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A POSSIBLE INVOLVEMENT OF VIRUS-ASSOCIATED PROTEASE IN THE FUSION OF SENDAI VIRUS ENVELOPES WITH HUMAN ERYTHROCYTES

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A proteolytic activity is shown to be associated with relatively purified preparations of intact Sendai virus particles or with their reconstituted envelopes which are vesicles containing mainly the viral glycoproteins. Intact Sendai virus as well as reconstituted Sendai virus envelopes have been shown to be able to hydrolyze various protein molecules such as the human erythrocyte membrane polypeptide designated as band 3 and soluble polypeptides such as histone and insulin B-chain. The results of the present work raise the possibility that a direct correlation exists between the virus-associated proteolytic activity and the ability of the virions to lyse cells, to fuse with their membranes, and to promote cell-cell fusion. Inhibitors of proteolytic enzymes such as phenylmethylsulfonyl fluoride, tosyllysinechloromethylketone and tosylamidephenylethylchloromethylketone, or combinations thereof, inhibit the virus-associated proteolytic activity concomitantly with inhibition of its hemolytic and fusogenic activities. Electron microscopic studies showed that the various inhibitors did not affect the binding ability of the virus preparations. The possible involvement of a protease in the process of virus-membrane fusion is discussed.

Introduction

Adsorption of enveloped viruses to host cell surfaces is considered to be specific, since it is mediated mainly by carbohydrate residues of membrane glycoproteins [1]. In some cases surface glycolipids have also been suggested to serve as virus receptors [2]. Following adsorption, the viral components can be introduced into the cell interior either after endocytosis of the whole virus particle [3] or via a virus-cell fusion process [4]. It is not surprising that enveloped viruses can be endocytised by living cells. Attachment of almost any particulate material to a variety of cell surface groups triggers a process of endocytosis at the end of which the absorbed material is enclosed within intracellular endocytic vacuoles [5]. On the other hand, fusion of virus envelopes with cell-plasma membranes is not a self-evident process and its details are still obscure.

Enveloped viruses belonging to the paramyxovirus group, among them Sendai virus, fuse with cell-plasma membranes at pH 7.4, a process which is followed by an increase in the leakage of small molecules and cell lysis [4]. Binding of Sendai virus particles to cell-surface receptors is mediated by the viral hemagglutinin/neuraminidase, while fusion of the viral envelope with the cell-plasma membrane is promoted by the viral fusion factor [1,6]. Both are glycoproteins which appear as

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; TLCK, tosyllysinechloromethylketone; TPCK, tosylamidephenylethylchloromethylketone; [^{3}H]H_2DIDS, 4,4′-diisothiocyano-2,2′-ditritiostilbenedisulfonic acid.

'spikes' of 70 Å length on the outer surface of the viral envelope. Numerous studies have shown that sialic acid residues of membrane glycoproteins and, to a lesser extent, of membrane glycolipids, serve as receptors of Sendai virus particles [1,4,6].

From previous studies [7], it is clear that the sialic acid-containing membrane glycoproteins are exposed on the outer side of the cell-plasma membrane, the polypeptides of which are about 100–150 Å in length. Therefore, it is conceivable that after attachment, the bilayer of the Sendai virus envelope should be about 150–200 Å apart from the bilayer of the cell-plasma membrane. Indeed, this was confirmed by electron microscopy studies [8,9]. Evidently, in order to fuse with the cell membrane, the viral envelope must 'cross' the crowded layer of the cell-surface glycoproteins which avoids direct contact between the viral and the cell-plasma membrane bilayers. It was suggested that removal of surface glycoproteins may be required in order to render the cell bilayer susceptible to the action of the viral fusion factor [4].

It has been proposed before that hydrolytic enzymes and especially proteases may be involved in the process of virus-cell fusion [10,11]. Following attachment, a virus-associated protease may locally hydrolyze cell-surface glycoproteins and thus unmask or expose the targeted membrane bilayer to the action of the viral fusion factor. A similar mechanism has been suggested previously for penetration of phages to bacteria [12].

In the present work we have shown that inhibitors of proteolytic enzymes such as PMSF, TLCK or TPCK, or a combination thereof, inhibit induction of lysis of human erythrocytes, promotion of cell-cell fusion and infection of embryonated eggs by Sendai virus. Furthermore, it has been shown that a proteolytic activity is associated with intact virus particles and with its reconstituted envelopes.

Inhibition of the virus-associated proteolytic activity is accompanied by a concomitant inhibition of the virus' hemolytic and fusogenic activities but not if its hemagglutinating and binding abilities.

Materials and Methods

Cells. Human blood type O, recently outdated, was washed three times in Solution A (160 mM NaCl buffered with 20 mM Tricine, pH 7.4), as previously described [13]. Human erythrocytes were radioactively labeled with 4,4′-diisothiocyano-2,2′-ditritiostilbene-disulfonic acid ($[^3H]H_2$DIDS) as described before [14].

Virus. Sendai virus was propagated, harvested, suspended in Solution A, and its hemagglutination, hemolytic and fusogenic activities were determined as described before [13].

Preparation of fusogenic reconstituted Sendai virus envelopes. Reconstituted Sendai virus envelopes were prepared essentially as previously described [15]. Triton X-100 was added to a suspension of Sendai virus particles to give a detergent : virus ratio of 2 : 1 (w/w). After 60 min incubation at room temperature, the detergent-solubilized viral preparation was centrifuged and the clear supernatant obtained contained the two main viral envelope glycoproteins. Triton X-100 was removed by dialysis for 72 h in Spectrapor membrane tubing (12000–14000 M.W. cut-off, Spectrum Medical Industries, Inc.), at the end of which fusogenic-reconstituted viral envelopes were collected by centrifugation.

Degradation of ^{125}I-labeled insulin B-chain by Sendai virus or by reconstituted Sendai virus envelopes. Insulin B-cahin was prepared and radioiodinated using $Na^{125}I$ by the iodine monochloride (ICL) method, as described by Kenny [16]. The specific activity of the [^{125}I]insulin B-chain was $2–4 \cdot 10^7$ cpm/mg protein.

Intact Sendai virus particles or reconstituted Sendai virus envelopes (up to 100 μg protein) were incubated at 37°C with 10 μg ^{125}I-labeled insulin in a final volume of 100 μl of Solution A. Proteolysis was terminated by the addition of 200 μl trichloroacetic acid (25%, w/v) and 25 μl casein (2%, w/v) as a carrier protein. After a 10 min incubation at 4°C, the suspension was centrifuged and the amount of radioactive material in the trichloroacetic acid soluble fraction was estimated in a gamma counter (Packard). The amount of ^{125}I-labeled insulin added to the system was considered 100%. The total amount of radioactive material found in the trichloroacetic acid-soluble fraction of control systems incubated in the absence of Sendai virus preparations never exceeded 2% of the total added radioactive material. Hydrolysis was estimated as described previously [16].

Radioiodination of histone. Histone (Type VII-S calf thymus, Sigma) was dissolved in water to give a concentration of 10 mg/ml. In order to inactivate any possible proteolytic activity associated with the histone preparation, the pH was adjusted to 2.0 and the resulting solution was incubated for 1 h at 70°C. At the end of the incubation period, the pH was readjusted to 7.4 by K_2HPO_4, the final concentration of which was 0.2 M.

Radioiodination of histones was performed by using chloramine-T and $Na^{125}I$, as described by Lefkowitz [17]. Briefly, the histone preparation (2–3 mg protein) was incubated with 400 μCi $Na^{125}I$ (carrier-free, Nuclear Research Center, Negev, Israel) and 0.2–0.5 mg chloramine-T, in a final volume of 1 ml of 0.2 M K_2HPO_4, pH 7.4. After 2 min at 4°C, the iodination reaction was terminated by the addition of 1 mg $N_2S_2O_5$ and 1 mg KI. The ^{125}I-labeled histone preparation obtained was dialyzed against Solution A for 36 h at 4°C, at the end of which the protein concentration was 1–2 mg/ml. The specific activity of the ^{125}I-labeled histone was about $1–2 \cdot 10^7$ cpm/mg protein.

Degradation of ^{125}I-labeled histone by Sendai virus. Sendai virus particles (up to 100 μg protein) were incubated, with gentle shaking, with 10 μg ^{125}I-labeled histone, in a final volume of 100 μl Solution A, at 37°C for 3–5 h. Proteolysis was terminated by the addition of 100 μl SDS-containing solubilization buffer (to give a final concentration of 0.1 M Tris-HCl, pH 6.8/1% SDS/10% glycerol/5% β-mercaptoethanol/0.015% pyronine-y). Samples were analyzed by slab gel electrophoresis (15% acrylamide) [18].

Degradation of the histone bands and accumulation of radioactively-labeled proteolytic products were analyzed either by autoradiography, using Agfa Gevaert (Curix) X-ray film, or after slicing stained-dried gels and counting the resulting slices in a gamma-counter (Packard). Protein was determined according to Lowry et al. [19] using bovine serum albumin as a standard.

Materials. Insulin (from bovine pancreas), pronase, PMSF, TLCK, TPCK and histone (Type VII-S from calf thymus) were purchased from Sigma. $Na^{125}I$ (carrier-free) and [^{3}H]H_2DIDS were obtained from the Nuclear Research Center, Negev, Israel.

Results

Quantitative analysis of Sendai virus-associated proteolytic activity

It has been well established that in intact human erythrocytes, only the polypeptide designated

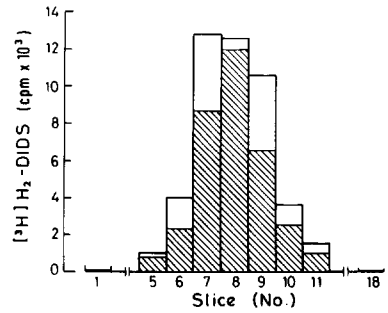

Fig. 1. Hydrolysis of human erythrocyte membrane polypeptide band 3 by Sendai virus particles. Intact human erythrocytes were radio-actively labeled by [^{3}H]H_2DIDS as described before [14], resulting in an erythrocyte preparation containing 50000 cpm/mg protein. PMSF (75 mM in methanol) was added to a virus suspension (10000–20000 hemagglutinating units in 250 μl Solution A) to give a final concentration of 5 mM, and the resulting suspension was incubated for 30 min at 37°C with gentle shaking. As PMSF and the other inhibitors of proteolytic enzymes (see Table I) are solubilized in methanol, a virus suspension (control) was incubated under the same conditions with 7% (v/v) of methanol. At the end of the incubation period, a volume of 8 ml Solution A was added to dilute the virus suspension which was then centrifuged at 100000 × *g* for 30 min. The pellet obtained was resuspended in 250 μl Solution A. [^{3}H]H_2DIDS-labeled human erythrocytes (150 μl of 25%, v/v) were mixed with 200 μl (80000 hemagglutinating units/ml) of untreated or PMSF-treated Sendai virus particles. After 150 min of incubation at 37°C, a volume of 0.5 ml of a solution containing 10% (w/v) SDS/0.8% (w/v) urea/10% (w/v) dithiothreitol was added to the virus-cell suspension, and the mixture obtained was immediately placed in boiling water for 2 min. A volume of 100 μl (containing about $1–2 \cdot 10^5$ cpm) was then loaded on disc electrophoretic gels (7.5% acrylamide) [29]. At the end of the electrophoresis period, the gels were cut into 3-mm slices which were then solubilized in 0.5 ml H_2O_2, containing 1% ammonium hydroxide (70°C, 12 h). After the addition of 5 ml scintillation liquid (picofluor), the samples were counted in a scintillation counter. Counts were found to be associated mainly with the 'band 3 zone' in the gels. Coomassie blue staining revealed that the band 3 polypeptides were located principally between slices 6–10 (data not shown). Clear (□) and hatched (▨) areas represent the amount of counts found to be associated with band 3 of human erythrocytes incubated with PMSF-treated and untreated virus particles, respectively. Experiments in which [^{3}H]H_2DIDS-labeled human erythrocytes were incubated in the absence of virus particles, gave identical results (data not shown) to those obtained after incubation with PMSF-treated Sendai virus particles.

as band 3 can covalently be labeled with [^{3}H]H_2DIDS [14]. Therefore, we have used it as a specific and quantitative marker of band 3. The results in Fig. 1 show that Sendai virus particles are able to hydrolyze the polypeptide band 3 when incubated with [^{3}H]H_2DIDS-labeled intact human erythrocytes (see also Ref. 20). Incubation with Sendai virus particles at 37°C led to a substantial decrease (about 30%) in the amount of band 3-associated [^{3}H]H_2DIDS. This was revealed by a quantitative analysis of the amount of [^{3}H]H_2DIDS found to be associated with band 3 after gel electrophoresis of control and Sendai virus-fused human erythrocytes (Fig. 1).

When incubated at 37°C with human erythrocytes, Sendai virus particles induce extensive hemolysis, resulting in the formation of polyghosts which are occasionally sheared into small fragments [13]. Therefore, in order to avoid any loss of [^{3}H]H_2DIDS band 3-containing membrane fragments, the entire reaction mixture was loaded on the gels (see legend to Fig. 1). It is thus conceivable that any reduction observed in the quantity of [^{3}H]H_2DIDS reflects a real decrease in the amount of band 3, probably by hydrolysis. No decrease in the amount of band 3-associated [^{3}H]H_2DIDS was observed when radioactively-labeled human erythrocytes were incubated with Sendai virus particles which were pretreated with inhibitors of proteolytic enzymes such as PMSF (Fig. 1).

The presence of a proteolytic activity in preparations of Sendai virus particles is also evident from experiments showing that, in addition to the human erythrocyte band 3, also the histone polypeptides (Fig. 2), insulin B-chain (Fig. 3) and fibrinogen (data not shown) were degraded by Sendai virus particles at 37°C. However, it should be mentioned that many proteins or polypeptides and protease substrates such as casein, bovine serum albumin, polylysine and glutaryl-phenylalanine-*p*-nitroanilide or benzoyl-arginine-*p*-nitroanilide were found to be resistant to the Sendai virus-associated proteolytic activity (data not shown).

The results in Fig. 2 show that when Sendai virus particles were incubated at 37°C with ^{125}I-labeled histone for various periods of time, a gradual accumulation of low molecular weight proteo-

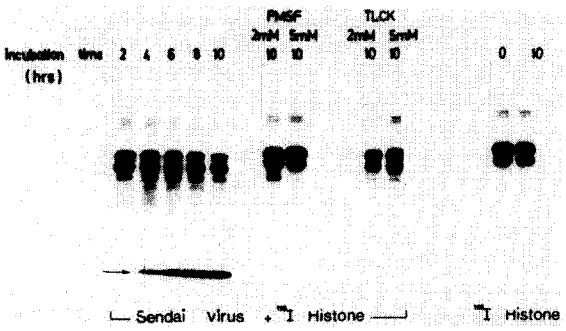

Fig. 2. Proteolysis of ^{125}I-labeled histone by Sendai virus particles: Analysis by autoradiography. Several systems containing Sendai virus particles (100 μg protein) were incubated with ^{125}I-labeled histone at 37°C, in a final volume of 100 μl Solution A, as described in Materials and Methods. Either PMSF or TLCK in methanol was added to the virus suspension before the addition of ^{125}I-labeled histone. The final concentration of methanol in the reaction mixture never exceeded 7%. At the time intervals indicated in the figure, the reaction was terminated by the addition of 100 μl solubilizing buffer, and the samples were loaded on gels, separated by gel electrophoresis (15% acrylamide) and analyzed by autoradiography, as described in Materials and Methods. Arrow indicates low molecular weight proteolytic products.

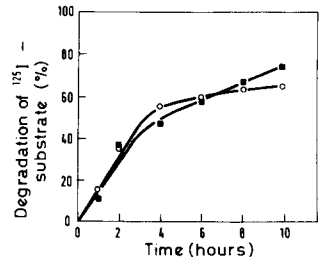

Fig. 3. Kinetics of degradation of ^{125}I-labeled histone and ^{125}I-labeled insulin B-chain by Sendai virus particles. Several systems containing Sendai virus particles (100 μg protein) were incubated with either ^{125}I-labeled insulin B-chain or ^{125}I-labeled histone, as described in Materials and Methods. Degradation of ^{125}I-labeled insulin was determined by estimating the amount of radioactivity present in the trichloroacetic acid soluble fraction, as described in Materials and Methods. Degradation of ^{125}I-labeled histone was determined as in Fig. 2 and in Materials and Methods. At the various time intervals, samples were withdrawn and low molecular weight proteolytic products were separated from the main histone band by slab gel electrophoresis (15% acrylamide). At the end of the run, the gels were sliced and the amount of radioactivity in the main histone bands in each slice was determined, as described in Materials and Methods. Estimation of the degree of hydrolysis was as described for ^{125}I-labeled insulin [16]. ■, ^{125}I-labeled insulin B-chain; ○, ^{125}I-labeled histone.

lytic products could easily be detected (see arrow at the bottom of the gels in Fig. 2). The increase in the amount of low molecular weight proteolytic products was correlated to a decrease in the amount of the main polypeptide histone bands (Fig. 2). Hydrolysis of ^{125}I-labeled histone by Sendai virus particles was inhibited by either 2–5 mM PMSF or by 2–5 mM TLCK. No hydrolytic products or decrease in the amount of the main polypeptide histone bands were noted in these systems, even after 10 h of incubation at 37°C (Fig. 2).

Fig. 3 demonstrates and compares the kinetics of degradation of the histone polypeptides and of the insulin B-chain. Proteolysis of both was linear for about 3 h, reaching a plateau after 5–6 h of incubation at 37°C. At that time, 60–70% of these substrates were hydrolyzed.

The protease was active at rather broad pH ranges, being optimal between pH 6.0 and 9.0 (Fig. 4A), whereas the optimal temperature was found to be between 37 and 40°C (Fig. 4B). The virus-associated proteolytic activity was found to be sensitive to high temperatures, being inhibited above 45–50°C. Preliminary experiments also indicated that bivalent metals such as Ca^{2+} or Mg^{2+} stimulate proteolysis, while EDTA (4 mM) and EGTA (4 mM) significantly inhibited it.

Reconstituted Sendai virus envelopes which are fusogenic membrane vesicles containing only the two main viral envelope glycoproteins [15], were also able to hydrolyze various polypeptides such as the insulin B-chain (Fig. 5) and histone (data not shown). The specific activity of the reconstituted Sendai virus envelope-associated protease is about 2–3-fold higher than that of the intact virus preparation, and its activity was linear with protein concentration (Fig. 5A). Both the Sendai virus and the reconstituted Sendai virus envelope-associated proteolytic activities were inhibited by PMSF (Fig.

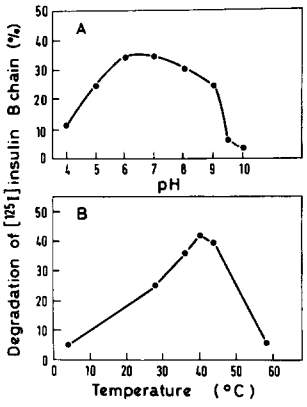

Fig. 4. Proteolysis of ^{125}I-labeled insulin B-chain by Sendai virus particles: (A) pH dependency; (B) effect of temperature. Several systems containing Sendai virus particles (100 μg protein) were incubated at different pH values and various temperatures with 7.5 μg ^{125}I-labeled insulin B-chain in a final volume of 100 μl, for 2 h at 37°C, as described in Materials and Methods. pH values of 4.0–7.0 were achieved by suspending a washed pellet of virus particles in 160 mM NaCl to which 25 mM sodium-acetate buffer adjusted to the different pH values was added. pH values of 7.0–9.0 were obtained by using a solution containing 160 mM NaCl, adjusted to the different pH values by 25 mM Tricine/NaOH. pH 10.0 was obtained by the use of 25 mM carbonate/sodium bicarbonate buffer. The degree of proteolysis was estimated as described in Materials and Methods.

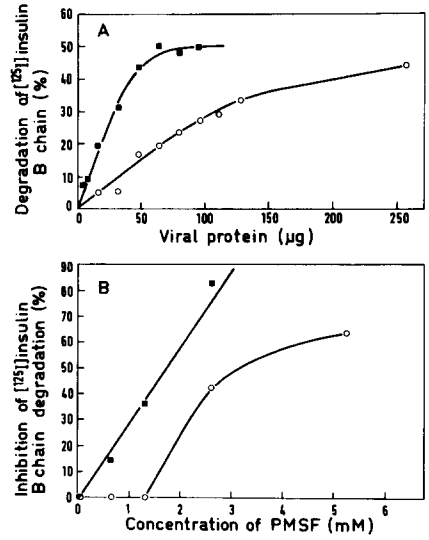

Fig. 5. Degradation of ^{125}I-labeled insulin B-chain by Sendai virus and reconstituted Sendai virus envelopes: (A) dependence on the viral protein concentration; (B) inhibition by increasing concentratoins of PMSF. (A) Intact Sendai virus particles or reconstituted Sendai virus envelopes at different protein concentrations were incubated with 7.5 μg ^{125}I-labeled insulin B-chain for 3.5 h at 37°C, in a final volume of 100 μl Solution A. (B) Different amounts of PMSF solution (75 mM in methanol) were added to a suspension containing either 32 μg intact virus or reconstituted Sendai virus envelopes in a 100 μl volume of Solution A. The resulting mixture was incubated for 30 min at 37°C, after which 7.5 μg of the ^{125}I-labeled insulin were added and incubated with the virus suspension at 37°C for 3.5 h. Degradation of ^{125}I-labeled insulin was estimated as described in Materials and Methods. ■, reconstituted Sendai virus envelopes; ○, intact Sendai virus particles.

5B). However, as can be seen (Fig. 5B), the reconstituted Sendai virus envelope-associated protease is more sensitive to inhibition by PMSF than the Sendai virus-associated protease.

A possible correlation between the virus-associated protease and its ability to fuse and hemolyse human erythrocytes

A possible correlation between the viral-associated proteolytic activity (tested on histone as substrate) and its ability to fuse or hemolyse human erythrocytes is suggested by the results in Table I. Virus particles, whose proteolytic activity was inhibited with inhibitors of proteolytic enzymes by more than 60–70%, were unable to fuse human erythrocytes (Table I). Inhibition of the virus-fusogenic activity was always accompanied by a significant reduction in its hemolytic activity (Table I). It is evident that relatively high concentrations of inhibitors were required to inhibit virus-induced fusion or hemolysis. However, the same concentrations were required to inhibit significantly the virus-associated protease (Table I, see also Fig. 3). As much as 5 mM PMSF, 6 mM TLCK, a combination of both (0.5 mM PMSF/3.75 mM TLCK) or a combination of 3.75 mM TPCK/3.75 mM TLCK were needed to inhibit induction of hemolysis, membrane fusion and virus-associated protease (Table I) *. It is noteworthy that agglutination (Table I), which occurs after binding of virus particles to the human erythrocyte surface, and

* Since the various inhibitors are solubilized in methanol, a control system in which a virus suspension was treated with methanol, was always added (see legend to Fig. 1). Under the conditions used here, methanol up to 8% had very little effect, if any, on the different Sendai virus activities.

TABLE I

THE CORRELATION BETWEEN THE EFFECT OF VARIOUS INHIBITORS OF PROTEOLYTIC ENZYMES ON SENDAI VIRUS-ASSOCIATED PROTEASE AND ON ITS ABILITY TO LYSE AND FUSE HUMAN ERYTHROCYTES

Washed, intact human erythrocytes (0.5 ml of 2.5%, v/v) in Solution A were incubated with 10–20 μl (30–60 μg protein, giving about 400–800 hemagglutinating units) Sendai virus particles for 10 min at 4°C, and subsequently incubated at 37°C for 30 min with gentle shaking. Agglutination and degree of cell-cell fusion were estimated by observation in phase microscope [13]. Hemolysis was determined by estimating the absorbance at 540 nm of the supernatant obtained after centrifugation of the various systems subsequent to a 15 min incubation at 37°C [13]. Total hemolysis was obtained by addition of 20 μl of a 28% ammonia solution to a human erythrocyte suspension. Sendai virus particles were treated with the various inhibitors of proteolytic enzymes, as described for treatment of Sendai virus particles with PMSF in Fig. 1. The degradation of ^{125}I-labeled histone by Sendai virus was as described in Materials and Methods and in Fig. 3. The degree of proteolysis was estimated after 180 min of incubation at 37°C, during which 40% (maximum proteolysis) of the total amounts of added histone was hydrolyzed by the control, untreated virus. Degree of cell-cell fusion: + + + Polyghosts of 5–15 cells were obtained and 60–80% of the cells in the population were fused. + + Polyerythrocytes or polyghosts of 5–10 cells were obtained and 40–60% of the cells in the population were fused. ± Very little cell-cell fusion, if any. About 1–5% of the cells in the population were fused. Essentially the same results were obtained from three independent experiments, however, the data in the table represent the results obtained from one of these experiments.

Inhibitor	mM	Virus activities			Degradation of ^{125}I-labeled histone (percent of maximum)
		Agglutination	Lysis (% of total)	Cell-cell fusion	
None [a]		+	96	+ + +	100
PMSF	0.5	+	90	+ +	60
PMSF	3.0	+	20	—	38
PMSF	5.0	+	0	—	29
TLCK	3.75	+	30	±	50
TLCK	6.0	+	20	—	35
PMSF + TLCK	0.5 + 3.75	+	0–5	—	14
TPCK	3.75	+	90	+ +	90
TPCK + TLCK	3.75 + 3.75	+	0–5	—	10

[a] A virus suspension was incubated with 7% (v/v) methanol, as described in Fig. 1, for PMSF. Under the conditions used, methanol-treated virus behaved similarly to control, untreated virus.

TABLE II

THE EFFECT OF PMSF ON THE RECONSTITUTED SENDAI VIRUS ENVELOPE-ASSOCIATED PROTEASE AND ON ITS ABILITY TO PROMOTE CELL-CELL FUSION

Intact Sendai virus particles or reconstituted Sendai virus envelopes were treated with 5 mM PMSF, as described for intact Sendai virus in Fig. 1. Induction of cell-cell fusion was as described in Table I, except that 25 μg reconstituted Sendai virus envelopes were used. Proteolysis of ^{125}I-labeled insulin by reconstituted Sendai virus envelopes and by intact Sendai virus was performed and determined as described in Materials and Methods. For proteolysis, 25 μg reconstituted Sendai virus envelopes and 100 μg intact Sendai virus were incubated with ^{125}I-labeled insulin B-chain for 2 h at 37°C. Symbols of cell-cell fusion as in Table I.

System	Treatment	Cell-cell fusion	Proteolysis (%)
Sendai virus	None	+ + +	26.5
Sendai virus	PMSF	—	11.2
Reconstituted Sendai virus envelopes	None	+ + +	28.7
Reconstituted Sendai virus envelopes	PMSF	—	5.7

disagglutination, a process that reflects the activity of the virus-associated neuraminidase, were unaffected by the various inhibitors. Indeed, determination of the viral neuraminidase activity shows that it was unaffected by the various inhibitors (data not shown).

A possible correlation between the virus-fusogenic and proteolytic activities (this time using insulin B-chain as substrate) was also evident from experiments using reconstituted Sendai virus envelopes. Treatment of the envelopes with 4 mM PMSF completely blocked their fusogenic ability and significantly inhibited their proteolytic activity (Table II). Similar to the observation with intact virus particles (Table I), the fusogenic activity of the reconstituted Sendai virus envelopes was also blocked only when the proteolytic activity of the viral envelope preparations was inhibited by more than 60–70% (Table II).

PMSF-treated Sendai virus particles: Infectivity and morphological studies

Table III shows that incubation of Sendai virus particles with PMSF blocked their infectivity, as tested by their ability to multiply in the allantoic fluid of embryonated eggs. Injection of as many as 80 hemagglutinating units/egg of PMSF-treated virus particles did not yield any significant amount of new virions, while injection of only two hemagglutinating units/egg of untreated virions was sufficient to yield 25000 hemagglutinating units/infected egg (Table III).

Examination by electron microscopy (Fig. 6) showed that treatment with PMSF did not cause any noteworthy structural changes in the virions, and their morphology is identical to that of control untreated viruses. PMSF-treated virus particles were able to bind to and promote agglutination of human erythrocytes (Fig. 6A and B). From the electron microscopic studies, it is apparent that, as opposed to control, untreated virus particles, the PMSF-treated virions were unable to establish tight contact with the erythrocyte membranes. The envelopes of the PMSF-treated virus particles remained, even after long incubation at 37°C, about 150–200 Å apart from the erythrocyte membrane (Fig. 6B). Evidently, the erythrocytes which were incubated with PMSF-treated virus (Fig. 6A and B), remained intact and retained all of their hemoglobin, while untreated virus particles readily fused and hemolysed human erythrocytes (Fig. 6C) [8,13,20].

These electron microscopic studies support the view that proteolysis of surface membrane proteins might be required to allow close contact between the virus envelopes and the recipient cell membrane, and especially with its phospholipid bilayer. A means to test this possibility was by the

TABLE III

INHIBITION OF SENDAI VIRUS INFECTIVITY BY TREATMENT WITH PMSF

Sendai virus was treated with 5 mM PMSF, as described in Fig. 1. Different amounts of PMSF-treated or untreated virus particles were injected into 25 embryonated chicken eggs, as previously described [13]. After 48 h of incubation at 37°C, the eggs' allantoic fluid was harvested and the virus titer checked, as described before [13].

	Sendai virus particles (hemagglutinating units/egg)	
	Input multiplicity (hemagglutinating units/egg)	Yield (hemagglutinating units/egg)
Untreated Sendai virus	2	$25 \cdot 10^3$
	20	$25 \cdot 10^3$
	80	$51 \cdot 10^3$
PMSF-treated Sendai virus	2	< 10
	20	< 10
	80	< 10

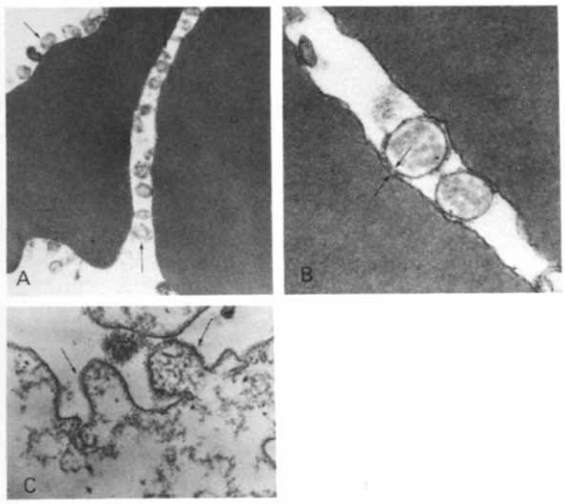

Fig. 6. The interaction between PMSF-treated Sendai virus particles and human erythrocytes at 37°C: Electron microscopy studies. Sendai virus particles were treated with PMSF and incubated with human erythrocytes, first at 4°C for 10 min and then at 37°C for 60 min, as described in Fig. 1 and Table I. At the end of the incubation period, samples were fixed, embedded in Epon, and prepared for electron microscopic observations as previously described [13]. (A) Many intact Sendai virus particles (PMSF-treated) are seen attached (arrows) to the agglutinated human erythrocytes. Note that the human erythrocytes are intact (loaded with hemoglobin), despite the fact that they were incubated with the virus particles for 60 min at 37°C. Magnification × 10240. (B) A higher magnification, showing interaction between PMSF-treated Sendai virus particles and human erythrocytes. Note that the virus envelope and the erythrocyte membrane (arrows) are about 200–250 Å apart from each other, even after 60 min incubation at 37°C. Magnification × 56000. (C) The same experimental conditions as in A but with control, untreated virus. Due to promotion of lysis by the active virus, only erythrocyte membranes are seen in the electron microscope field. In some places (arrows) fusion between a virus particle and the erythrocyte membrane can be detected. Magnification × 33600.

use of pronase-treated human erythrocytes which are devoid of surface glycoproteins [21] and to examine their susceptibility to PMSF-treated Sendai virus particles. PMSF-treated Sendai virus particles failed to hemolyse or fuse pronase-treated erythrocytes (data not shown). It should be noted that both control and PMSF-treated virus particles were able to agglutinate the pronase-treated human erythrocytes. Identical results were obtained after treatment of human erythrocytes with a variety of proteolytic enzymes such as trypsin, chymotrypsin and pepsin (data not shown). The inability of the glycoprotein-free human erythrocytes to be fused by PMSF-treated Sendai virus particles may suggest that, if proteolysis is required, it is local and a very specific process.

Discussion

The interaction between Sendai virus particles and human erythrocytes has been used by us as a convenient system to study the detailed mechanism of virus-cell penetration and membrane fusion. Since under the conditions used in the present work the human erythrocytes are not capable of endocytic activity, it is conceivable that viral envelopes which cannot be displaced from the erythrocyte membrane after incubation at 37°C,

are fused with it. Fusion of the viral envelopes with the erythrocyte membrane leads, in most cases, to induction of cell lysis and promotion of cell-cell fusion [1,4,6]. Evidently, fusion between the viral envelope and the erythrocyte membrane is a process of membrane-membrane fusion, as the viral envelope resembles in its structure and composition a typical biological membrane [6]. Based on various approaches and the use of different fusogenic agents, both of viral [22] and chemical [23] origin, it has been proposed that fusion between biological membranes occurs by intermixing of protein-free phospholipid bilayers. This implies that before the step of membrane-membrane fusion occurs, the phospholipid bilayers have to be exposed, thus allowing molecular attachment between phospholipid groups of adjacent membranes. However, it was well established that both the phospholipid bilayer of the viral envelopes and the erythrocyte membranes are masked by a heavy layer of membrane glycoproteins [1,6,7].

The Sendai virus envelope's hemagglutinin/neuraminidase glycoprotein serves as a binding protein whose presence is essential for the specific attachment of the viral particle to the sialic acid residue of cell surface glycoproteins, whereas the fusion factor-viral glycoprotein is essential for promotion of fusion between the viral envelope and the cell-plasma membrane. Based on previous observations [24,25], it has been proposed that fusion of the viral envelope with the targeted membrane is mediated by the hydrophobic part of the fusion factor-glycoprotein.

The results of the present work also suggest that a virus-associated protease may be required for the process of virus-cell fusion. We propose that a specific and local hydrolysis of cell-surface glycoproteins is required to allow the fusion factor-glycoprotein to reach its targeted membranes. Through this mechanism, specific membrane glycoproteins are removed and the membrane phospholipids may become, at these areas, exposed to the action of the viral-fusion factor.

Several lines of evidence support the above assumption. The results of the present work demonstrate the presence of Sendai virus-associated protease which is able to hydrolyze membrane glycoproteins such as the erythrocyte band 3 polypeptide. Inhibition of the virus proteolytic activity by several known protease inhibitors, blocks the virus-hemolytic and fusogenic activities without interfering with the binding ability. Only alkylating agents which have been shown to block the activities of serine proteases, were effective in inhibiting both the proteolytic and fusogenic activities of Sendai virus particles. Other inhibitors such as leupeptin, pepstatin or soybean trypsin inhibitor failed to inhibit the above activities. This may be due to lack of susceptibility of the virus-associated protease to these reversibly bound inhibitors. As relatively high concentrations of inhibitors were required to inhibit the virus fusogenic and hemolytic activities, it can be assumed that the inhibitors exert their effect by unspecific inactivation via chemical modification of the viral fusion factor. However, it is striking that the same high concentrations of inhibitors were required to inhibit the virus-associated protease. This may imply a correlation between the two activities and that the virus-associated protease plays an active role in the entire virus-cell fusion process.

Support for this view was obtained from experiments using reconstituted Sendai virus envelopes which are reconstituted viral envelopes containing mainly the fusion factor- and hemagglutinin/neuraminidase-glycoproteins [15]. Our experiments showed that these relatively pure, reconstituted membrane preparations possess a proteolytic activity (Fig. 5). As expected, the specific activity of the reconstituted Sendai virus envelope-associated protease was higher than that of the intact virus particles. Inhibition of the reconstituted Sendai virus envelope-associated protease was always accompanied by blocking of its fusogenic and hemolytic activities.

The observation that the protease is associated with the reconstituted Sendai virus envelopes obtained after removal of a detergent (Triton X-100) [26] from the virus-solubilized glycoproteins, may imply that the protease is a membrane protein tightly associated with the viral lipid bilayer. This might explain the degradation of the viral components observed in the absence of PMSF during the reconstitution process [27] and the need for relatively high concentrations of protease inhibitors for inhibition of the viral protease and its hemolytic and fusogenic activities.

The possibility that the virus-associated pro-

tease is a contamination carried with the virus from the chicken egg allantoic cells, cannot be excluded. Indeed, the presence of a protease in the chicken egg allantoic membrane preparation on which the virus is propagated, was recently reported [28]. However, preliminary results in our laboratory showed that a proteolytic activity can also be detected in a biologically-active and pure preparation of the viral fusion factor-glycoprotein (data not shown). Addition of inhibitors such as PMSF to the pure preparation of the fusion factor-glycoprotein, completely blocks its hemolytic activity. Experiments are currently conducted in our laboratory to further study the correlation between the biological activity of the pure preparations of the fusion factor-glycoprotein and their ability to hydrolyze membrane polypeptides.

It is tempting to speculate that the difference between enveloped viruses which penetrate cells by endocytosis and those which fuse with the cells' plasma membranes, is the presence of an active proteolytic enzyme in the latter. Experiments to examine this possibility are currently being pursued in our laboratory.

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