Sendai virus envelopes devoid of hemagglutinin-neuraminidase but containing the fusion protein (F-virosomes) were prepared. F-virosomes exhibited discernible serine protease activity at neutral pH. Electrophoretic analysis of the protein profile of F-virosomes under nonreducing conditions, by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing, led to the identification of a previously unknown glycoprotein with a relative molecular weight of 45,000 (45K protein) associated with the F protein. The identity of the 45K protein, as distinct from F protein, was established by Western blot analysis with F- and 45K-specific antibodies. This 45K protein forms a nexus with the F protein through noncovalent hydrophobic interactions, as proved by its sensitivity to urea treatment, and it is essential for the proteolytic activity of the F-virosomes as well as for the fusion of the viral envelope with host cell membrane. N-terminal sequence analysis (first 11 amino acids) of this protein showed strong homology (>90%) to flavivirus NS3 serine proteases but no similarity to any of the Sendai viral proteins. On the basis of the N-terminal sequence, oligonucleotides were designed corresponding to the sense and antisense DNA sequences. Dot blot hybridization and primer extension with these oligonucleotides with the viral and the host genome confirmed the host origin of this protein. Further, the limited proteolytic digestion of the target membrane resulted in significant inhibition of viral fusion. With it. On the basis of these results, we postulate a model for the molecular mechanism of F protein-induced membrane fusion, which may provide a rationale for other paramyxoviruses.

Sendai virus, with a negative-strand RNA genome, belongs to the family of paramyxoviruses and fuses at neutral pH with the cellular plasma membrane (11). The viral genome is known to encode two transmembrane glycoproteins, hemagglutinin-neuraminidase (HN) and fusion factor (F). While HN protein helps the virus to attach to its target cell membrane, by binding to terminal sialic acid-containing-receptors, F protein is essential for the fusion of the viral envelope with the host cell membrane, leading to the internalization of the viral genome. The F protein is synthesized as a precursor, F0, which is subsequently cleaved by host proteases into its biologically active form, composed of F1 and F2 subunits, with relative molecular weights of 45,000 and 14,000 (45K and 14K proteins), respectively, linked by disulfide bonds (35). As a result of this cleavage, an extremely hydrophobic domain, termed fusion peptide, is exposed at the amino terminus of the F1 subunit, which probably undergoes a conformational change during fusion (25). This fusion peptide is highly conserved among paramyxovirus F proteins and is known to directly catalyze membrane fusion (32). However, the molecular mechanism underlying membrane fusion induced by paramyxovirus fusion protein is not yet fully understood. It was suggested earlier that proteolytic enzymes may be involved in the fusion process at neutral pH (29). This is consistent with serine protease-like activity associated with Sendai virus and its reconstituted envelopes (F- and HN-virosomes) with both F and HN glycoproteins (21). We have recently established that Sendai virus F protein in F-virosomes can induce complete membrane fusion, provided that the latter are bound tightly to the target cell membrane (5, 9, 23). Our results are consistent with the studies on other paramyxoviruses which show F protein in absence of HN can induce syncytium formation (2, 19). However, unlike influenza virus (and other enveloped animal viruses which exhibit pH-dependent membrane fusion) (39), the trigger for the paramyxovirus F protein-induced fusion of lipid bilayers is not known. Although induction of membrane fusion through a conformational change in the F protein is an attractive hypothesis (25), it is difficult to comprehend how this change occurs at neutral pH under normal physiological conditions.

To shed some light on the mechanism of Sendai virus F protein-induced membrane fusion, we prepared F-virosomes as reported by us previously (5) and identified a distinct serine protease activity associated with them. We demonstrate that a previously unknown glycoprotein (45K, distinct from F protein) is attached with the F protein by noncovalent hydrophobic interactions which copurifies with the F protein. Hence, the F-virosome preparations described herewith contains both the F and 45K glycoproteins. Our results suggest a direct involvement of the protease activity of the F-45K complex in the virus-target cell membrane fusion. Viral envelopes deficient in 45K protein were found to be devoid of proteolytic and fusogenic activities. The dot blot hybridization with oligonucleotides derived from the N-terminal sequence of the 45K protein and the primer extension analysis led to the conclusion that this protein is encoded by the host genome. In addition, our studies on the fusion of viral envelope with protease-treated target cell membrane indicate the existence of a putative proteaceous receptor for Sendai virus. These results are discussed in the context of fusion active conformational changes in the F protein under the influence (trigger) of the 45K partner.
Fusion mechanism of Sendai virus.

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FUSION MECHANISM OF SENDAI VIRUS

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Reagents.

Octadecylrhodamine (R18) was purchased from Molecular Probes (Eugene, Ore.). SM2 Bio-Beads were obtained from Bio-Rad (Richmond, Calif.).TX-100 was obtained from Aldrich (Milwaukee, Wis.). Histones (type IIs, from calf thymus), 2-mercaptoethanol, diethiothreitol, PMSF, RCMA, anti-rabbit antisera, and NP-40 were procured from Sigma Chemical Co. (St. Louis, Mo.). Freund’s adjuvants (complete and incomplete) were purchased from Difco Laboratories (Detroit, Mich.). T4 polynucleotide kinase was purchased from New England Biolabs Inc. (Beverly, Mass.). M-MuLV reverse transcriptase was obtained from United States Biochemical (Cleveland, Ohio). Dulbecco’s modified Eagle’s medium, Dulbecco’s phosphate-buffered saline, fetal calf serum, trypsin-EDTA, buffer-saturated phenol, and positively charged nylon membranes were purchased from Life Technologies Inc. (Grand Island, N.Y.). APMSF and proteinase K were obtained from Boehringer GMhH (Mannheim, Germany). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Corp. (Bedford, Mass.). Carrier-free Na125I and y-32P-ATP were obtained from BARC, Bombay, India. Monoclonal antibodies against Sendai virus F protein were kind gifts from Allen Portner (St. Jude Children’s Research Hospital, Memphis, Tenn.). All other reagents used were of analytical grade.

Cells.

HeLa and MDCK cells obtained from the American Type Culture Collection (Rockville, Md.) were grown in MEM supplemented with 5% newborn calf serum, 100 units/ml of penicillin, 100 δg of streptomycin sulfate per ml in 25-cm² plastic bottles (Falcon, Becton Dickinson, Paramus, N.J.).

Membrane fusion of F-virosomes.

Sendai virus (Z) was grown in the allantoic sac of 10-day-old embryonated chicken eggs incubated at 37°C (the normal temperature of propagation) or at 38.5 or 40°C. The virus was harvested and gradient purified as described elsewhere (31). Reconstituted Sendai virus envelopes containing F glycoprotein (F-virosomes) were prepared as described by Bagai et al. (5). These F-virosomes were used to raise antibodies in rabbit or else radiolabeled with Na125I by the chloramine-T method (41).

Electrophoretic analysis and isoelectric focusing.

Sendai virus and F-virosoemes were resolved by SDS-PAGE in a separating gel containing 10% acrylamide and 0.27% bisacrylamide in the presence of 1.0% SDS under reducing and nonreducing conditions in the presence of 2% NP-40 and 9 M urea (22). The glycoproteins of F-virosomes and reconstituted F-virosomes were resolved by isoelectric focusing in a disc gel with a pH range of 5.5 to 7.5. The glycoproteins were visualized by Coomasie blue staining (0.25% Coomasie blue R-250 in 35% methanol-10% acetic acid), Western blotting (38) with anti-F-virosome antibodies, or autoradiography (5) with 125I-labeled F-virosomes. Isoelectric focusing was carried out with ampholytes in the pH range 3.5 to 10.0 in a separating gel containing 3.8% acrylamide and 2% bisacrylamide under nonreducing conditions in the presence of 2% NP-40 and 9 M urea (22). The glycoprotein bands were checked by the silver staining procedure (23) and by Western blotting with RCA, binding followed by development with anti-rabbit antibodies and protein A-horseradish peroxidase conjugate.

Development of anti-F and anti-45 K polyclonal sera.

Following SDS-PAGE of F-virosomes and F-virosoomes after having been subjected to radiolabeling with Trichloroacetic acid, the gel was sliced and the bands were excised. The excised bands were applied to a column of Bio-Gel P-100 and eluted with 10 mM Tris. The fractions were collected and counted for radioactivity.

Electrotransfer of total RNA.

Total RNA from both the infected and mock-infected HeLa cells was prepared by SDS and proteinase K digestion followed by ethanol precipitation as described previously (33). Total cellular RNA from uninfected and Sendai virus-infected CAM, as well as HeLa and MDCK cells and Sendai viral genomic RNA were prepared by the acid guanidine phenol method as described previously (14).

Design and synthesis of oligonucleotides.

From the determined N-terminal sequence, two 28-mer degenerate oligonucleotides were prepared, keeping in view the viral codon usage (11), with the sequence 5’-GAGGGGCTGGTCACTACACTG(T)/GTCGATCA(A)-3’ (sense sequence, oligonucleotide 1) and its complementary sequence (oligonucleotide 2). These oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems) and purified on an Oligo Pac column.

Protein blot hybridization. Dot blot hybridization of infected CAM DNA and total RNA, and the viral genomic RNA with 32P-labeled oligonucleotides (labeled at their 5’ termini by transfer of y-32P from [y-32P]ATP with T4 polynucleotide kinase [33]) was performed for 12 h at 37°C in 6x NET (1 NAME_10 = 150 mM NaCl, 15 mM Tris-HCl [pH 8.0], and 1 mM EDTA) containing 0.1% SDS, 5x Denhardt’s reagent, 20 mM sodium phosphate (pH 7.0) and 200 δg of herring sperm DNA per ml. The filters were washed five times for 5 min each with 6x SSC (1x SSC = 150 mM NaCl plus 15 mM sodium citrate) containing 0.1% SDS at room temperature and then once with the same buffer at 42°C before exposure to X-ray film.

Primers.

The 32P-labeled oligonucleotide 1 (homologous to the 45K mRNA sequence) and oligonucleotide 2 (complementary to the mRNA sequence) were annealed to 20 mg of total RNA from both the infected and uninfected CAM, uninfected HeLa and MDCK cells, and genomic RNA of

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Sendai virus in 50 mM Tris-HCl (pH 8.3) containing 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 500 μM dithranucleoside triphosphates, and 1,000 U of RNasin (Sigma) per ml. Annealed primer was extended with 300 U of M-MuLV reverse transcriptase (United States Biochemicals) at 37°C for 1 h (33). The products were analyzed by 5% acrylamide–8 M urea gel electrophoresis followed by autoradiography.

RESULTS

Electrophoretic analysis of Sendai virus and F-virosomes.

The protein composition of the F-virosomes was examined by SDS-PAGE in the presence and absence of 2-mercaptoethanol. Under reducing conditions, the Coomassie blue staining of the gel revealed two bands (Fig. 1A, lane 2) corresponding to 45K and 14K, respectively, while in the nonreducing gel of the F-virosomes, two bands of 60K and 45K were visible (lane 1). Similar profiles were obtained when the bands were visualized by Western blotting with anti-F-virosome serum (Fig. 1B) or when 125I-labeled F-virosomes were resolved by SDS-PAGE under similar conditions followed by autoradiography (Fig. 1C). Under nonreducing conditions, F protein migrates as a band of 60K comprising of both the subunits F₁ and F₂. Here, a discernible band of 45K was also visible, but F₂ was not visualized even by Western blotting and autoradiography. Under reducing conditions, the 45K protein comigrates with the F₁ subunit of F protein. The increase in the intensity of the 45K band under reducing conditions, where F₁ and 45K comigrate, is smaller than expected, since F₂ protein (attached to F₁ under nonreducing conditions) dissociates from F₁ under reducing conditions and migrates as a distinct band of 14K. Purified Sendai virions (egg grown), when electrophoresed under similar conditions, exhibited an additional protein band corresponding to 45K in the absence of reducing agent (Fig. 2, lane 2). As observed for F-virosomes, this 45K protein comigrates with F₁ under reducing conditions (lane 1). Similar electrophoretic analysis of Sendai virus grown in MDCK cells, by the method of Paterson and Lamb (30), also revealed the presence of the 45K protein (data not shown). Further evidence in support of the 45K protein is provided by isoelectric focusing of the F-virosomes under nonreducing conditions, which revealed an additional protein at pI 3.7 distinct from F protein (pI 4.9) (36) (Fig. 3). The 45K band was stained by PAS staining for sugars, confirming that it was a glycoprotein (Fig. 4B). The proteins from F-virosomes were resolved by SDS-PAGE under nonreducing conditions, transferred to nitrocellulose paper, and tested for binding with RCA₁, followed by Western blotting with anti-RCA₁ antibodies (Fig. 4A). The 45K protein is specifically recognized by the lectin, revealing that its oligosaccharide chains contain a terminal galactose moiety.

Establishment of the identity of the 45K protein. In our attempt to confirm that this 45K protein is not F₁ or some fragment of the F protein, we have raised polyclonal antisera against the purified F and 45K proteins in mice and verified our results further with the help of known anti-F monoclonal antibodies. It is clear from the Western blot analysis of purified virosomes under nonreducing conditions that neither polyclonal anti-F sera (Fig. 5A, lane 1) nor anti-F monoclonal antibodies (lane 3) could bind to the 45K protein. On the other hand, polyclonal anti-45K sera (lanes 5 and 6) was not able to light up the band containing F protein. However, both anti-F polyclonal (lane 2) and monoclonal (lane 4) antibodies could bind to F₁ polypeptide, as is evident from the Western blot analysis of intact virus under reducing conditions. Western blot analysis of purified F and 45K proteins and F-virosomes under nonreducing conditions also exhibited no cross-reaction between F- and 45K-specific antibodies (Fig. 5B). This kind of specificity...
of immune interaction supports the notion of 45K being the additional virus-associated protein.

Characterization of the protease activity in F-virosomes. F-virosome-induced proteolytic degradation of 125I-histone was quantitated by decrease in the TCA-precipitable radioactivity. F-virosome-induced 125I-histone degradation was also recorded by autoradiography (data not shown). The F-virosome preparation exhibited protease activity, with an optimum temperature of 37°C and pH 7.0 ± 0.5 (data not shown). This protease activity was shown to be inhibited (>90%) by serine protease inhibitors (7 mM PMSE and 2 mM APMSF) and by incubation of F-virosomes at 56°C for 20 min. The optimum conditions for protease activity were similar to those required for virus-cell fusion, and the treatments inhibiting it are also known to inhibit F protein-mediated membrane fusion (5). F-virosomes incubated with 6 M urea solution lead to the dissociation of the 45K protein (Fig. 6a, lane 3). The 45 K protein could also be dissociated from Sendai virus by similar treatments (data not shown). Urea (3 M), KCl (1.0 M), EDTA (10 mM [pH 10]), and freeze-thaw cycles failed to dissociate F-45K complex from the virosomal and viral membrane. Such 45K-deficient F-virosomes exhibited markedly lower proteolytic activity (Fig. 6b). During reconstitution, the 45K protein reassociated with the F protein to a significant extent, as revealed by SDS-PAGE (Fig. 6a, lane 4) and virosomes regained their proteolytic activity significantly (>75% of the original activity) (Fig. 6b). Sendai virus also followed a similar trend in terms of hemolytic activity (70% of the native activity) (Fig. 6c). The data obtained with intact virus emphasize the role of the 45K protein in fusion (which is directly related to hemolysis) in the presence of the natural attachment protein of the virus (HN). Taken together, these data indicate that the 45K protein is an extrinsic membrane protein which is associated tightly with the F protein by noncovalent hydrophobic interactions and that its presence, along with F protein, is essential for the proteolytic and hemolytic (fusion) activities of the F protein.

Temperature dependence of the appearance of the 45K glycoprotein in the Sendai virus membrane. Sendai virus grown at 38.5 or 40°C showed a considerable decrease in the amount of the 45K glycoprotein compared with that of its normal counterpart (grown at 37°C); this was reflected in both the intact viruses and their corresponding F-virosomal preparations (Fig. 7a). The 45K-deficient Sendai viruses contained normal amounts of all other proteins as checked by the hemagglutination titer, SDS-PAGE (Fig. 7a), and Western blotting, with anti-whole Sendai virus antiserum (data not shown). Such deficient virus stocks were completely devoid of infectivity. F-virosomes prepared from Sendai virus grown at higher temperatures of propagation exhibited markedly lower (>80% decrease) proteolytic activity (Fig. 7c) compared to those from virus grown at the normal temperature (37°C). Moreover, Sendai virus grown at temperatures higher than 37°C showed a profound decrease (>90%) in membrane fusion activity (Fig. 7b), as assayed by a direct fusion assay, despite having cleaved F (F1 and F2 subunits). The data obtained with intact virus correlate with those obtained with F-virosomes, emphasizing the importance of the 45K protein in the fusion process in its natural environment. Also, it shows that the cleavage of F, to F1 and F2, is not sufficient for virus to become fusion active.

Effect of limited proteolysis of the target membrane on its fusion with Sendai virus. HRBC, treated with proteinase K, exhibited a significant reduction in hemolysis with increasing times of incubation at 37°C. About 50% inhibition of hemolytic activity was obtained within 30 min of incubation (compared with untreated control cells) when HRBC were incubated with 2 µg of Sendai virus, while incubation for 2 h led to more than 80% inhibition (Fig. 8). However, the binding activity, in terms of the hemagglutination titer, remained unaltered (about 2×).

N-terminal sequence homology. The N-terminal 11-amino-acid sequence of the 45K protein was determined and then subjected to a sequence homology search. It did not show appreciable homology to any region of the sequences of known Sendai virus or host proteins but exhibited a remarkable (>90%) homology to a highly conserved sequence in the serine protease domain of the flavivirus NS3 proteins (Table 1). The ninth amino acid, histidine, is considered to be a part of the putative catalytic triad.
Origin of the 45K glycoprotein. From the N-terminal sequence of the 45K glycoprotein, oligonucleotides corresponding to the mRNA (oligonucleotide 1) and its complementary sequence (oligonucleotide 2) were designed. Both the oligonucleotides hybridized specifically with the genomic DNA from the host cells (CAM from 10-day-old chicken eggs) (Fig. 9A, dots 4 to 7, and Fig. 9B, dot 3), while total RNA from CAM hybridized only with oligonucleotide 2 (Fig. 9B, dots 1 and 2). On the other hand, genomic RNA from Sendai virus exhibited no hybridization signal with either of the oligonucleotides (Fig. 9A, dots 8 and 9, and Fig. 9B, dot 4), indicating that the gene coding for the 45K protein resides in the host cells and not in the viral genome. Hybridization signals were also obtained with genomic DNA samples from HeLa and MDCK cells (data not shown). DNA samples from lambda bacteriophage and herring sperm were used as negative controls (Fig. 9A, dots 1 to 3).

Primer extension with total cellular RNA from uninfected CAM, MDCK and HeLa cells, and infected CAM with oligonucleotide 2 (antisense) (Fig. 9C, lanes 6 to 9) and negative-stranded Sendai virus RNA with oligonucleotide 1 (sense) (Fig. 9C, lane 5), using M-MuLV reverse transcriptase, resulted in extended products ranging from 200 to >764 bases with all the RNA samples except viral genomic RNA. Appropriate controls (Fig. 9C, lanes 2 to 4) were used to justify the primer, RNA, and enzyme dependence of the extended cDNA products.

**DISCUSSION**

Nearly two decades have elapsed since the early proposition about the involvement of hydrolytic enzymes, especially proteases, in the process of enveloped virus-cell fusion (29). Although Israel et al. (21) reported such proteolytic activity in intact Sendai virus particles or with their reconstituted envelopes (F- and HN-virosomes) and studied its involvement in virus-membrane fusion, a detailed molecular characterization of the protease, in terms of the structure-function relationship, has remained unexplored. The role of the virus-associated proteases was surmised for the hydrolysis of cell surface glycoproteins for unmasking of the targeted phospholipid bilayer to the action of the viral fusion factor following a tight attachment. A similar role has been recently postulated for muscle cell fusion during the formation of multilamellar myotubes (42). In this regard, the metalloprotease domain of meltrin-α (a transmembrane protein) is believed to promote cell-cell interactions, leading to membrane fusion by degrading some extracellular matrix. We have recently shown that F-virosomes (prepared from Sendai virus grown in the allantoic sac of 10- to 11-day-old embryonated chicken eggs at 37°C) can bind and fuse to hepatoblastoma cells (HepG2 cells) in culture (and liver cells in vivo) through a strong interaction between the terminal galactose moiety of F protein and asialoglycoprotein receptor on the membrane of the HepG2 (liver) cells (5–7, 9). It has also been observed that HN glycoprotein is not essential for F-induced membrane fusion, provided that F can be tightly bound to the target membrane, either through a heterologous membrane ligand like influenza virus hemagglutinin (8) or through carbohydrate-carbohydrate interactions (23), and that Sendai virus and F-virosomes treated with PMSF and APMSF (specific serine protease inhibitors) failed to catalyze membrane fusion.

To explore the mechanism underlying the inhibition of membrane fusion by PMSF and APMSF, we initiated systematic electrophoretic analysis of F-virosomes as well as their proteolytic activity. By using the improved molecular sieving properties (26) of classical Laemmli SDS-PAGE (10% gels) under reducing conditions, two Coomassie blue stained bands, 45K and 14K, were visualized (Fig. 1A, lane 2), which correspond to the F1 and F2 subunits of F protein, respectively (35). On the other hand, under nonreducing conditions, two bands of 60K (accounting for intact F protein) and 45K were observed (Fig. 1A, lane 1). The Western blot pattern (Fig. 1B) and autoradiograph (Fig. 1C) under similar nonreducing conditions also reveal the additional 45K band. This 45K band remained unnoticeable, because it comigrated with the F1 band in the presence of reducing reagents. This notion is strengthened by the protein profile of purified Sendai virus, whereby a distinct band corresponding to M, 45,000 is visible under nonreducing conditions (Fig. 2). Further evidence regarding the
identity of the 45K band as distinct from F1 comes from iso-electric focusing, which revealed that its pI was 3.7 (Fig. 3). This is at variance with the reported value (36) for F protein (pI 4.9). The glycoprotein nature of the 45K species and the presence of the terminal β-galactose moiety in its oligosaccharide chain were confirmed by PAS staining (Fig. 4B) and Western blotting with anti-RCAl antibodies (Fig. 4A), respectively. The unique identity of the 45K protein is bolstered by the absence of a cross-reaction between polyclonal antisera against F and 45K alone and the failure of anti-F monoclonal antib-

![FIG. 7. Temperature dependence of the formation of biologically active F-45K glycoprotein complex on the Sendai viral membrane. (a) Electrophoretic analysis: Purified Sendai virus grown at 37°C (lane 1, 60 μg of protein), 38.5°C (lane 2, 60 μg of protein), and 40°C (lane 3, 60 μg of protein) was resolved by SDS-PAGE under nonreducing conditions, as described in the legend to Fig. 1, and visualized by Coomassie blue staining. F-virosomes prepared from Sendai virus grown at 37°C (lane 4, 5 μg of protein), 38.5°C (lane 5, 5 μg of protein) and 40°C (lane 6, 5 μg of protein) were resolved by SDS-PAGE under nonreducing conditions on a 10% gel and visualized by Western blotting with anti-F-virosome serum. (b) Fusion activity. Sendai virus from each lot was labeled with R18 as described in the text. The kinetics and extent of fusion activity of Sendai virus, grown at different temperatures of propagation with HRBC, were monitored by the fluorescence dequenching assay for membrane fusion as described in Materials and Methods. The points, corrected with data from appropriate controls, are averages of three independent experiments. (c) Proteolytic activity. Degradation of 125I-histone by F-virosomes, prepared from Sendai virus (grown at various temperatures), was determined as described in Materials and Methods. Each point is an average of three independent experiments.]

![FIG. 8. Effect of protease K treatment of HRBCs on their subsequent lysis by Sendai virus. Limited proteolytic digestion of HRBC membrane was carried out as described in the text. The virus-cell fusion was measured as percent hemolysis of the RBCs as detected by the release of hemoglobin from the lysed cells (5). The amount of hemoglobin released in the presence of 0.3% TX-100 was taken as 100% lysis, and the percent inhibition of hemolysis was determined. HRBCs incubated at 37°C for the same times as the experimental samples were taken as positive controls. Each point is mean of three independent determinations.]

<table>
<thead>
<tr>
<th>Virus or protein</th>
<th>Distance from N terminus</th>
<th>Sequence</th>
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<td>E GV F H T L W H d X T</td>
</tr>
<tr>
<td>JEV</td>
<td>43</td>
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<tr>
<td>TBE</td>
<td>46</td>
<td>K GV L H T M W H V T</td>
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Aligned sequence of the N-terminal 11 amino acids of the 45K protein and flavivirus NS3 protein serine protease domain.

Abbreviations for the names of the viruses: JEV, Japanese encephalitis virus; KUN, Kunjin virus; MVE, Murray valley encephalitis virus; WNV, West Nile virus; DEN2, Dengue virus type-2; DEN4, Dengue virus type-4; YF, Yellow fever virus; TBE, tick-borne encephalitis virus (viral sequences are taken from the review by Chambers et al. [13]).

Numbers refer to the distance of the first residue shown from the N termini of the proteins.

The amino acid component (histidine) of the putative catalytic triad.
ties to bind to the 45K protein (Fig. 5). These data indicate that the 45K glycoprotein is an integral constituent of the Sendai viral membrane distinct from F protein.

We have demonstrated a significant level of proteolytic activity in F-virosomes (Fig. 6b) with both the F and 45K glycoproteins, comparable to that observed in the Sendai virus and its reconstituted envelopes (F- and HN-virosomes) previously (21). A pH optimum of 7.0 ± 0.5 and a maximal activity at 37°C conform to the conditions required for virus-cell interactions (5). Moreover, the APMSF sensitivity of the F-virosome-induced 125I-histone degradation, in addition to PMSF and heat-inactivated buffer, supports its serine protease nature.

Similar treatments are known to inhibit the viral hemolytic and heat-induced inhibition, supports its serine protease nature. The limited proteolysis of the target cell membrane (HRBCs) with proteinase K led to its decreased susceptibility to fusion (exhibited by lower hemolysis) (Fig. 8) but did not affect the binding with virus (shown by unaltered hemagglutination), strongly suggesting the existence of a poorly characterized proteinaceous membrane component (substrate for protease) through which the F-45K complex could associate and lead to fusion, in agreement with a previous observation that proteolytic cleavage of RBC membrane components occurs during Sendai virus-induced hemolysis (29).

The limited proteolysis of the target cell membrane (HRBCs) with proteinase K led to its decreased susceptibility to fusion (exhibited by lower hemolysis) (Fig. 8) but did not affect the binding with virus (shown by unaltered hemagglutination), strongly suggesting the existence of a poorly characterized proteinaceous membrane component (substrate for protease) through which the F-45K complex could associate and lead to fusion, in agreement with a previous observation that proteolytic cleavage of RBC membrane components occurs during Sendai virus-induced hemolysis (29).

The N-terminal 11-amino-acid sequence of the 45K protein (Table 1) exhibited no homology to any known Sendai virus protein sequences or to those of the host cell proteins (chicken). However, the 45K N-terminal sequence exhibits a strong homology to the N-terminal region of several flavivirus NS3 proteins (Table 1) known as serine proteases (13). Furthermore, the ninth amino acid, histidine, of the sequence is identical to that of other flavivirus NS3 proteins, which is considered to be a part of the putative catalytic triad of serine proteases. NS3 serine protease is known to be required for hepatitis C virus polyprotein processing during its replication and maturation (37). An analogous role for the 45K protein is improbable in the context of our present knowledge of paramyxovirus biology. However, this homology certainly upholds our belief that the F-45K complex is a serine protease.

To determine the origin of the 45K protein, degenerate oligonucleotides, corresponding to the sense (oligonucleotide 1) and antisense (oligonucleotide 2) sequences were synthesized.
sized. Oligonucleotide 1, which is same as the sense sequence of the gene encoding the 45K protein, failed to hybridize with the negative-strand Sendai virus genomic RNA (Fig. 9B, dot 4). On the other hand, both oligonucleotides 1 and 2 (antisense sequence) hybridized efficiently with the genomic DNA of CAM (host cells) (Fig. 9A, dots 4 to 7 and 9B, dot 3). Moreover, oligonucleotide 2 hybridized with the total cellular RNA from the host cells (Fig. 9B, dots 1 and 2). These results point toward a host origin of the 45K protein. Since hybridization signals were also observed with other host DNA samples, namely, from HeLa and MDCK cells, we conclude that this protein may be ubiquitous in nature. This contention is further supported by the observation that culture-grown Sendai virus (in MDCK cells) also exhibits a protein of 45K in its membrane analogous to its corresponding counterpart from egg-grown virus. The primer extension with these oligonucleotides plus the host total RNA or viral genomic RNA exhibited extended products for host RNA samples but not for viral RNA (Fig. 9C), further strengthening the notion of its host origin. Total cellular RNA from uninfected cells also yielded extended products, although the intensity of these bands was lower than that of bands due to RNA from infected cells (Fig. 9C). This leads to the conclusion that the 45K protein is a host protein produced normally to carry out some unknown host cellular process and that its synthesis by the host cells is increased during Sendai virus infection. Since virus/virosome-cell fusion events are closely related processes (10); this conforms to the notion that this protein is essential for the infectivity of the virus produced.

Although Rapaport and Shai (32) have described the fusion of liposomal membranes induced by synthetic fusion peptides corresponding to a sequence of Sendai virus F protein, such studies do not necessarily reflect a biologically relevant fusion process. Moreover, collective data suggest that enveloped viruses with pH-independent fusion activity may require host cell factors distinct from the virus receptor for optimal fusion (40). In support of this notion, a recent review (25) has speculated on the role of a putative target membrane receptor, “maybe a cellular protein,” in the docking of F protein as a prelude to a conformational change required for fusion. However, the molecular trigger for this conformational change is difficult to comprehend (10). The 45K protein being held tightly with the F protein through hydrophobic interactions may account for the stability of the F-45K complex on the viral surface as well as may act as “trigger” for the required fusion-active conformational change at the right place and at the right time (Fig. 10). This proposed model conforms to our earlier observation that fusion between membranes of Sendai virus and erythrocyte ghost occurs at a 30-fold-higher rate than with liposomes (34). Here, too, some unidentified target membrane component (protein factor X) was postulated to play a vital role in biologically significant Sendai virus-induced membrane fusion. The results of our present study on the reduction of hemolysis
(not hemagglutination) of proteinase K-treated HRBCs strongly suggest the existence of a putative coreceptor through which the F-45K complex could associate by virtue of being a protease (Fig. 10). An analogous model has been postulated whereby host proteins associated with the HIV envelope form an “adhesion patch” preceding the virus-cell fusion (4). This is further bolstered by the observation that molecules other than CD4 may be required for membrane fusion induced by the HIV-1 envelope (15, 16). Membrane fusion is believed to be triggered by the docking of this F-45K complex to some target membrane protein component via a putative protease-substrate interplay. Protease activity of the F-45K complex may be held responsible for the simultaneous removal of surface glycoproteins and the concomitant exposure of the “fusion peptide” of F protein to permit fusion between apposing lipid bilayers. This process is independent of the binding of intact virus to the target membrane through HN protein, as exhibited by inhibition of fusion (hemolysis) but not hemagglutination with proteinase K-treated HRBCs (Fig. 8). Overall, this process may be considered analogous to the dissociation of gp120 from HIV-1 virions induced by CD4 binding, which finally leads to the fusion-active conformational changes in gp41 (16). The detailed molecular mechanism of membrane fusion, in the light of this 45K moiety, can now be addressed. It will be of interest to discuss and critical consideration of the manuscript. We thank S. Shankar for help in N-terminal sequencing. We also thank Poonam Chhikara for help in raising polyclonal antisera. We are grateful to A. B. Mukherjee and G. C. Kundu for their generous help. This work was supported by the Council of Scientific and Industrial Research and Department of Biotechnology, Government of India. M.K. is a recipient of a senior research fellowship from the University Grants Commission, Government of India.

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