Activation of the Sendai Virus Fusion Protein (F) Involves a Conformational Change with Exposure of a New Hydrophobic Region*

Ming-chu Hsu, Andreas Scheid, and Purnell W. Choppin
From The Rockefeller University, New York, New York 10021

The F protein of paramyxoviruses is actively involved in the induction of membrane fusion. This fusion may be between viral and cellular membranes, as in the initiation of infection or in virus-induced lysis of erythrocytes, or between the plasma membranes of different cells. The F protein is activated by proteolytic cleavage to yield two disulfide-linked polypeptides (F1 and F2); however, its mechanism of action is not clear.

In the present study, the conformations of the inactive, uncleaved precursor glycoprotein (F0), and the active, cleaved form (F1,2) have been compared. The UV circular dichroism spectra of the two forms of the F protein indicate that cleavage results in a conformational change. Detergent-binding studies by velocity sedimentation analysis of Triton X-100-protein complexes revealed an increase in exposed hydrophobic surface of the protein on cleavage. The inactive F0 bound an estimated 27 molecules of Triton X-100/F polypeptide; these molecules are presumably bound to the hydrophobic region of the glycoprotein that anchors the spike-like protein in the virus membrane and that is common to both forms of F. The active form, F1,2, bound 67 molecules of Triton X-100. This increase in the number of detergent binding sites upon F protein activation indicates the presence of a hydrophobic region that is peculiar to the active form, and that may be of functional significance in the membrane fusion reaction.

The membrane of paramyxoviruses contains two glycoproteins, HN and F, which form spike-like projections on the external surface of the virion (1). The HN protein has neuraminidase activity and is responsible for attachment of virions to cell surfaces (2, 3). The F protein is involved in the membrane fusion that joins the viral membrane with target membranes, an event which is reflected in the expression of several biological activities of the virus, i.e. virus penetration, virus-induced cell fusion, and hemolysis (4–7). Virions that express these activities contain the F protein in the form of a disulfide-linked complex (F1,2), consisting of two glycopolypeptides (F1 and F2) which are derived by proteolytic cleavage of an inactive precursor (F0) by a host cell enzyme. F0 is present on inactive virions that are produced by cells which lack a suitable protease for F protein activation (4–6, 8).

Efforts have been made to study the fusion reaction in systems that are chemically better defined than are the natural reactants, i.e. intact virions and biological membranes. Fragments of the viral envelope (9) and glycoprotein extracts reconstituted with lipid are active in the fusion reaction (10–13). The isolated lipid-free F1,2 protein cannot induce membrane fusion; however, when the isolated F1,2 protein is reconstituted with phosphatidylcholine into a membrane, fusion can be obtained, if a mechanism is provided to attach the F1,2-containing vesicles to the target membrane (13). This attachment mechanism can be provided not only by the HN protein, but also by wheat germ agglutinin (13). Evidence has been obtained that the viral membrane can fuse with multilamellar liposomes (14), suggesting that the protein of the natural target cell membranes may not be required.

The mechanism of F protein action is a matter of great interest, and some information has begun to emerge recently. One possible mechanism, i.e. the association of a phospholipase activity, was excluded previously (15), and other enzymic activities, such as a protease activity (16), are difficult to reconcile with the apparent lack of need for protein in the target membrane. Another possibility would involve a direct hydrophobic interaction of the F protein with the target membrane, and this has been discussed in the light of the finding of an unusually hydrophobic polypeptide sequence at the NH2 terminus of F1, which is created by the activating cleavage (17–20). That this region of the molecule at the cleavage site is important for activity is suggested by the conservation of the NH2-terminal sequence at the cleavage site among different paramyxoviruses and virus mutants (18, 19), and by the fact that oligopeptides with a structure resembling this region inhibit the action of the F protein (20).

The present study provides direct evidence for hydrophobic binding sites that are present on the active form of the F protein, but not on the inactive form, and that may represent a functionally important feature of the F protein. These experiments involve a comparison of the active and inactive F proteins with regard to secondary structure and capacity for binding of the nonionic detergent Triton X-100. The active form, F1,2, was isolated from wild type Sendai virus grown in embryonated chicken eggs. The inactive form, F0, was obtained from a Sendai virus mutant, pa-c1, grown in the same host. This mutant can be cleaved and activated by proteases such as chymotrypsin or elastase, but not by the proteases available in the chorioallantoic membrane of the chick embryo (6). This source of precursor protein was chosen because it yields F0 preparations that contain no F1,2, in contrast to the presence of some cleaved F protein in preparations of wild type virus grown in cultured cells, and because the use of the same host avoids variations in the host-specified carbohydrate component. The only known difference between the F proteins of the egg-grown wild type and mutant viruses is that the mutant virus F protein is not cleaved in the egg, presumably because of a point mutation at the cleavage site (18).

*This research was supported by research grants PCM-85-10991 from the National Science Foundation, CA-18213 from the National Cancer Institute, and AI-05600 from the National Institute of Allergy and Infectious Diseases. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Hydrophilic Sites of Sendai Virus F Protein

EXPERIMENTAL PROCEDURES

Materials—Triton X-100 was obtained from New England Nuclear (Boston, MA); Emulphogene BC-720, from GAF Corp. (New York, NY); and sodium cholate, from Calbiochem (Los Angeles, CA). Phos- phatidylcholine was prepared from egg yolk (21, 22). Ovalbumin and human γ-globulin were purchased from Sigma (St. Louis, MO), bovine serum albumin, from Miles Laboratories, Inc. (Kankakee, IL), and density gradient-grade sucrose, from Schwarz/Mann (Orangeburg, NY). [3H]Leucine was obtained from Amer- sham (Arlington Heights, IL) and D2O (99.79 D), from Merck, Sharp and Dohme (Montreal, Canada).

Virus—Wild type Sendai virus and the mutant pa-cl were grown in the allantoic sac of 10-day-old embryonated eggs, purified by repeated pelleting, and stored at −70°C.

Protein Preparation—For detergent-binding experiments, viral glycoproteins were selectively solubilized with 2% Triton X-100 in 10 mM Na phosphate, pH 7.5, and insoluble protein removed by centrifuga- tion at 100,000 × g for 45 min (2, 3). For circular dichroism (CD) measurements, the F glycoprotein was prepared as follows. Virions were disrupted in 2% Triton X-100, 10 mM Na phosphate, 1 mM NaCl, pH 7.5. The nucleocapsid and M protein were removed by centrifuga- tion and dialysis against low salt buffer, respectively (3, 5). The HN and F glycoproteins in the supernatant were separated and purified by column chromatography on Sephacore 4B with covalently linked fetuin (5, 23) as described (13).

Circular Dichroism—The F protein obtained from chromatog- raphy on fetuin-Sepharose was further purified and transferred from the UV-absorbing Triton X-100 solution by sedimentation into a 10-25% sucrose gradient containing 0.1% Emulphogene BC-720 and 10 mM Na phosphate, pH 7.5. Pooled fractions were passed through a Sephadex G-10 column (0.9 x 50 cm) equilibrated with the same buffer to remove sucrose. The final concentration of protein used for CD measurements was estimated by a modified Lowry method (24) that is not affected by the presence of detergents or lipid in the samples.

Reconstituted particles containing F protein and phosphatidylcholine were prepared as described (13). F protein obtained by chromatog- raphy on fetuin-Sepharose was transferred from the Triton X-100 solution into cholate-containing buffer by sedimentation into a 10-25% sucrose gradient with 2% sodium cholate, 1 mM NaCl, and 10 mM phosphate, pH 7.5. F protein-containing fractions were pooled and mixed with phosphatidylcholine (protein/lipid = 2:1, w/w) and cholate was removed by dialysis against 10 mM Na phosphate, 0.15 M NaCl, pH 7.2, at 4°C for 36-48 h.

Circular dichroism spectra were recorded with a Cary 60 spectro- polarimeter equipped with a CD attachment which was generously made available by Dr. M. Sonenberg at the Sloan-Kettering Institute, New York. An added diaphragm between the sample cell and the detector allows changes in the angle of detection by variation of the size of the diaphragm, and this enables one to determine effects from light scattering of the sample (25). Cells of 1 cm path length were used. Before each run the spectropolarimeter was cali- brated with a solution of d-10-camphorsulfonic acid (Eastman Kodak), twice recrystallized from ethyl acetate. Spectra were taken at room temperature with constant purging of nitrogen. The ellipticity curves obtained were analyzed on the basis of reference spectra derived by Greenfield and Fasman (26) and by Chen et al. (27, 28).

Density Gradient Centrifugation—Sedimentation coefficients ($s_{20,w}$) and partial specific volumes ($\gamma$) of protein-detergent complexes were determined using the procedure described by Smigel and Fleischer (29). Continuous sucrose gradients in cellulose nitrate tubes, $\gamma$ x 2% inches, were formed from solutions of 10% and 25% sucrose (w/w) in 10 mM Na phosphate and 0.1% Triton X-100, pH 7.5, in H2O or D2O. Triplicate aliquots of viral glycoprotein solutions, prepared in low salt buffer as described above under “Protein Preparation,” or of standard protein solutions (10 mg/ml), 0.2 ml/gradient, were overlaid on the gradients. After centrifugation at 55,000 rpm for 4 h (H2O gradient) or 6 h (D2O gradients) in a Spinco SW 60 rotor in a Beckman L2-65B centrifuge with the temperature set at 4°C, the run was terminated by deceleration with the brake off. Eight-drop fractions collected from the bottom, and refractive indices of every other fraction were read at 20°C in a Bausch and Lomb Abbé 3L refrac- tometer immediately after fractionation. Fractions of gradients with standard proteins were analyzed for protein content (24), and viral glycoprotein fractions were analyzed for radioactivity by mixing ali- quots with 0.5 ml of water and 4 ml of toluene/Triton X-100/Liquid- fluor (1600:1000:116) and measurement in a liquid scintillation counter.

The measured refractive indices were used to determine the density and the viscosity at each point along the gradient. The numerical values for density and viscosity, resulted in a series of $s_{20,w}$ and $\gamma$ values for the protein-detergent complex in H2O and D2O (29). The intersection of the two curves determines the $s_{20,w}$ and $\gamma$ values.

Because of the difficulty in determining the exact temperature of the gradients during centrifugation, the temperature that gave the correct $s_{20,w}$ for the standard proteins was used for all calculations (29). The corrected temperature was within 1°C of the setpoint. The computer program used by Smigel and Fleischer (29) was kindly made available by them and adapted to IBM 360 and PDP-11 computers with the generous help of Dr. James S. Murphy (from The Rockefeller University).

Polyacrylamide Gel Electrophoresis—Slab gels of 1.5 mm thick- ness with 10% polyacrylamide and 0.25% bisacrylamide in the separ- ating gel were prepared as described by Laemmli (30). Gels were run at 12 mA for 18 h and stained with 0.2% Coomassie blue in 50% methanol and 7% acetic acid.

Neuraminidase Assays—Neuraminidase assays were done by the procedure of Aminoff (31) as described previously (5) in 125 mM sodium acetate, pH 5.5.

RESULTS

Circular Dichroism of Cleaved and Uncleaved F Proteins—Fig. 1 shows the polyacrylamide gel analysis of the isolated F proteins used for these experiments. The F protein, isolated from egg-grown wild type Sendai virions and purified by column chromatography followed by preparative velocity sedimentation in sucrose gradients (see "Experimental Pro- cedures"), contains the two glycopolypeptides, F1 and F2, which are separated when run after reduction with dithiothreitol (lane B), but migrate as a disulfide-linked complex, F1,F2, in the absence of reducing reagent (lane C) (8). The F protein isolated from the mutant pa-cl grown in eggs is not cleaved (6), and is seen in the gel as the uncleaved precursor

FIG. 1. Polyacrylamide gel analysis of F proteins isolated from wild type Sendai virus and from mutant pa-cl. Pooled fractions of sucrose gradients containing F protein from wild type (lanes B and C) or mutant pa-cl (lanes D and E) virions were dialyzed, and aliquots were precipitated with 90% 1-butanol and denatured with sodium dodecyl sulfate in the presence (lanes B and E) or absence (lanes C and D) of 10 mM dithiothreitol. The outermost lanes show unfraccionated virion polypeptides of wild type (lane A) and mutant pa-cl (lane F) virions, including the major nonglycosyl- ated polypeptides, NP, M, and P, and the glycoproteins HN, F2, and F. Under the conditions used, F, migrated in the dye front. Gels were stained with Coomassie blue.
Hydrophobic Sites of Sendai Virus F Protein

F₀ (lanes D and E). A slight difference in the migration of reduced and unreduced F₀ (lanes D and E) has been ascribed to interchain disulfide bonds (8).

For measurement of the circular dichroism of the F protein in solution, nonionic detergent must be present to prevent aggregation, and because of the strong UV absorption of the Triton X-100 which was used for F protein isolation, it was necessary to transfer the protein into Emulphogene BC-720, a nonionic detergent without the UV-absorbing benzene ring of Triton X-100. The transfer was accomplished by sedimentation of the isolated F protein out of the Triton X-100 solution into gradients containing Emulphogene BC-720 as described under “Experimental Procedures.”

The CD spectra of the cleaved and the uncleaved F protein in 0.1% Emulphogene BC-720 solution (Fig. 2) show the negative ellipticity bands around 208 and 222 nm that are characteristic of a predominantly α-helical conformation. The comparison of the spectra indicates a greater α-helical component in the cleaved form of the F protein, as is evident from the greater negative ellipticity and from the position of the minima. A best fit analysis of the ellipticity curves using the reference spectra of Chen et al. (27, 28) gave the following distribution of the conformational components: For F₀, 60% α-helix, 15% β-sheet, and 25% random coil; and for F₁₀, 75% α-helix and 25% random coil. The reference spectra used for these calculations take into account the coupling of the transition moments along very long α-helices, and they yielded the curves that were closest to the observed ellipticities. With a carbohydrate content of the F protein of 15% (32), no significant contribution of carbohydrate to the CD spectrum in this wavelength range would be expected (33). The quantitative analysis of the circular dichroism spectra made use of protein concentrations that were determined by a modified Lowry method which eliminates the error from detergent and lipid (24). Without the knowledge of the primary structure of the F protein, a more precise determination of protein concentration is not possible at present, and the above numbers therefore do not indicate the absolute secondary structure of the protein. However, the inherent uncertainty of the Lowry procedure (34) applies equally to the cleaved and the uncleaved forms of the F protein, and would therefore not affect the observed qualitative differences.

To analyze the ellipticity of the F protein in the absence of detergent and in a state closer to its natural conformation in the virus envelope, the isolated F proteins were reconstituted with phosphatidylcholine (13). Under the conditions used and with a phosphatidylcholine and F protein ratio of 1:2 (w/w), reconstitution yielded vesicles of 40 to 80 nm diameter, with glycoproteins present as spikes that are similar in shape and arrangement to the spikes in the virus envelope (Fig. 3) (13).

With particles of this size, light scattering could contribute to the observed ellipticities, however variation of the acceptance angle of the detector between 8°C and 60°C did not influence the observed CD spectra, and this eliminates light scattering as a source of error. There is a small but consistent difference in the ellipticity curves of the cleaved and uncleaved proteins in the reconstituted membranes as is evident from the shape of the curves (Fig. 4). The quantitative analysis of the ellipticity curves (27, 28) resulted in 90% α-helix and 10% random coil for F₀, and 95% α-helix and 5% random coil for F₁₀. It has been reported that “bunching” of protein can result in distortion of the CD curve, diminishing the intensity at 208 nm more than at 222 nm (35), however in virions and in reconstituted membranes the spikes are spaced well apart, and there is no difference in the packing density of cleaved and uncleaved F spikes (13).

It is unlikely that bunching through aggregation of spikes is a problem, because at the low protein concentrations used, i.e. less than 50 μg/ml, the proteins sediment homogeneously in sucrose gradients (see below).

It is apparent that cleaved and uncleaved forms of F show a greater content of α-helical structure in the reconstituted particles than in detergent solution (cf. Fig. 4 and Fig. 2). The reason for this is not yet clear; however, it may be related to the fact that when associated with the membrane, the F protein spikes are oligomers, as is evident from the size of the spike, and by analogy to the oligomeric structure of spike

The transfer was accomplished by sedimentation of the isolated F protein out of the Triton X-100 solution into gradients containing Emulphogene BC-720 as described under “Experimental Procedures.”

The CD spectra of the cleaved and the uncleaved F protein in 0.1% Emulphogene BC-720 solution (Fig. 2) show the negative ellipticity bands around 208 and 222 nm that are characteristic of a predominantly α-helical conformation. The comparison of the spectra indicates a greater α-helical component in the cleaved form of the F protein, as is evident from the greater negative ellipticity and from the position of the minima. A best fit analysis of the ellipticity curves using the reference spectra of Chen et al. (27, 28) gave the following distribution of the conformational components: For F₀, 60% α-helix, 15% β-sheet, and 25% random coil; and for F₁₀, 75% α-helix and 25% random coil. The reference spectra used for these calculations take into account the coupling of the transition moments along very long α-helices, and they yielded the curves that were closest to the observed ellipticities. With a carbohydrate content of the F protein of 15% (32), no significant contribution of carbohydrate to the CD spectrum in this wavelength range would be expected (33). The quantitative analysis of the circular dichroism spectra made use of protein concentrations that were determined by a modified Lowry method which eliminates the error from detergent and lipid (24). Without the knowledge of the primary structure of the F protein, a more precise determination of protein concentration is not possible at present, and the above numbers therefore do not indicate the absolute secondary structure of the protein. However, the inherent uncertainty of the Lowry procedure (34) applies equally to the cleaved and the uncleaved forms of the F protein, and would therefore not affect the observed qualitative differences.

To analyze the ellipticity of the F protein in the absence of detergent and in a state closer to its natural conformation in the virus envelope, the isolated F proteins were reconstituted with phosphatidylcholine (13). Under the conditions used and with a phosphatidylcholine and F protein ratio of 1:2 (w/w), reconstitution yielded vesicles of 40 to 80 nm diameter, with glycoproteins present as spikes that are similar in shape and arrangement to the spikes in the virus envelope (Fig. 3) (13).

With particles of this size, light scattering could contribute to the observed ellipticities, however variation of the acceptance angle of the detector between 8°C and 60°C did not influence the observed CD spectra, and this eliminates light scattering as a source of error. There is a small but consistent difference in the ellipticity curves of the cleaved and uncleaved proteins in the reconstituted membranes as is evident from the shape of the curves (Fig. 4). The quantitative analysis of the ellipticity curves (27, 28) resulted in 90% α-helix and 10% random coil for F₀, and 95% α-helix and 5% random coil for F₁₀. It has been reported that “bunching” of protein can result in distortion of the CD curve, diminishing the intensity at 208 nm more than at 222 nm (35), however in virions and in reconstituted membranes the spikes are spaced well apart, and there is no difference in the packing density of cleaved and uncleaved F spikes (13).

It is unlikely that bunching through aggregation of spikes is a problem, because at the low protein concentrations used, i.e. less than 50 μg/ml, the proteins sediment homogeneously in sucrose gradients (see below).

It is apparent that cleaved and uncleaved forms of F show a greater content of α-helical structure in the reconstituted particles than in detergent solution (cf. Fig. 4 and Fig. 2). The reason for this is not yet clear; however, it may be related to the fact that when associated with the membrane, the F protein spikes are oligomers, as is evident from the size of the spike, and by analogy to the oligomeric structure of spike

FIG. 2. Mean residue ellipticity of cleaved (F₁₀) and uncleaved (F₀) F proteins in solution with Emulphogene BC-720. Spectra, indicated by the lines, were taken on cleaved (- - -) F protein, 0.036 mg/ml, and uncleaved (-----) F protein, 0.025 mg/ml, in 10 mM sodium phosphate and 0.1% Emulphogene BC-720, pH 7.5. Calculated best fitting spectra are indicated by the circles, (○) for cleaved and (●) for uncleaved F protein. The error bars represent the standard deviation calculated from spectra of three different protein preparations.
glycoproteins on enveloped viruses, whereas the F protein in detergent solution is present in monomeric form.

**Determination of Detergent Binding of F Proteins by Velocity Sedimentation in Sucrose Gradients**—In the course of preparative purification of F proteins by velocity sedimentation on Triton X-100-containing sucrose gradients, it became apparent that the cleaved F protein consistently sedimented at a faster rate than the uncleaved form of F. In a co-sedimentation analysis of the glycoproteins isolated from virions containing $[^{3}H]F_{0}$ and $[^{14}C]F_{1,2}$ (Fig. 5), the HN proteins co-sedimented, whereas the cleaved F protein sedimented distinctly ahead of the uncleaved form. A similar difference in the sedimentation rate was also observed when Emulphogene BC-720 was used instead of Triton X-100 (data not shown). This difference cannot be explained on the basis of size of the glycoproteins because cleavage of the F protein has been shown to entail no significant loss of polypeptide (8), and because the cleaved form sediments faster. Analysis of the glycoproteins by gel filtration on Sepharose 6B in the presence of Triton X-100 showed elution of $F_{1,2}$ before $F_{0}$ (data not shown), indicating that the faster sedimentation of the cleaved form of F was due to an increase in the size of the F protein-detergent complex, rather than to a change in the frictional coefficient.

To determine the basis for the change in sedimentation properties of the cleaved F protein, it was important to determine the amount of Triton X-100 in the protein-detergent complexes. Procedures for this using radioactively labeled Triton X-100 did not work well because they require the protein to be present at high concentrations, at which the protein aggregates even in the presence of detergent. An alternate procedure described by Smigel and Fleischer (29) employs velocity sedimentation of detergent-protein complexes in media of different density, i.e. sucrose-$H_{2}O$ and sucrose-$D_{2}O$, to obtain values for both the sedimentation coefficients and the partial specific volumes of the complex, and this permits the calculation of the proportion of protein and detergent in the complex as described below.

**Sedimentation of the unfractionated glycoprotein extract**

![Fig. 5. Sedimentation of Sendai virus glycoproteins into sucrose density gradients containing Triton X-100. The continuous sucrose density gradients (10-25%) contained 0.1% Triton X-100 and 10 mM sodium phosphate, pH 7.5. Virions (2 mg of viral protein/ml) with cleaved ($F_{1,2}$) and uncleaved ($F_{0}$) F protein, labeled with $[^{14}C]$-amino-acids and $[^{3}H]$leucine, respectively, were mixed and the glycoproteins solubilized with 2% Triton X-100 in 10 mM sodium phosphate, pH 7.5, yielding solubilized HN and F at approximately 0.2 mg/ml each (2, 3, 5). Two hundred µl of the supernatant was overlaid on continuous sucrose-$H_{2}O$ and sucrose-$D_{2}O$ density gradients containing 0.1% Triton X-100 and subjected to centrifugation for 18 h at 50,000 rpm. Fractions were analyzed for radioactivity and neuraminidase activity.](image-url)

**Fig. 6. Analysis of the sedimentation of the cleaved $F_{1,2}$ protein into sucrose-$H_{2}O$ (A) and sucrose-$D_{2}O$ (B) density gradients containing Triton X-100.** Viral glycoproteins were extracted with 2% Triton X-100 from wild type Sendai virus (2 mg of viral protein/ml) as described in legend Fig. 5 and "Experimental Procedures." Two hundred µl of virus glycoprotein or standard proteins (10 mg/ml) in 10 mM sodium phosphate, pH 7.5, and 0.1% Triton X-100 were overlaid on continuous sucrose-$H_{2}O$ and sucrose-$D_{2}O$ gradients containing 0.1% Triton X-100 and 10 mM sodium phosphate, pH 7.5, and subjected to centrifugation for 15 h (sucrose-$H_{2}O$) or 26 h (sucrose-$D_{2}O$) at 50,000 rpm in a Beckman SW 60 rotor. Fractions were collected and analyzed for radioactivity or protein content. The standard proteins used were ovalbumin (--- O---), bovine serum albumin (O--- O---) and human γ-globulin (A--- A---).

![Fig. 4. Mean residue ellipticity of cleaved ($F_{1,2}$) and uncleaved ($F_{0}$) F proteins in reconstituted membranes. Spectra, indicated by the lines, were taken on phosphatidylcholine vesicles containing the cleaved F protein (---), 0.037 mg/ml, and the uncleaved F protein (--.--), 0.030 mg/ml, suspended in 10 mM sodium phosphate, pH 7.5. The membranes were reconstituted with protein and phosphatidylcholine at an initial ratio of 2.1, w/w (10). Calculated best fitting spectra are indicated by the circles, (C) for cleaved and (O) for uncleaved F protein. The error bars represent the standard deviation calculated from spectra of three different protein preparations.](image-url)
containing both the HN and the F proteins yielded bands that were as sharp as those of standard proteins, indicating that the glycoprotein-detergent complex sediments as homogeneous species in the H2O- and D2O-sucrose gradients (Fig. 6). The peak position of the F protein is independent of glycoprotein concentration over a range of 100 to 400 μg of glycoprotein applied (Fig. 7). In such experiments, the size of the faster sedimenting peak was found to increase with protein concentration. Analysis of the proteins in the two peaks (Fig. 8) indicated that the slower sedimenting peak contained only F protein; however, the faster sedimenting peak contained not only HN, but, at high protein concentrations, also some F protein, presumably in an aggregated form. Fig. 8 also shows that this aggregation occurred only with the cleaved and not with the uncleaved form of the F protein.

As outlined by Smigel and Fleischer (29), and using their computer program, the sedimentation coefficient (s20,W) as a function of the partial specific volume (i) was calculated from the position of the peaks in the gradient, and the density and viscosity through the gradient as calculated from refractive index measurements (cf. “Experimental Procedures”). Sedimentation through two different density gradient media, sucrose in H2O and sucrose in D2O, gives the s20,W and i simultaneously. Fig. 9 illustrates this for the cleaved F1, Triton X-100 complex. Analysis of the detergent complexes of the cleaved and uncleaved F protein resulted in s20,W and i values listed in Table I. The reproducibility of the determinations is shown by the small standard error among different experiments (Table I). Among the triplicate gradients within a given experiment, the standard error was even smaller (not shown).

The foremost practical problem in this procedure is the determination of the exact temperature in the gradients. Following the procedure of Smigel and Fleischer (29), we circumvented this problem by using the gradient temperature which gave the correct s20,W value for the standard proteins.

The increase in the partial specific volume from 0.750 cm³/g for the uncleaved to 0.789 cm³/g for the cleaved F protein can be explained only by an increase in the amount of Triton X-100 in the glycoprotein-detergent complex. The amount of detergent in the complexes can be calculated because the partial specific volume of the complex is assumed to be determined by the weight fractions and partial specific volumes of the constituents:

\[ i = iF(1-dD) + iD(dD) \]  

where \( iF, iD, \) and \( dD \) are the partial specific volumes for the glycoprotein-detergent complex, the glycoprotein, and the detergent, and \( dD \) is the weight fraction of the detergent in the complex.
TABLE I
Sedimentation coefficients and partial specific volumes of complexes of Triton X-100 and cleaved and uncleaved F proteins

| Experiment | A | B | C | D | Average |
|------------|---|---|---|---|---------|
| F₁₂ₛₐₛ | 7.05 | 6.75 | 6.80 | 7.05 | 6.91 ± 0.16 |
| F₁₂ | 0.794 | 0.787 | 0.787 | 0.793 | 0.789 ± 0.004 |
| F₀ | 6.32 | 6.80 | 6.51 | 6.00 | 6.41 ± 0.10 |
| 0.750 | 0.738 | 0.761 | 0.750 ± 0.010 |

volumes of glycopeptides with low (<20%) carbohydrate content (39), i.e., fetuin, plasminogen, transferrin, α₂-glycoprotein, and α₁-antitrypsin.

Using Equation 1 and the experimentally determined values for the partial specific volumes of the F protein-detergent complexes (Table I), the weight of Triton X-100 in the F₁₂ detergent complex was calculated to be 21% of the total, and in the F₁₂ₛₐₛ-detergent complex, 40%. Taking 65,000 as the molecular weight for F₀ and F₁₂ (8) and 644 for Triton X-100 (29), the calculated molar ratios of Triton X-100 to glycoprotein in the complex were 27 for F₀ and 67 for F₁₂ₛₐₛ.

An alternate approach to the calculation of the partial specific volume of glycoproteins is the procedure described by Gibbons (39), which uses the partial specific volumes of individual carbohydrate moieties for the calculation. This procedure has been shown to give satisfactory values for several glycoproteins with very high carbohydrate content; however, with proteins containing less carbohydrate, we found it to result in discrepancies between calculated and observed partial specific volumes, and we therefore concluded that the values used above are more accurate. However, we did calculate detergent binding using the partial specific volumes reported by Gibbons, and the carbohydrate composition as reported by Kohama et al. (32), and obtained for F₀ and F₁₂ partial specific volumes of 0.722, and molar ratios of detergent to protein of 21 and 59, respectively. Thus, using these values, the differences between the two forms of the F protein are of the same magnitude as found above.

The method of Smigel and Fleischer (29) involves a number of assumptions. In addition to the difficulty in determining the actual temperature of the gradients during the run, and to calculating the partial specific volumes of the glycoprotein as discussed above, it is assumed that the binding of the detergent is independent of sucrose concentration and that it is the same in H₂O and D₂O. Also, it is assumed that the partial specific volumes of protein and detergent are the same in the complex state as in the free state. In the present study, we compared two forms of the same protein, and therefore errors in these assumptions would not alter the conclusion drawn, i.e. the approximate doubling of Triton X-100 binding with F protein cleavage. In the course of the calculation, we have also assumed that cleavage of the F protein does not alter the hydration of the protein. If the observed difference in the partial specific volume of the F₀ and the F₁₂ₛₐₛ were due to differential hydration, then F₁₂ₛₐₛ would have to bind 0.2 g of H₂O/g of protein more than that already bound by F₀. Considering the extent of hydration for a typical soluble protein, 0.2 g of H₂O/g protein (40), this seems to be unlikely.

Discussion

The present results document changes in the conformation of the F protein that accompany the proteolytic cleavage of this protein. Because they correlate with the active and inactive state of the F protein, these changes may provide clues to the possible mode of its action. The outcome of F protein activity is membrane fusion between the viral membrane and other membranes; however, the precise mechanism by which this occurs has not been identified. The reaction requires close contact of viral and target membranes, which is provided by the attachment of the receptor-binding protein, HN, to neuraminic acid residues on the target membrane (23), and the F protein then interacts with the target membrane to induce fusion between the bilayers. Conversion of the inactive precursor to the active F protein by proteolytic cleavage does not entail a significant loss of amino acids, suggesting that the activation that results from cleavage involves a change in the conformation of the F protein (8). A conformational change has now been directly demonstrated by the CD spectra of the active and inactive proteins. The hemagglutinating protein of influenza virus is also cleaved to yield two disulfide-linked polypeptides (41), and the cleavage activates the infectivity of the virus (42, 43). A change in the conformation of the hemagglutinating protein on cleavage has been reported previously (44), however the change in the CD spectrum was seen only in the near UV and not in the far UV, as found with the F protein in the present study.

One aspect of the change in the F protein conformation is the increase in detergent binding described here, which presumably represents the exposure on cleavage of additional hydrophobic sites at the surface of the active F protein. Significant binding of nonionic detergents such as the 27 molecules of Triton X-100/F₀ polypeptide found in the present study has been observed only with membrane proteins (29, 36, 37, 45, 46). The implication from this general observation is that Triton X-100 binds to sites of the protein that in the native configuration interact with lipid, and this can readily be extended to the F protein, which is inserted in the lipid of the virus membrane by a hydrophobic portion at the base of the spike. The hydrophobic anchor has been shown with other viral glycopeptides to represent only a small portion of the molecule; thus, with the HN protein of paramyxoviruses the hydrophobic base accounts for less than 8% of the protein (18), and for the hemagglutinating protein of influenza A virus less than 6% (47). The maximum area of F₀ that would be occupied by 27 molecules of Triton X-100 (0.5 nm²/molecule) (48) would be 10% if the protein-detergent complex were spherical, and with the actual dumbbell shape of the spike this hydrophobic area could be considerably smaller than 10%.

The mode of attachment of the F spike to the viral membrane by hydrophobic interaction with the viral lipid applies to both the cleaved and the uncleaved form of the protein, and even though one cannot be certain that this interaction is quantitatively identical for both forms of F, it is highly unlikely that the observed increase from 27 molecules of Triton X-100 in the F₀ to 67 in the cleaved F protein reflects a change in this portion of the molecule. The likely explanation for the increase in detergent binding is the exposure of additional hydrophobic sites on another region of the cleaved protein. These sites may represent a portion of the protein that could engage in hydrophobic interaction with other membranes, and such an interaction may be involved in the membrane-fusing activity of the protein.

The possibility that the mechanism of action of the F protein involves a hydrophobic interaction with the target membrane has been suggested previously, based on the structural features of the protein at the cleavage site. The polypeptide sequence at the NH₂-terminal portion of the cleavage site is unusually hydrophobic (7, 17-20), e.g. a stretch of at least 26 uncharged amino acids, and conserved among different paramyxoviruses (18-20). Furthermore, the structure of this NH₂-terminal region of the active F protein has been shown to be important for activity, because peptides that mimic the NH₂-terminal sequence are inhibitors of F protein-
induced membrane fusion (20). However, it remains to be established conclusively that, as the results suggest, the hydrophobic polypeptide sequence at the cleavage site is itself part of an active site or of the detergent binding region.

The present evidence for a newly exposed hydrophobic region of the active F protein sheds light on the general question of why the proteins that are involved in virus penetration, i.e., the F proteins of paramyxoviruses and the hemagglutinating proteins of myxoviruses are first synthesized as inactive precursors which must be cleaved by a suitable cellular protease to be activated. The present findings suggest that this may provide a means of preventing the F protein from engaging in hydrophobic interactions with intracellular membranes before it is assembled into the viral envelope. Proteolytic cleavage is a late event in the virus assembly, and has been shown to take place at the plasma membrane (41). In keeping with this, the hemagglutinating protein associated with the microsomal fraction of infected cells is present in the uncleaved form in most virus-cell systems (49, 50), and in the one system where cleaved protein has been found in the microsomal fraction (51), it is not known whether those cleaved proteins are still suitable for virus assembly.

Acknowledgments—We thank Drs. Martin Sonenberg and Alan S. Schneider at the Sloan-Kettering Memorial Cancer Center for making available the spectrophotometer, Drs. Murray Snigol and Sidney Fleischer for providing the computer program, and Dr. James S. Murphy at The Rockefeller University for his generous help in adapting the computer program. We appreciate the excellent technical assistance of Robert A. Chase and Akhtar H. Samad.

REFERENCES
1. Choppin, P. W., and Comans, R. W. (1975) in Comprehensive Virology (Fraenkel-Conrat, H., and Wagner, R. R., eds) Vol. 4, pp. 95-178, Plenum Press, New York.
2. Scheid, A., Caliguiri, L. A., Comans, R. W., and Choppin, P. W. (1972) Virology 50, 640-652.
3. Scheid, A., and Choppin, P. W. (1973) J. Virol. 11, 263-271.
4. Homma, M., and Ohuchi, M. (1973) J. Virol. 12, 1457-1465.
5. Scheid, A., and Choppin, P. W. (1974) Virology 57, 475-490.
6. Scheid, A., and Choppin, P. W. (1976) Virology 69, 265-277.
7. Choppin, P. W., and Scheid, A. (1980) Rev. Infect. Dis. 3, 40-61.
8. Scheid, A., and Choppin, P. W. (1977) Virology 90, 54-66.
9. Hosaka, Y. (1970) J. Gen. Virol. 8, 43-54.
10. Hosaka, Y., and Shimizu, Y. K. (1972) Virology 49, 627-639.
11. Hosaka, Y., and Shimizu, Y. K. (1972) Virology 49, 640-646.
12. Volsky, D. J., and Loyter, A. (1978) FEBS Lett. 92, 190-194.
13. Hsu, M.-c., Scheid, A., and Choppin, P. W. (1979) Virology 95, 476-491.
14. Haywood, A. M. (1974) J. Mol. Biol. 83, 427-436.
15. Elsbach, P., Holmes, K. V., and Choppin, P. W. (1969) Proc. Soc. EXP. Biol. Med. 131, 651-657.
16. Neurath, A. R., Vernon, S. K., Hartzell, R. W., Wiener, F. P., and Rubin, B. A. (1970) J. Gen. Virol. 19, 21-36.
17. Getting, M.-J., White, J. M., and Waterfield, M. D. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2737-2740.
18. Scheid, A., Graves, M. C., Silver, S. M., and Choppin, P. W. (1978) in Negative Strand Viruses and the Host Cell (Mahy, B. W. J. and Barry, R. D., eds) pp. 183-191, Academic Press, New York.
19. Scheid, A., Hsu, M.-c., and Choppin, P. W. (1980) in Membrane-Membrane Interactions (Gilula, N. B., ed) Society of General Physiologists 34, pp. 119-130, Raven Press, New York.
20. Richardson, C. R., Scheid, A., and Choppin, P. W. (1980) Virology 105, 205-222.
21. Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. L. (1985) J. Am. Oil Chem. Soc. 42, 53-56.
22. Alstiel, L., and Landsberger, F. R. (1977) Nature 269, 70-72.
23. Scheid, A., and Choppin, P. W. (1974) Virology 62, 125-133.
24. Markwell, M. A. K., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) Anal. Biochem. 87, 206-210.
25. Schneider, A. S., and Harnatts, D. (1976) Biochemistry 15, 4158-4162.
26. Greenfield, N., and Fasman, G. D. (1969) Biochemistry 8, 4108-4116.
27. Chen, Y.-H., Yang, J. T., and Martinez, H. M. (1972) Biochemistry 11, 4120-4131.
28. Chen, Y.-H., Yang, J. T., and Chau, K. H. (1974) Biochemistry 13, 3350-3355.
29. Smigel, M., and Fleischer, S. (1977) J. Biol. Chem. 252, 3689-3696.
30. Laemmli, U. K. (1970) Nature (Lond.) 227, 680-685.
31. Aminoff, D. (1966) Biochemistry 51, 384-392.
32. Kohama, T., Shimizu, K., and Ishida, N. (1978) Virology 90, 226-234.
33. Stone, A. L. (1969) Biopolymers 7, 173-188.
34. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1961) J. Biol. Chem. 193, 265-275.
35. Holtzworth, G. (1972) in Membrane Molecular Biology (Fox, C. F., and Keith, A. D., ed) pp. 228-286, Sinauer Associates, Stamford, Conn.
36. Helenius, A., and Simons, K. (1972) J. Biol. Chem. 247, 3656-3661.
37. Clarke, S. (1975) J. Biol. Chem. 250, 5459-5469.
38. Tanford, C., Nozaki, Y., Reynolds, J. A., and Makino, S. (1974) Biochemistry 13, 2369-2376.
39. Gibbons, R. A. (1966) in Glycoproteins (Gottschalk, A., ed) pp. 29-95, Elsevier, Amsterdam.
40. Tanford, C. (1961) Physical Chemistry of Macromolecules, pp. 317-399, John Wiley & Sons, New York.
41. Lazarowitz, S. G., Comans, R. W., and Choppin, P. W. (1971) Virology 46, 830-843.
42. Lazarowitz, S. G., and Choppin, P. W. (1975) Virology 68, 440-444.
43. Klenk, H.-D., Rott, R., Orlich, M., and Blodorn, J. (1975) Virology 68, 440-454.
44. Planagan, M. T., and Skehel, J. J. (1977) FEBS Lett. 80, 57-60.
45. Fleischer, B., and Smigel, M. (1978) J. Biol. Chem. 253, 1632-1638.
46. Oswald, R. E., and Freeman, J. A. (1979) J. Biol. Chem. 254, 3419-3426.
47. Geising, M.-J., Bye, J., Skehel, J., and Waterfield, M. (1980) in Structure and Variation of Influenza Virus (Laver, G., and Air, G., eds) pp. 1-10, Elsevier/North-Holland, New York.
48. Neer, E. J. (1974) J. Biol. Chem. 249, 6527-6531.
49. Stanley, P., Gandhi, S. S., and White, D. O. (1973) Virology 53, 92-106.
50. Comans, R. W. (1973) Virology 51, 56-70.
51. Klenk, H.-D., Wollert, W., Rott, R., and Scholtissek, C. (1974) Virology 57, 28-41.