Enhancement of membrane-fusing activity of Sendai virus by exposure of the virus to basic pH is correlated with a conformational change in the fusion protein

(paramyxovirus/cell fusion/hemolysis/virus penetration/circular dichroism)

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ABSTRACT The effect of pH on the membrane-fusion activity of Sendai virus was examined (pH 5.0–9.5) by using, as assays of activity, hemolysis of chicken erythrocytes and the fusion of baby hamster kidney (BHK-21) cells. Exposure of virus to basic pH increased fusion activity; the optimum pH was found to be ≈ 9.0 . All assays were carried out at pH 7.0, and the virus retained enhanced fusion activity after it was exposed to basic pH and returned to neutral pH. The enhanced fusion activity was correlated with an irreversible conformational change in the fusion protein (F protein) of the virus, as demonstrated by a change in the circular dichroism spectrum of the protein.

The fusion protein (F protein) of paramyxoviruses, which forms spike-like projections on the surface of the virus, is involved in the fusion of the viral membrane with target cell membranes (1–3). This fusion event is responsible for several biological activities of the virus—i.e., virus penetration, cell fusion, and hemolysis. The active F protein consists of two disulfide-linked polypeptides, F_1 and F_2 , which are derived from an inactive precursor, F_0 , by proteolytic cleavage by a host cell enzyme (4). Cells that lack a protease capable of cleaving F_0 produce fusion-inactive, noninfective virions (1–3). We have found (5) that isolated F protein reconstituted into membranes with phosphatidylcholine showed fusion activity, if a mechanism for attachment of the F protein to cell membranes was provided. This could be provided not only by the receptor-binding protein (virus hemagglutinin and neuraminidase protein; HN protein) of the virus but also by a lectin such as wheat germ agglutinin.

Much effort has been directed to the understanding of the mechanism of virus-induced membrane fusion. Phospholipase activity in the virus was excluded in early studies (6). The possibility that fusion involved proteolytic activity of the F protein (7) on the target membrane was made unlikely by the finding of Haywood (8) that virions could fuse with liposomes lacking proteins. The relevance of fusion of virus with such vesicles *in vitro* to fusion of virions with cell membranes was recently established by our finding that virus containing cleaved F protein, but not those with uncleaved F_0 , could fuse with liposomes containing phospholipids and cholesterol (unpublished experiments).

A direct hydrophobic interaction between the F protein and the target membranes was proposed on the basis of (i) the finding of a long hydrophobic amino acid sequence at the NH_2 terminus generated by the cleavage and (ii) the fact that this sequence is highly conserved among paramyxoviruses (9–11). In addition, oligopeptides with sequences resembling this NH_2 -terminal sequence were found to specifically inhibit the fusion action of the F protein (11). A fusion mechanism involving a hydrophobic interaction was further supported by our finding that the activating cleavage of the F protein involves a confor-

mational change with exposure of a new hydrophobic region on the protein (12).

The membrane-fusion activity of paramyxoviruses occurs over a wide pH range; however, an optimum pH of 8.4–8.5 was found for cell fusion and hemolysis by the paramyxovirus SV5 (13, 14). We have investigated the effect of pH on the membrane-fusing activity of Sendai virus and have confirmed that the fusing activity is enhanced at basic pH; we also found that this elevated activity remains when the virus is returned to neutral pH. An irreversible change in the conformation of the F protein was found by measuring the circular dichroism (CD) of the protein, which correlated with the increased membrane-fusing activity of the virus.

MATERIALS AND METHODS

Virus. The RU and Z strains of Sendai virus were grown in 10-day-old embryonated chicken eggs, harvested 48 hr after infection, and purified by repeated pelleting as described (5). Virus was suspended in 10 mM phosphate/150 mM NaCl, pH 7.2, and stored at -70°C . Viral protein concentration was determined by a modified Lowry procedure (15).

Hemolysis Assays. Virus pelleted from stock virus suspensions (2 mg of viral protein per sample) was resuspended in 1 ml of each of the following buffers: 25 mM Na acetate, pH 5.0, 5.5, and 6.0; 25 mM Na phosphate, pH 6.5, 7.0, and 7.5; 25 mM Tris-HCl, pH 8.0, 8.5, and 9.0; and 25 mM NaHCO_3 , pH 9.5. Virus suspensions were kept on ice for 1 hr and then loaded onto discontinuous sucrose gradients consisting of 2 ml of 50% sucrose and 8 ml of 20% sucrose in 10 mM Na phosphate/150 mM NaCl, pH 7.0 (P_i/NaCl), and centrifuged at 28,000 rpm for 3 hr in a Spinco SW 41 rotor. The virus was collected at the 50%/20% sucrose interface and dialyzed against P_i/NaCl overnight; then the hemagglutination titer and viral protein concentration were determined. Chicken erythrocytes, 2–4 days old, were washed twice and resuspended in P_i/NaCl to a final concentration of 0.5%. Virus samples exposed to each pH (1.2 μg of viral protein in 0.5 ml of P_i/NaCl containing 16 hemagglutinating units) were mixed with 0.5 ml of erythrocytes in P_i/NaCl , pH 7.0, kept on ice for 20 min, and shifted to a 37°C water bath. Multiple samples of virus exposed to each pH were prepared, and one set of samples was removed from the water bath at each time point, chilled on ice for 5 min, and spun in an Eppendorf centrifuge at 4°C for 7 min. The clear supernatants were removed, and their absorbance at 545 nm was read in a Zeiss PM6

Abbreviations: NP protein, virus nucleocapsid protein; F protein, virus fusion protein; HN protein, virus hemagglutinin and neuraminidase protein; BHK-21, baby hamster kidney cells; P_i/NaCl , 10 mM sodium phosphate/150 mM NaCl, pH 7.0; CD, circular dichroism.

spectrophotometer. Readings of control samples of erythrocytes without virus were taken for each time point and subtracted from the readings of virus-containing samples.

Permeability of the Virus Envelope to Macromolecules and Salt. After the virus was exposed to different pHs and purified as described above, possible damage to the viral envelope was examined by the accessibility of the viral nucleocapsid protein (NP protein) to trypsin added to the virus suspension and by penetration of uranyl acetate stain into the virus as visualized with electron microscopy (16). Virus suspensions, 40 μ g of viral protein in 40 μ l of P_i /NaCl were incubated with 0.04 μ g of trypsin at 37°C for 20 min. Soybean trypsin inhibitor (0.05 μ g) was added, followed by 10 μ l of 250 mM Tris-HCl, pH 6.8/10% NaDodSO₄ and 5 μ l of 1.5% dithiothreitol. The samples were boiled for 2 min and subjected to electrophoresis in a 10% polyacrylamide gel with 0.25% *N,N'*-methylenebisacrylamide as described (5). To detect stain-penetrable virus particles, virus suspensions were fixed with 2% formaldehyde of the same pH on grids coated with Formvar film and carbon, washed in distilled water, and stained with 2% uranyl acetate. Specimens were examined on a Philips EM 300 electron microscope.

Fusion of Baby Hamster Kidney (BHK-21) Cells. Pellets of the Z strain of Sendai virus (2.2 mg of viral protein) obtained from stock suspensions were resuspended in 0.5 ml of 25 mM Tris-HCl at pH 7.0 or 9.0. Virus suspensions were kept on ice for 1 hr, and the hemagglutinating units were adjusted to 12,800 units/ml with 7.5 ml of reinforced Eagle's medium with 2% calf serum. Confluent monolayers of BHK-21 cells in 10 cm² plastic Petri dishes containing 2.4-cm round cover slips were washed twice with ice-cold isotonic phosphate-buffered saline, pH 7.2 (17); 400 μ l of virus (5,120 hemagglutinating units) was added to each dish, which was placed immediately into a 37°C incubator. Duplicate dishes were removed at each time point and washed three times with ice-cold phosphate-buffered saline. Cells were fixed with 1 ml of methanol at room temperature for 20 min and stained with Giemsa's stain (Fisher) for 5–10 min. Cover slips were removed from the Petri dishes and mounted with Gurr UVinert Mountant (Hopkin and Williams, Essex, England). Photographs were taken with a Zeiss light microscope and Kodak Plus X film, and the extent of cell fusion was expressed as the percentage of total nuclei in polynucleated cells.

CD Measurements. The F protein was purified from Sendai virions as described (5). In brief, the F, HN, and M proteins were solubilized with 2% Emulphogene BC-720/0.5 M NaCl/10 mM Na phosphate, pH 7.4. The M protein was precipitated after dialysis against water. The F and the HN proteins were then separated by column chromatography on a fetuin-Sepharose column in 10 mM phosphate/citrate buffer (pH 5.5) containing 0.1% Emulphogene BC-720. The HN protein adsorbed to the column and the F protein came through. The fractions containing the F protein were identified by protein determinations, and the purity of the protein was determined by polyacrylamide gel electrophoresis (5).

Before CD measurements, 3 ml of the F protein solution collected from the fetuin-Sepharose column was dialyzed against 10 mM Na phosphate buffer, pH 7.0/0.1% Emulphogene BC-720, and the dialysate was divided into three equal portions: two were titrated to pH 9.0 with 1 M NaOH; after 1 hr on ice, one of the pH 9.0 solutions was titrated to pH 7.0 with 1 M HCl. The volume of the NaOH and HCl solutions used to adjust the pH was $\approx$ 1% of the total volume. Circular dichroism measurements were taken on the three F protein samples with a Jobin-Yvon Auto-Dichrograph, model Mark V, interfaced with a computer capable of averaging multiple scanings. This instrument was generously made available to us by E. Breslow

of the Biochemistry Department, Cornell University Medical College. Before each run, the spectropolarimeter was calibrated with *d*-10-camphorsulfonic acid (Eastman) twice recrystallized from ethyl acetate). A cell of 1-mm path length was used. Spectra were taken at room temperature with constant purging with nitrogen gas. To ensure the reliability of the spectra taken at the far UV wavelength, the CD spectrum of myoglobin was taken periodically and compared with the known spectrum.

RESULTS

Effect of Exposure of Virus to Different pHs on Virus-Induced Hemolysis. Previous studies in this laboratory showed that hemolysis and cell fusion by the paramyxovirus SV5 occur at neutral pH and over a wide pH range; however, these activities are enhanced at basic pH, with an optimum at 8.4–8.5 (13, 14). Investigation of the effect of hemolytic activity of Sendai virus that had been exposed to various pHs and then returned to pH 7.0 showed that exposure to basic pH caused an irreversible increase in hemolytic activity. Virus that had been exposed to pH 9.0 showed the highest hemolytic activity when compared to virus exposed to other pHs (Fig. 1). Ten minutes after the virus/erythrocyte mixture was shifted to 37°C, the amount of hemoglobin released by virus exposed to pH 9.0 buffer was $\approx$ 2 times that of virus exposed only to pH 7.0. It should be noted, however, that virus exposed to acid or neutral pH did cause some hemolysis; A readings from control erythrocyte samples without virus were subtracted from the values obtained with virus. Thus, unlike Semliki Forest virus and influenza virus, which hemolyze well only at acidic pH (18–20), Sendai virus is active over a pH range from 5 to 9, and shows no significant difference in hemolysis between pH 5 and 7. The hemagglutinating titer per unit of viral protein was determined for each virus preparation after exposure to different pHs, and no difference in hemagglutinating activity was found (data not shown). Because all hemolysis assays were carried out at pH 7.0 in P_i /NaCl, an effect of pH on the erythrocytes could not have contributed to the observed enhanced hemolysis. These results suggest that an irreversible, basic pH-induced structural change in the virus had occurred that did not affect hemagglutination but did result in enhanced hemolytic activity.

FIG. 1. Enhancement of Sendai virus-induced hemolysis of chicken erythrocytes by exposure of the virus to basic pH. Virus was treated with buffers at different pHs and purified in a sucrose gradient. Equal amounts of virus were mixed with erythrocytes in P_i /NaCl, pH 7.0, and incubated on ice for 20 min and then at 37°C for 10 min. A was measured after the pelleting of erythrocytes, and measurements from control samples without virus were subtracted.

Enhanced Hemolysis Is Not Due to Damage to the Viral Membrane. Hemolysis of erythrocytes by Sendai virus follows fusion of the viral membrane with the plasma membrane of the erythrocytes (21, 22); however, Homma and co-workers (16, 23) showed that fusion of the membranes alone did not necessarily lead to the hemolysis. Sendai virus harvested 24 hr after infection of embryonated chicken eggs fused with erythrocytes without causing hemolysis, whereas virus harvested 48 hr after infection not only fused with but also hemolyzed the cells. Only the late-harvest virus was seen with the electron microscope to be penetrated by uranyl acetate (16). Damage to the viral membrane appeared to provide channels for the stain and for the hemoglobin molecules.

To determine if basic pH damaged the Sendai virus envelope and if this were responsible for the enhanced hemolysis, we added trypsin to the virus suspension. When the nucleocapsid of paramyxovirus is not protected by the viral envelope, trypsin cleaves the NP protein to a polypeptide with a M_r of $\approx 34,000$ and to smaller fragments (24, 25). Fig. 2 shows that exposure of Sendai virus to basic pH did not render the viral NP protein susceptible to the trypsin that was added to the virus suspension, although, as expected, digestion of the NP protein did occur if the virus was disrupted with Triton X-100. A small amount of free nucleocapsid from disrupted virions is present in all virus preparations, as shown (Fig. 2, lanes b) by the appearance of t4, which is the M_r 34,000 polypeptide derived from NP protein. However, the amount of trypsin-digestible NP protein did not increase on exposure of the virus to basic pH. In the control Triton X-100-disrupted virus, there was complete degradation of NP protein and other viral proteins (Fig. 2, lanes c).

Possible damage to the viral membrane at basic pH was further studied by investigating the penetration of uranyl acetate stain into the virus. Table 1 shows the percentage of viral particles that are penetrated by uranyl acetate as visualized with the electron microscope. Cycling freshly harvested virus and frozen and thawed virus from pH 7.0 to pH 9.0 and back to pH 7.0 did not increase the number of uranyl acetate-containing particles; indeed, there was a slight decrease. As found by Shi-

Table 1. Penetration of uranyl acetate stain into Sendai virus exposed to different pHs

Virus*	pH	% virus particles penetrated†
Not frozen	7‡	13, 15
	7-9-7§	5, 2
Frozen and thawed	7*	26, 29
	7-9-7‡	21, 20

* Virus was harvested 2 days after inoculation, purified, and used directly or after freezing and thawing.

† The two values were results with different preparations of the same virus harvest. Each percentage value represents 200-300 virus particles counted.

‡ Virus was exposed only to P_i /NaCl.

§ The pH 7.0 virus suspension was titrated with 1 M NaOH to pH 9.0, held on ice for 1 hr, and then titrated back to pH 7.0 with 1 M HCl.

mizu *et al.* (16), freezing and thawing increased the number of virus particles penetrated by the stain. These results indicate that the enhanced hemolysis caused by exposure of the virus to alkaline pH could not be explained on the basis of increased permeability of the viral envelope.

Effect of Exposure of Virus to Basic pH on Virus-Induced Cell Fusion. To determine if Sendai virus exposed to basic pH also shows higher cell-fusing activity for cultured cells, BHK-21 cells were treated with the Z strain of Sendai virus. Virus previously exposed to pH 9.0 caused a more rapid progression of cell fusion than virus exposed only to pH 7.0 (Fig. 3). After 2 hr, fusion had progressed to such an extent in both cases that no difference could be detected.

Effect of Exposure of Virus to Basic pH on the Conformation of the F Protein. The above findings suggested that a change in the F protein was responsible for the observed increased fusion activity of Sendai virus upon exposure to basic pH. We have shown (12) that a conformational change in the F protein accompanied the activation of fusion by proteolytic cleavage. CD measurement of the purified F protein in the nonionic detergent Emulphogene BC-720 after exposure to different pHs showed that another conformational change did

FIG. 2. Lack of susceptibility of the NP protein of Sendai virus to trypsin digestion after exposure of the virus to basic pH. Sendai virus was treated with buffer at different pHs and purified in a sucrose gradient. Trypsin was added, the virus suspensions were held at 37°C for 20 min, and aliquots of each virus sample were examined by electrophoresis in a polyacrylamide gel. Lanes: a, virus treated with buffer at each pH; b, virus in lane a treated with trypsin; c, virus in lane a treated with trypsin in the presence of 0.1% Triton X-100. t4 is a trypsin cleavage product of the NP protein; t1, t2, and t3 are unidentified cleavage products. The F_2 polypeptide of the F protein comigrated with the dye front in the presence of dithiothreitol.

FIG. 3. Fusion of BHK-21 cells by Sendai virus exposed to different pHs, expressed as the percentage of the total nuclei in polynucleated cells. Virus was treated with buffer at pH 7.0 (○) or pH 9.0 (●) and diluted in reinforced Eagle's medium with 2% calf serum. Confluent monolayers of BHK-21 cells on coverslips in 10-cm² Petri dishes were inoculated with 5,120 hemagglutinating units of virus, incubated at 37°C for the indicated times, and fixed and stained. Photographs were taken, and 250-350 nuclei were counted for each point.

FIG. 4. CD of the F protein in 0.1% Emulphogene BC-720 solution at pH 7.0 (—), after the titration of the pH 7.0 solution to pH 9.0 (---), and after the titration of the pH 9.0 solution back to pH 7.0 (.....). The concentration of F protein was 37 $\mu\text{g}/\text{ml}$. A cell of 1-mm path length was used, and the spectra were recorded after 30 accumulated scans.

indeed result from exposure to basic pH, and that this change was irreversible (Fig. 4). When the pH of the F protein solution was raised from pH 7.0 to 9.0, a change in the CD spectra was seen at wavelengths below 200 nm. As the pH was shifted from pH 9.0 back to 7.0, the F protein continued to show the CD spectrum characteristic of pH 9.0. Thus, there is a correlation between a conformational change in the F protein and the irreversible, enhanced fusion activity of the virus after exposure to basic pH and return to neutral pH. No difference in the CD spectra of the F protein at wavelengths of 200–250 nm was observed at the pHs studied. We have found previously that the F protein has a rather high helical content that is increased on cleavage—i.e., 75% for the cleaved active F protein in Emulphogene BC-720 solution (12). Because the helical structure of proteins contributes more to the CD spectrum at 200–250 nm than do the β -sheet and the random-coil structures of proteins (26, 27), the helical content of the F protein is probably not altered greatly by exposure of the protein to basic pH. The observed change in CD spectra at <200 nm is likely to be due to change in the β -sheet, random-coil, or other nonspecified protein structure. Satisfactory quantitative analysis of the change is not possible because of the conflicting reports on the intrinsic CD spectra of the β sheet and the random coil of proteins at this wavelength (26, 27). The conformational change that accompanies the proteolytic activation of the F_0 precursor protein and is detected by CD at 200–250 nm results in increased binding of Triton X-100 to the cleaved active F protein, as demonstrated by the technique of sedimentation of the protein-detergent complex in gradients of different density (12). The pH-induced conformational change of the cleaved active F protein described here did not result in any detectable further increase in detergent binding, as demonstrated by the finding that the complex of Triton X-100 and F protein that had been exposed to pH 9.0 sedimented precisely with the complex of Triton X-100 and F protein that had been exposed only to pH 7.0 (data not shown).

DISCUSSION

These studies have shown that once exposed to basic pH, Sendai virus maintains an increased membrane-fusing activity after the virus has been returned to neutral pH, and this increased fusion

activity is correlated with an irreversible conformational change of the F protein. This increased fusion activity cannot be explained by an effect on virus attachment, which is mediated by the viral HN protein, or by increased permeability of the viral envelope. Thus, the observed parallel change in the conformation of the F protein appears to be involved in the enhanced fusing activity. We reported earlier (12) that the activating proteolytic cleavage of the F protein involves a conformational change in the protein with exposure of a new hydrophobic region, which supported the concept of a direct hydrophobic interaction of the F protein with the target membrane during fusion. In the present study, no further increase in surface hydrophobicity of the F protein was detected at pH 9.0, as measured by the amount of Triton X-100 bound to the protein. The available evidence suggests that an interaction of the F protein spikes with the target membranes leads to the fusion of the lipid bilayers of the virus and the target cell membranes. How the conformation of the F protein at basic pH enhances this fusion process remains to be determined.

It should be emphasized that paramyxoviruses can fuse with plasma membranes at physiological pH; thus, although exposure to basic pH enhances fusion activity, high pH is not a requirement. Evidence was reported recently that three other enveloped viruses—Semliki Forest virus, vesicular stomatitis virus, and influenza virus—fuse with membranes at acidic pH (28–30). For these viruses, the lysosome has been suggested as the site where membrane fusion and penetration of the viral genome into cytoplasm occurs. With the exception of influenza virus, in which the hemagglutinating protein has been implicated in the fusion of the membranes, evidence for direct involvement of a specific viral protein in the fusion event at acid pH has not been clearly established. After our demonstration that the activating cleavage of the F protein of Sendai virus was accompanied by a conformational change in the protein with exposure of the new hydrophobic region (12), a similar observation on the hemagglutinating protein of influenza virus was reported by Skehel *et al.* (31). The influenza hemagglutinating protein also requires proteolytic cleavage for its activation (32–34), and a hydrophobic region is exposed at acidic pH (31). A similar mechanism of hydrophobic interaction could mediate the fusion of the different enveloped viruses with cell membranes, with the variation among viruses depending on the pH at which the viral protein involved in membrane fusion acquires the optimum conformation for fusing activity.

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