On the other hand, the proteases play a crucial role in many steps of SV infection, since they not only operate post-translational proteolytic activation on mature viral particles [3], but also determine SV organ tropism [10,11].

Here we show that SV, after mild proteolytic treatments, dramatically enhances the fusion rate with erythrocyte membranes. The lag time occurring after the binding of treated virus and before its fusion is drastically shortened.

2. Materials and methods

2.1. Virus

Sendai virus, Z strain, was propagated in 10-day-old chicken embryos. After 72 h, the virus was harvested and purified by differential centrifugation as in [12].

2.2. Hemolytic assay

SV hemolytic activity was detected by incubating in 20 mM Tris-HCl, 140 mM NaCl (TBS) pH 7.4, at the temperatures indicated in the single experiment descriptions, viral particles with 2% (v/v) human red blood cells (type 0, Rh+), obtained from Centro Trasfusionale, Università La Sapienza, Rome from healthy donors who had given informed consent. Samples were then diluted 5-fold with cold TBS, pH 7.4, and intact cells were removed by centrifugation in a bench centrifuge. The released hemoglobin was evaluated as supernatant absorbance at 413 nm [12]. 100% hemolysis was determined by addition of Triton X-100, 1% final concentration, to an identically treated sample.

2.3. Ghost preparation

Ghosts were prepared by hypotonic lysis of human erythrocytes in 5 mM sodium phosphate, pH 8.0 at 4°C, as in [13]. The amount of ghosts is expressed as mg of protein.

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Abbreviations: BPTI, bovine pancreas trypsin inhibitor; F, fusion protein; FRE, fusion rate enhancement; HN, hemagglutinin-neuraminidase; RBC, human red blood cells; RSVE, reconstituted Sendai virus envelope; R18, octadecyl rhodamine B chloride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SV, Sendai virus; SVtry, Sendai virions pre-treated with trypsin; TBS, TRIS-buffered saline

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2.4. Octadecyl rhodamine B chloride (R18) dequenching assay

The assay is based on the properties of R18 fluorescent probe. Because of the lipid nature of the R18, when incubated with enveloped viral particles, it becomes firmly embedded into the viral envelope. If the loading occurs at a high probe concentration, the fluorescence is quenched. Upon fusion, the mixing of the viral envelope lipids with the lipids of erythrocyte ghosts brings about the dilution of R18 which goes along with an increase in fluorescence (dequenching). Therefore, by a continuous monitoring of the fluorescence intensity increase, we followed the fusion kinetics. Virus labeling with R18 was performed as in [14]. Fusion between labeled virus and erythrocyte ghosts was continuously detected as fluorescence increase (excitation at 560 nm, emission at 590 nm) by a Perkin-Elmer LS-5B spectrofluorometer. Fusion was initiated by mixing, directly in the spectrofluorometric cuvette, the labeled virus with 1 mg ghost in 3 ml of TBS at pH 7.4. The fluorescence detected by addition of Triton X-100 (1% final concentration) represents 100% dequenching.

2.5. Trypsin removal by affinity chromatography

Trypsin removal was achieved by affinity chromatography on a Sepharose 4B CNBr resin (Pharmacia) conjugated to bovine pancreas trypsin inhibitor (BPTI). The conjugation procedure was derived from that described in [15]. Briefly, 1 ml of drained Sepharose 4B CNBr was incubated at 4°C for 14 h with 5 mg of BPTI under gentle stirring in 5 ml of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3. At the end of the incubation period the reaction was blocked by adding 0.2 M glycine at pH 8.0. 3.2 mg of BPTI resulted, covalently conjugated to 1 ml of drained gel. Trypsin activity was evaluated with the chromogenic trypsin substrate N_α-benzoyl-DL-arginine-4-nitro-anilide hydrochloride (BAPNA) [16]. Briefly, 200 μl of the sample to be tested was mixed with 0.2 mg of BAPNA in a final volume of 1.2 ml of TBS, pH 7.8. After incubation at 25°C for 15 min, the reaction was stopped by adding 0.8 ml of acetic acid 30% (v/v) and the absorbance at 410 nm was measured.

2.6. N-terminal determination

After treatment with trypsin (30 min at 4°C) viral proteins were separated by SDS-PAGE under reducing conditions, and blotted onto Immobilon membrane (Millipore). Protein bands were stained with Coomassie blue R. HN and F bands were excised and subjected to amino-terminal sequencing in an Applied Biosystems liquid phase sequencer 477A.

2.7. Chemicals

Trypsin, TPCK treated, and BPTI were purchased from Merck. Endoproteinases Arg-C (endo-Arg) and Lys-C (endo-Lys), sequencing grade, were from Boehringer Mannheim. R18 was from Molecular Probes Europe BV. Triton X-100 and Surfactant-Amps purified detergents were obtained from Pierce Chemical Co. All other reagents were purchased locally from Sigma-Aldrich.

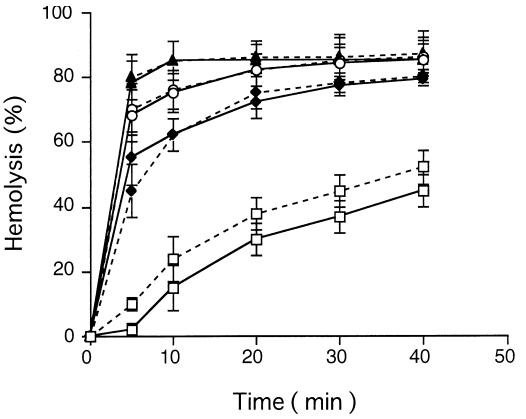

Fig. 1. Effect of trypsin pre-treatment on the kinetics of Sendai virus hemolytic activity. Either 500 μg of Sendai virus or 50 μg of reconstituted Sendai virus envelope (RSVE) was incubated at 4°C for 15 min with or without 1% trypsin (w/w) in 1 ml TBS pH 7.8; at the end of the incubation period trypsin was inhibited by 2-fold weight excess of BPTI. The hemolysis was performed by incubating 1 μg Sendai virus or 0.1 μg RSVE with 200 μl of 2% human erythrocytes suspended in TBS pH 7.4 at (-□-) 25°C: virus; (--□--) 25°C: RSVE; (-◆-) 25°C: virus preincubated with trypsin; (--◆--) 25°C: RSVE preincubated with trypsin; (-○-) 37°C: virus; (--○--)37°C: RSVE; (-▲-) 37°C: virus preincubated with trypsin; (--▲--) 37°C: RSVE preincubated with trypsin. The hemolysis was stopped at the indicated time by adding 0.8 ml of chilled TBS pH 7.4 and determined as described in Section 2. The bars represent the standard deviations calculated from the data of five different experiments performed with four different batch preparations.

3. Results and discussion

Previous works have shown that trypsin treatments of mature infective SV at 37°C for 1 h split the F_1 subunit into the two fragments F_{32} (32 kDa) and F_{19} (19 kDa) [7,17], resulting in F inactivation and loss of the fusogenic property of SV. The digestion with trypsin is restricted to F as the HN resists this protease. Surprisingly, during the course of a study using limited proteolysis of F, we found that brief pre-treatment of SV with trypsin results in a fusion rate enhancement (FRE). Fig. 1 shows that trypsin treatment of SV (SVtry), 15 min at 4°C, dramatically increases the SV hemolytic activity. After 5 min hemolysis is consistently one order of magnitude higher than in the control. At 37°C the hemolytic activity of SVtry

Table 1
The effect of trypsin removal on the duration of the fusion rate enhancement property acquired by trypsin-pre-treated Sendai virions

Virus	Trypsin	BPTI-Seph.	% Trypsin activity[a]	Recovered hemolytic units[c] ± S.D.[d] after incubation at 25°C			
				10 min	2 h	8 h	18 h
+	−	−	0.0	1[c]	0.88 ± 0.1	0.77 ± 0.2	0.57 ± 0.2
+	+	−	100	5.4 ± 0.7	4.9 ± 1.1	2.6 ± 0.7	0.61 ± 0.2
	+	+	0.0	nd	nd	nd	nd
−	+	−	65 ± 8.0[b]	nd	nd	nd	nd
−	+	+	0.0	nd	nd	nd	nd
+	−	+	0.0	0.92 ± 0.2	0.90 ± 0.2	0.83 ± 0.2	0.65 ± 0.2

nd, not determined.
[a]100% of trypsin activity is that resulting after incubation at 4°C for 10 min of 5 μg of trypsin with 500 μg of Sendai virions in 1.0 ml 20 mM Tris-HCl, 140 mM NaCl, pH 7.8, incubated for 5 min at 4°C with 50 μl drained Sepharose 4B gel, then rapidly passed through a Poly-Prep column (Bio-Rad) in a cold room. Aliquots of 200 μl were assayed in triplicate for trypsin activity as described in Section 2. 0.0% of trypsin activity results when the above described trypsin-virus mixture is incubated with 50 μl of drained Sepharose 4B containing covalently conjugated 160 μg bpti.
[b]Incubation of trypsin in the condition described above produces autoproteolysis.
[c]Hemolytic unit is the hemolysis produced upon incubation at 25°C for 10 min of 1 μg of Sendai virus with 200 μl of 2% human erythrocytes, the hemolysis is stopped by adding 0.8 ml of chilled TBS pH 7.4 and determined as described in Section 2.
[d]The standard deviations are calculated from five different experiment using four different batch virus preparations.

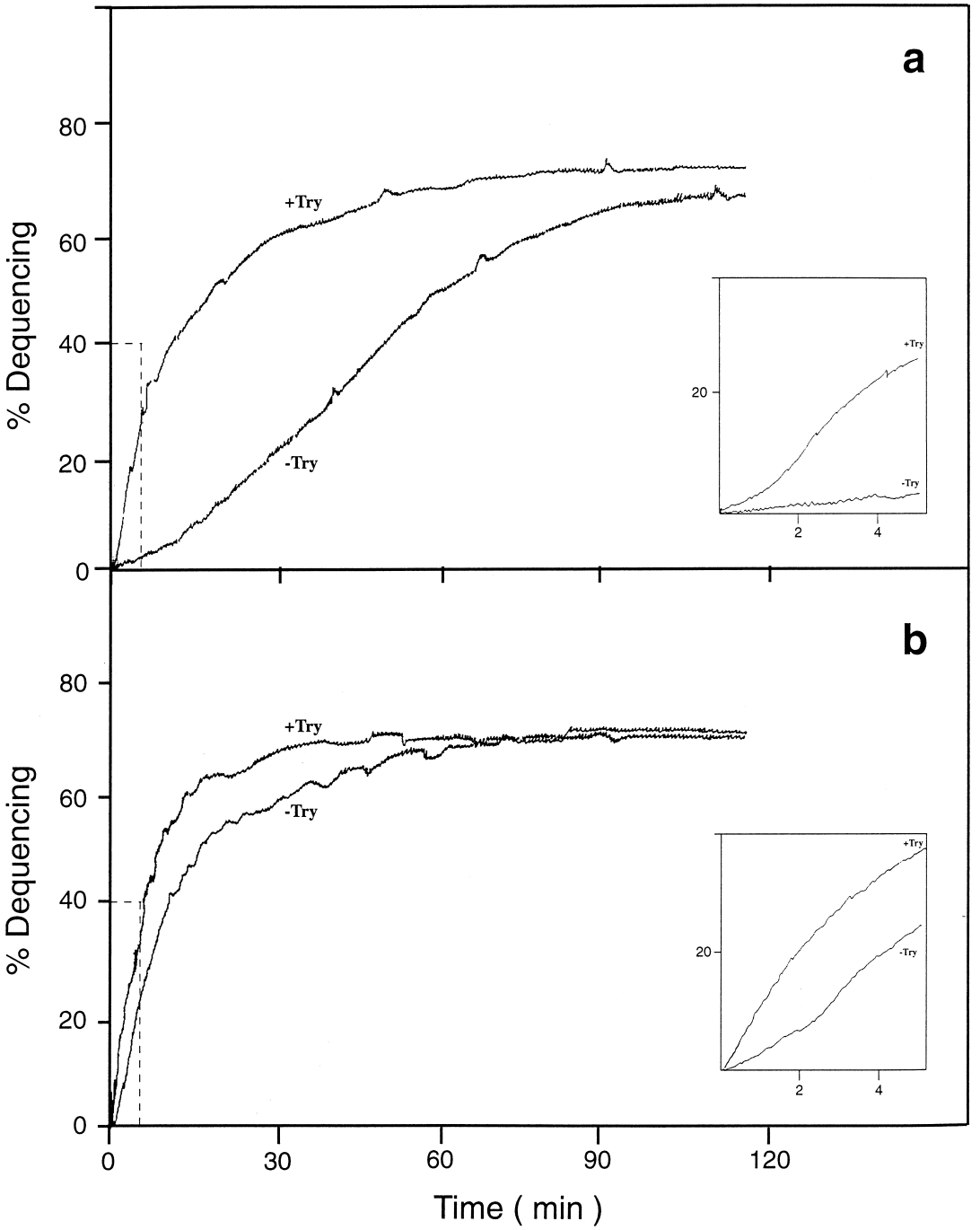

Fig. 2. Effect of trypsin pre-treatment on the kinetics of fluorescence dequenching of R18-labeled Sendai virions upon fusion with erythrocyte ghost membranes at (a) 25°C or (b) 37°C. Sendai virus particles were first tagged with R18 as reported in Section 2, then incubated with 1% trypsin (w/w) at 4°C for 15 min in TBS pH 7.8; at the end of the incubation period trypsin was inhibited by 2-fold weight excess of BPTI. The fluorescence dequenching was continuously monitored upon incubation with 1 mg human erythrocyte ghosts of 5 μg R18-labeled virions either pre-treated with trypsin (+Try) or not treated (−Try) as described in Section 2. The figure is representative of one out of three distinct experiments performed with three different batch preparations.

appears not significantly higher than untreated SV. Furthermore, Fig. 1 shows that similar data are obtained by performing identical experiments on reconstituted Sendai virus envelope (RSVE), prepared as reported by Moscufo et al. [7]. This indicates that only the Sendai envelope proteins are involved in the FRE phenomenon.

To document the FRE in the first minutes of SV fusion we used the R18 fluorescence dequenching method (the rationale of the R18 assay is reported in Section 2). A typical experiment obtained by monitoring fusion of R18-labeled SV with erythrocytes ghosts is depicted in Fig. 2a,b. The fluorescence dequenching was recorded both at 25°C (Fig. 2a) and at 37°C (Fig. 2b). SV fusion starts being detectable after a lag time of 2 min at 37°C (Fig. 2b) and after 5 min at 25°C (Fig. 2a). After mild trypsin treatment of SV the lag time appears to be abolished at 37°C (Fig. 2b) or reduced to 2 min when the

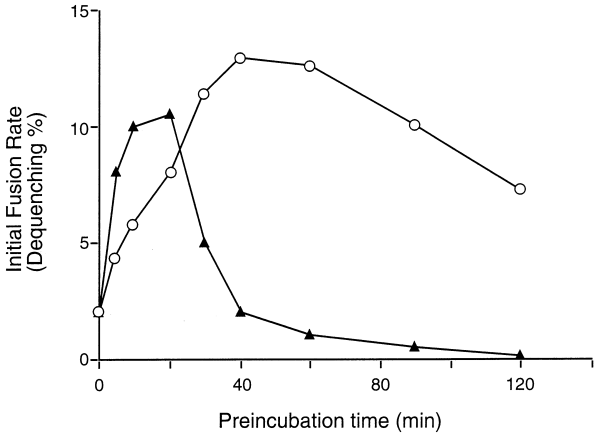

Fig. 3. Initial fusion rate of Sendai virus with erythrocyte ghosts is affected by the pre-incubation time and temperature conditions of trypsin. Sendai virions were first tagged with R18, then incubated with 1% (w/w) trypsin in TBS pH 7.8 either at 25°C (▲) or at 4°C (○), for the time period indicated in the figure. At the end of incubation, trypsin was inhibited by 2-fold weight excess of BPTI. The initial fusion rate was calculated by the slope of fluorescence dequenching continuously monitored upon incubation of 5 μg R18-labeled virus with 1 mg protein of human erythrocyte ghost membranes as described in Section 2. The single data numbers represent the average of three different experiments performed using three batch virus preparations, maximal variations was within 12%.

assay is performed at 25°C (Fig. 2a). Remarkably, at 25°C the fusion kinetics of SVtry closely resemble that of SV at 37°C, the optimal temperature for SV fusion. At 37°C the maximum dequenching was reached in approximately 30 min with SVtry, half the time compared to untreated SV (Fig. 2b). Previous work has reported that effective SV receptors (100–200) are about 10% of total SV particles bound to the erythrocyte membrane [13]. Therefore the FRE might be due to an increase in the number of virions able to fuse per single erythrocyte ghost. Since the fluorescence dequenching reaches the same maximum value for both SV and SVtry, we infer that FRE is not correlated with the increase of effective membrane receptors.

The fact that mild proteolysis reduces the lag time to such an extent suggests that mild proteolysis induces in SV a ready-to-fuse state as low pH does in influenza virus, where under low pH the viral fusion takes about 1 s [18].

A possible involvement of trypsin molecules, present during the fusion assay, could not be ruled out, therefore experimental procedures were designed to remove trypsin by BTPI affinity chromatography. Table 1 shows that after complete trypsin removal SVtry maintains intact its ability to fuse at a higher rate. Furthermore, Table 1 shows that SVtry loses the FRE property after incubation at 25°C for 18 h. These results indicate that trypsin produces FRE by acting on SV prior to SV-target membrane interaction and that FRE is a transient acquired property of SVtry.

Characterization of FRE has been carried out with respect to the temperature and time parameters. Fig. 3 shows that maximum FRE is obtained by pre-treatment of SV with 1% trypsin for 20 and 40 min at 25°C and 4°C, respectively, as determined by R18 dequenching assay. By incubating the SV with trypsin for a longer period of time, a decrease of FRE, followed by fusion inactivation, is observed. The latter is the result of specific cleavage of the F_1 subunit into the two fragments F_{32} (32 kDa) and F_{19} (19 kDa) [7,17]. However, in the conditions used for producing FRE, the SDS-PAGE Coomassie pattern is identical to that of the native virus. To further establish whether new N-termini were produced under conditions that elicit FRE, N-terminus determination was performed on F and HN extracted from SVtry. Also in this case no differences were detected with respect to the untreated viral particles. These findings seem to imply a very low level of proteolysis of SV envelope proteins, still able to induce the FRE phenomenon. For example, if the cleaved envelope proteins are less in number than the limit of detectable amount of the assay (2–4%), the lack of proteolytic evidence is feasible. How the cleavage of a limited number of proteins might affect the SV fusion process is completely obscure. In an attempt to explain it, we note that, if proteolysis of a limited number of envelope glycoproteins increases their lateral mobility, this might facilitate the fusion process as suggested by Henis et al. [19].

To get some insight into the nature of the cleavage site, we treated SV with either chymotrypsin, endoproteinases Lys-C or Arg-C and analyzed their ability to induce FRE. As shown in Table 2, Lys-C is a strong enhancer of SV rate fusion although weaker than trypsin. Arg-C also shows a slight enhancing property. Interestingly, chymotrypsin, which in native virus specifically directs its action to the HN glycoprotein, is able to cause a 3-fold increase in SV hemolytic activity. The

Table 2
Different proteases produce an enhancement of Sendai virus rate of hemolysis

Protease	Protease/virus ratio (%, w/w)	Hemolytic units ± S.D.[d]
None	–	1
Trypsin[a]	1	5.9 ± 0.6
Chymotrypsin[a]	1.25	2.7 ± 1.1
Lys-C[b]	1.25	4.7 ± 0.9
Arg-C[c]	3.15	1.8 ± 0.4

Sendai virions were incubated at 25°C for 20 min at the indicated virus/protease ratio, except for trypsin, which was incubated at 4°C.
[a]Incubation was performed in TBS pH 7.8. Trypsin and chymotrypsin were inhibited with BPTI at 2-fold weight excess.
[b]Incubation was performed in TBS pH 8.4. Endoproteinase Lys-C was inhibited with 2-fold weight excess of leupeptin.
[c]Before incubation with Sendai virions endoproteinase Arg-C was first incubated with 0.5 mM DTT, 1 mM $CaCl_2$ in TBS pH 7.8, then diluted in TBS pH 7.8 containing Sendai virus to get the protease/virus weight ratio indicated. The Arg-C was inhibited by adding $HgCl_2$ to 10 mM final concentration.
[d]Hemolytic units were assayed as described in Table 1 but first it was assessed that each buffer solution used for individual proteases did not affect the virus-induced hemolysis.
The standard deviation was calculated from the data of five different experiments using four batch virus preparations.

lack of protease specificity suggests that an entire polypeptide rather than a particular site might be involved in FRE induction.

In conclusion, after mild proteolysis treatments, SV virions acquire an ability to fuse, as though they had already circumvented the rate-limiting step of the entire fusion process. Therefore this treatment might be helpful to study the intermediates of the SV fusion reaction.

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