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Detection of an Interaction between the HN and F Proteins in Newcastle Disease Virus-Infected Cells

JUDITH STONE-HULSLANDER AND TRUDY G. MORRISON*

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

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For many paramyxoviruses, including Newcastle disease virus (NDV), syncytium formation requires the expression of both surface glycoproteins (HN and F) in the same cell, and evidence suggests that fusion involves a specific interaction between the HN and F proteins. Because a potential interaction in paramyxovirus-infected cells has never been demonstrated, such as interaction was explored by using coimmunoprecipitation and cross-linking. Both HN and F proteins could be precipitated with heterologous antisera after a 5-min radioactive pulse as well as after a 2-h chase in nonradioactive medium, but at low levels. Chemical cross-linking increased detection of complexes containing HN and F proteins at the cell surface. After cross-linking, intermediate- as well as high-molecular-weight species containing both proteins were precipitated with monospecific antisera. Precipitation of proteins with anti-HN after cross-linking resulted in the detection of complexes which electrophoresed in the stacker region of the gel, from 160 to 300 kDa, at 150 kDa, and at 74 kDa. Precipitates obtained with anti-F after cross-linking contained species which migrated in the stacker region of the gel, between 160 and 300 kDa, at 120 kDa, and at 66 kDa. The three to four discrete complexes ranging in size from 160 to 300 kDa contained both HN and F proteins when precipitated with either HN or F antisera. That cross-linking of complexes containing both HN and F proteins was not simply a function of overexpression of viral glycoproteins at the cell surface was addressed by demonstrating cross-linking at early time points postinfection, when levels of viral surface glycoproteins are low. Use of cells infected with an avirulent strain of NDV showed that chemically cross-linked HN and F proteins were precipitated independent of cleavage of F_0. Furthermore, under conditions that maximized HN protein binding to its receptor, there was no change in the percentages of HN and F_0 proteins precipitated with heterologous antisera, but a decrease in F_1 protein precipitated was observed upon attachment. These data argue that the HN and F proteins interact in the rough endoplasmic reticulum. Upon attachment of the HN protein to its receptor, the HN protein undergoes a conformational change which causes a conformational change in the associated F protein, releasing the hydrophobic fusion peptide into the target membrane and initiating fusion.

The paramyxovirus Newcastle disease virus (NDV) encodes two surface glycoproteins, the hemagglutinin-neuraminidase (HN) protein and the fusion (F) protein. The HN protein has neuraminidase activity, attachment activity, and an undefined role in fusion. The only known function of the F protein is to mediate fusion. As in several paramyxovirus systems (reviewed in reference 10), it has previously been shown that the HN and F proteins of NDV are both necessary and sufficient for fusion. Other viral attachment proteins such as influenza virus HA (hemagglutinin) cannot complement the NDV F protein to permit fusion (15). Early research with reconstituted Sendai virus envelopes found that only envelopes containing both HN and F proteins were able to fuse with membranes or vesicles (2). Furthermore, the circular dichroism spectra of vesicles containing both HN and F proteins differed from the spectra obtained with F protein alone, HN protein alone, or vesicles containing F protein or HN protein that were mixed, suggesting a conformational change, and therefore an interaction, when both proteins are present in the same membrane. More indirect support for the idea of an interaction came from work which showed that HN and F proteins must be from the same virus for fusion to result (8). Recent work from several laboratories supports the idea that the stalk domain of the HN protein determines F protein specificity for fusion (4, 26, 30). The first direct demonstration of an interaction was in the measles system, where Malvoisin and Wild (12) used a vaccinia virus expression system to assay for possible HA-F protein interactions. Using this system, Malvoisin and Wild chemically cross-linked a complex at the cell surface which contained HA and F_1 proteins. Recently, homotypic HN and F proteins were coprecipitated from the surfaces of cells expressing these parainfluenza virus proteins by using vaccinia virus vectors as well (32).

Because of evidence which suggested an interaction between HN and F proteins, we have explored their association during NDV infection since there have been no reports of such an interaction in naturally infected cells. We were able to demonstrate an interaction between the HN and F proteins in NDV-infected cells by using coimmunoprecipitation and chemical cross-linking. We report that a complex between HN and F proteins could be immunoprecipitated with antisera against either protein, and this complex could be stabilized by chemical cross-linking. Precipitation of this complex was not dependent on cleavage of F_0 (uncleaved fusion protein) into F_1 and F_2 (cleaved forms of fusion protein), the complex did not dissociate immediately after HN protein attachment to uninfected cells, and it was not dependent on attachment of the HN protein to its receptor.

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Ave., Worcester, MA 01655. Phone: (508) 856-6592. E-mail: Trudy.Morrison@BANYAN.UMMED.EDU.

MATERIALS AND METHODS

Cells and viruses. Cos-7 cells, obtained from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle medium (DMEM)

supplemented with nonessential amino acids, vitamins, penicillin-streptomycin, and 10% fetal calf serum.

NDV strain Australia-Victoria (AV) and NDV strain B1 were grown and purified as previously described (6).

Infections. A total of 5×10^5 Cos-7 cells were plated on 35-mm-diameter tissue culture plates in DMEM; 16 to 20 h after plating, the cells were infected with either the AV or B1 strain of NDV at a multiplicity of infection of 10 (17). The virus was adsorbed for 30 min at 37°C before DMEM supplemented with $CaCl_2$ was added. The infection was allowed to proceed for an additional 4.5 h at 37°C.

Antibodies. Monoclonal antibodies specific for NDV HN were a generous gift of Ron Iorio. Antibodies used were anti-1c, anti-2b, anti-3a, and anti-4a (9). Polyclonal antiserum specific for NDV F was raised against a peptide with the sequence of the carboxy terminus of the F protein (24). Polyclonal antisera specific for NDV HN was a mixture of antisera raised against peptides with the sequence from amino acids 117 to 515 and amino acids 117 to 268 of the HN protein (24).

Radiolabeling, lysis, and immunoprecipitation of protein. At 5 h postinfection, cells were radiolabeled for 15 min at 37°C in DMEM containing 70% of the cysteine of standard medium and lacking methionine. The labeling medium contained 11 mCi of [^{35}S]methionine-cysteine (EXPRE^{35}S^{35}S; New England Nuclear) per ml. The cells were then chased in nonradioactive medium for 2 h. For 5-min pulse-chase experiments, the cells were incubated in medium lacking methionine for 20 min prior to the label. These cells were then chased in nonradioactive medium containing 0.1 mg of cycloheximide per ml and 2 mM nonradioactive methionine for various lengths of time. At the end of the chase period, cells were washed twice in phosphate-buffered saline (PBS) and lysed.

Two different lysis conditions were used for coimmunoprecipitation and chemical cross-linking experiments. For coimmunoprecipitation, the monolayer was lysed in reticulocyte standard buffer (0.01 M Tris-HCl [pH 7.4], 0.01 M NaCl) containing 0.5% sodium deoxycholate, 2.5 mg of *N*-ethylmaleimide per ml, 2 mg of iodoacetamide per ml, and 13 mM [3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate] (CHAPS) on ice (23). After cross-linking, the monolayer was lysed in reticulocyte standard buffer containing 0.5% sodium deoxycholate, 2.5 mg of *N*-ethylmaleimide per ml, 2 mg of iodoacetamide per ml, and 1% Triton X-100. After lysis, the nuclei were removed by centrifugation.

For immunoprecipitation of NDV proteins, infected cell lysates were incubated with antisera for 1 h at room temperature. Fixed, killed *Staphylococcus aureus* cells (Boehringer Mannheim) resuspended in PBS–0.5% polyoxyethylenesorbitan monolaurate–1 mg of bovine serum albumin per ml were added to the lysate in the presence of 0.15 to 0.4% sodium dodecyl sulfate (SDS) and incubated at room temperature with agitation for 30 min. The *S. aureus* cells were pelleted, and the supernatants were removed. The pellets were washed three times with PBS–1% Triton X-100–0.5% sodium deoxycholate–0.1% SDS. The *S. aureus* cells were then resuspended in sample buffer and stored at −20°C until analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

Chemical cross-linking. Cross-linking was accomplished by a modification of the procedure described by Russell et al. (21). Radiolabeled, infected cell monolayers were washed two times in PBS (pH 8.5) and then incubated with 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP; Sigma) ranging from 0.05 to 1.0 mM in PBS (pH 8.5) for 1 h at 4°C. The cross-linker was quenched at 4°C with 74 mM glycine for 5 min, and the cells were washed with PBS (pH 8.5) and lysed with Triton X-100 containing lysis buffer at 4°C. Extracts were immunoprecipitated as described above and analyzed by SDS-PAGE. For analysis of large, cross-linked species, two-dimensional SDS-PAGE was used. Immunoprecipitates were electrophoresed under nonreducing conditions, and individual lanes were cut from the gel and incubated in run buffer containing 10% β-mercaptoethanol (βME) for 5 min. The proteins in the gel slice were then electrophoresed under reducing conditions.

Cleavage of cell surface fusion protein. Radiolabeled, NDV strain B1-infected cells were washed two times with OptiMem (Gibco/BRL). The cells were then treated or mock-treated with acetylated trypsin (Sigma) (0.75% in OptiMem) for 10 min at 37°C. The plates were washed with DMEM containing soybean trypsin inhibitor (Boehringer Mannheim) (1.5% in OptiMem) and lysed with Triton X-100 containing lysis buffer and 3% soybean trypsin inhibitor.

Attachment assay. At 150 min postinfection, the infected monolayer was treated or mock treated with neuraminidase (Sigma) (0.2 U in 1 ml of DMEM) and incubated at 37°C for an additional 150 min. During the pulse-label and the chase, the cells were treated or mock treated with neuraminidase. After the chase, approximately 10^6 uninfected Cos cells were added to half of the plates at 4°C.

RESULTS

Coimmunoprecipitation of HN and F. To investigate a potential interaction between the HN and F proteins of NDV (strain AV)-infected cells, coimmunoprecipitation of the two proteins was explored. At 5 h postinfection, a time at which there is significant synthesis of both proteins (18, 19), cells were subjected to a 15-min pulse-label and a 2-h chase in nonradioactive medium. Proteins present in infected cell lysates were immunoprecipitated with either a mixture of monoclonal antibodies against the HN protein or an antibody specific for the cytoplasmic tail of the F protein. Each of the monoclonal antibodies against the HN protein immunoprecipitated HN and coimmunoprecipitated F protein, but a mixture was used to ensure that all populations of HN protein were immunoprecipitated. Concentrations of SDS ranging from 0.15 to 0.3% were used during immunoprecipitation, since the absence of SDS resulted in high levels of nonspecific aggregation and failure to detect any specific precipitation. Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions (Fig. 1). At all SDS concentrations, proteins that comigrated with both F_0 and F_1 proteins could be precipitated with HN antisera. In addition, a protein that comigrated with HN protein could be precipitated with F antiserum. Very little nonspecific precipitation, particularly at SDS concentrations of 0.25% and higher, was found. The amount of HN, F_0, and F_1 proteins coprecipitated with heterologous antisera decreased with increasing SDS concentrations. In 0.25% SDS, immunoprecipitation with F antiserum resulted in the coprecipitation of approximately 6% of total HN protein, and immunoprecipitation with HN antisera resulted in the coprecipitation of 2 and 1% of total F_0 and F_1 proteins, respectively. Various lysis and immunoprecipitation conditions were investigated, without a significant change in the results.

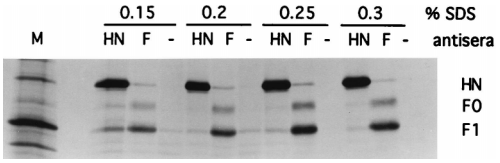

FIG. 1. Coimmunoprecipitation of HN and F from AV-infected cells. At 5 h postinfection, AV-infected Cos cells were pulse-labeled with [^{35}S]methionine-cysteine for 15 min and then chased in nonradioactive medium for 2 h. The cells were lysed in CHAPS buffer as described in Materials and Methods. Proteins present in extracts from 2×10^5 cells were precipitated with a mixture of monoclonal antibodies anti-1c, anti-2b, anti-3a, and anti-4a (HN antisera lanes), polyclonal antiserum against the cytoplasmic tail of F (F antiserum lanes), or no antiserum (−) in the presence of 0.15, 0.2, 0.25, or 0.3% SDS. The precipitated proteins were analyzed by SDS-PAGE in the presence of reducing agent. Lane M, virus-infected cell extract not immunoprecipitated.

AV infected cells were also subjected to a 5-min pulse and chased in nonradioactive medium for various lengths of time (Fig. 2). Coprecipitation with heterologous antisera was observed at all time points. Indeed, even after a 5-min pulse, a time at which the F protein should still be localized in the rough endoplasmic reticulum, extracts precipitated with HN

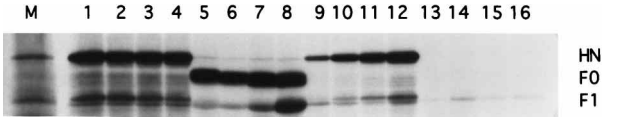

FIG. 2. Coimmunoprecipitation of HN and F after a 5-min pulse-label. At 5 h postinfection, AV-infected Cos cells were washed and incubated in medium lacking methionine for 20 min. Cells were pulse-labeled with [^{35}S]methionine-cysteine for 5 min and then chased in nonradioactive medium containing cycloheximide and excess methionine for 0 (lanes 1, 5, 9, and 13), 5 (lanes 2, 6, 10, and 14), 15 (lanes 3, 7, 11, and 15), or 30 (lanes 4, 8, 12, and 16) min as described in Materials and Methods. The cells were lysed in Triton buffer and precipitated with polyclonal antisera against amino acids 117 to 515 and 117 to 268 of the HN protein (lanes 1 to 4), polyclonal antiserum against the cytoplasmic tail of F protein (lanes 5 to 8), a mixture of monoclonal antibodies against HN protein (lanes 9 to 12), or no antiserum (lanes 13–16) in the presence of 0.25% SDS. The precipitated proteins were analyzed by SDS-PAGE in the presence of reducing agent. Lane M, virus-infected cell extract not immunoprecipitated.

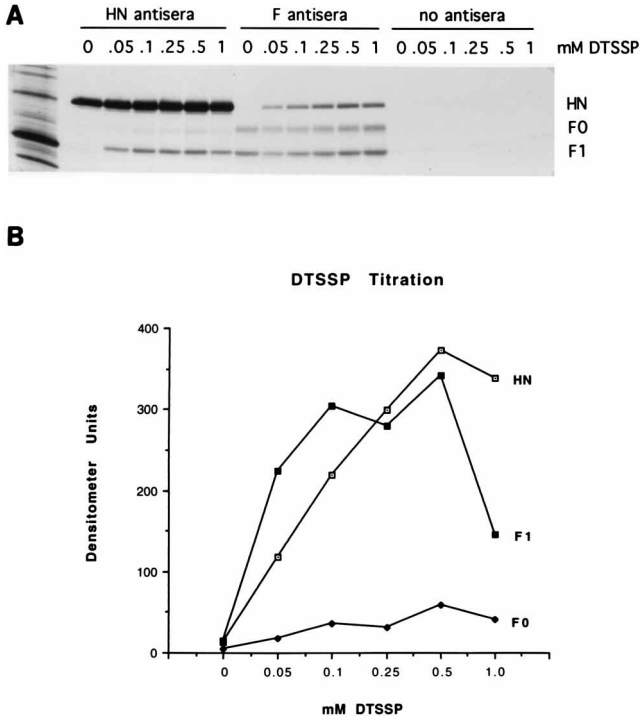

FIG. 3. Titration of the chemical cross-linker DTSSP. AV-infected cells were pulse-labeled as for Fig. 1. After the 2-h chase in DMEM, surface proteins were cross-linked in the presence of 0, 0.05, 0.1, 0.25, 0.5, or 1.0 mM DTSSP and lysed in Triton X-100 buffer as described in Materials and Methods. Proteins from the cytoplasmic extracts were precipitated with HN antisera, F antiserum at subsaturating levels, or no antiserum in the presence of 0.4% SDS and analyzed by SDS-PAGE under reducing conditions (A). (B) Panel A was scanned on a Molecular Dynamics densitometer, and the densitometer units of HN, F_0, and F_1 precipitated with heterologous antisera were plotted against increasing concentrations of DTSSP. Leftmost lane, virus-infected cell extract not immunoprecipitated.

antisera contained uncleaved F_0 protein, while F antiserum precipitated HN protein. Because the F protein is proteolytically cleaved in the trans-Golgi (19), this result implies that the potential HN-F protein interaction occurs in a subcellular compartment prior to the trans-Golgi along the exocytic pathway to the plasma membrane, before proteolytic processing of the F_0 protein.

Chemical cross-linking of HN and F. To stabilize the potential association of HN and F proteins on infected cell plasma membranes, use of the chemical cross-linker DTSSP was investigated. DTSSP is a membrane-impermeable cross-linker (25); therefore, incubation of intact cells with this cross-linker will link only proteins expressed on the cell surface. Furthermore, DTSSP is cleavable with reducing agents; thus, individual proteins in a cross-linked complex can be resolved on polyacrylamide gels after reduction in βME.

After a pulse-chase labeling protocol, monolayers were incubated with concentrations of cross-linker ranging from 0.05 to 1.0 mM. After lysis, proteins in the cytoplasmic extracts were precipitated with either HN antisera, F antiserum, or no antiserum under conditions more stringent than those used for Fig. 1 (see Materials and Methods). The precipitates were analyzed by SDS-PAGE under reducing conditions (Fig. 3A). All concentrations of cross-linker resulted in the precipitation of a cross-linked complex which contained proteins that comigrate with HN and F proteins. The amount of HN protein precipitated by F antiserum increased with increasing cross-linker concentrations up to maximal levels at 0.5 and 1.0 mM DTSSP (Fig. 3B). HN antisera precipitated an F_1-size molecule at all concentrations of cross-linker. This protein is likely F_1 protein and not NP, since DTSSP is not membrane permeable and increased amounts of this protein were precipitated with HN antisera in the presence of cross-linker. Furthermore, this material was in a cross-linked complex and not nonspecific aggregates of NPs, since no NP-size molecules were resolved under nonreducing conditions (see below). From 0.05 to 0.5 mM DTSSP, F_1 protein was precipitated in increasing amounts, while a decrease was observed at 1.0 mM DTSSP. F_0 protein was also precipitated in increasing amounts with HN antisera at cross-linker concentrations ranging from 0.1 to 0.5 mM, and a decrease was observed at 1.0 mM DTSSP. Six separate experiments at 0.5 mM DTSSP and at saturating levels of antisera (not shown) showed that approximately 28% of total labeled HN protein could be precipitated with F antiserum, while 5% of total labeled F_0 protein and 22% of total labeled F_1 protein were precipitated with HN antisera. Thus, cross-linking appeared to increase the detection of complexes containing HN and F proteins and resulted in precipitation of heterologous proteins at levels surpassing those observed by coimmunoprecipitation in the presence of low SDS concentrations (Fig. 1).

To determine sizes of the cross-linked species, immunopre-

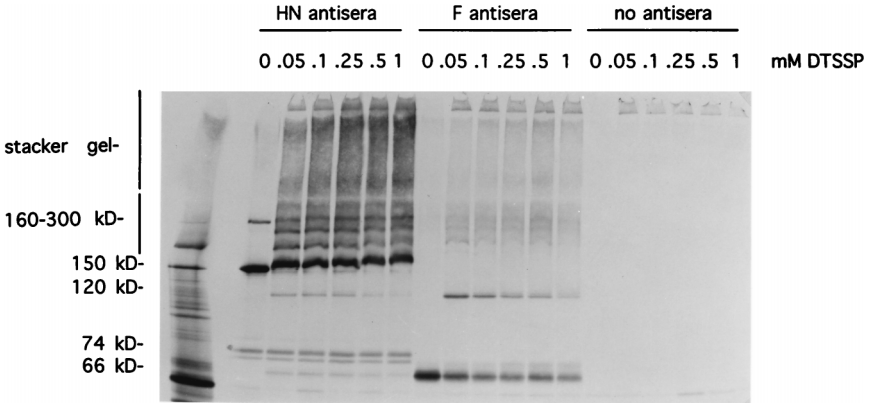

FIG. 4. Nonreduced cross-linked proteins observed in the presence of increasing concentrations of DTSSP. AV-infected cells were radiolabeled, cross-linked, lysed, and immunoprecipitated as for Fig. 2. The precipitates were analyzed by SDS-PAGE under nonreducing conditions. Molecular weights were assigned by using NDV strain AV-infected cell extracts as markers. Leftmost lane, virus-infected cell extract not immunoprecipitated.

cipitates were analyzed by SDS-PAGE under nonreducing conditions (Fig. 4). In the absence of cross-linker, HN antisera precipitated HN monomer (74 kDa), HN dimer (150 kDa), and HN tetramer (~300 kDa) (9). As the cross-linker concentration increased, the HN dimer migrated more slowly. Importantly, at all concentrations of DTSSP, HN antisera precipitated a 66-kDa species, a species with a molecular mass of approximately 150 kDa, four high-molecular-weight species with molecular masses ranging from approximately 160 to 300 kDa, and heterogeneous material with sizes of 120 kDa and greater. HN monomer was resolved as two species, and the levels observed remained unchanged with increasing cross-linker.

In the absence of cross-linking, F antiserum precipitated only a 66-kDa protein, the nonreduced, monomeric form of F protein (F_{nr}) (14, 31). With increasing cross-linker concentrations, there was a gradual decrease of the F monomer. In addition, we detected a species with an apparent molecular mass of approximately 120 kDa that comigrated with material precipitated with HN antisera. The amount of this species increased at 0.5 mM DTSSP but decreased when higher cross-linker concentrations were used, presumably because increasing cross-linker concentrations resulted in the formation of higher-molecular-weight complexes. Three larger complexes which migrated between 160 and 300 kDa as well as heterogeneous material of 120 kDa and greater were observed at all concentrations of cross-linker and comigrated with complexes precipitated with HN antisera. The amounts of these larger complexes increased as the concentration of DTSSP was increased to 0.5 mM and then decreased at 1.0 mM.

To identify proteins present in each cross-linked species, precipitated proteins were characterized by two-dimensional SDS-PAGE. The first dimension separated cross-linked complexes under nonreducing conditions as described for Fig. 4 (Fig. 5A), and the second dimension separated these complexes under reducing conditions. Such analysis of cross-linked complexes immunoprecipitated with HN antisera (Fig. 5B) showed that material present in the stacker gel as well as intermediate-size complexes with molecular masses of 160 to 300 kDa contained F_0 and F_1 proteins as well as HN protein. The species which comigrated with both the HN dimer as well as the monomer contained primarily HN protein. The species which migrated near 120 kDa resolved primarily into F_1 protein, with minimal amounts of HN protein and F_0 protein present. The distribution of HN protein (Table 1) among these species was 15% in the stacker gel, 32% at 160 to 300 kDa, 42% at 150 kDa, and 11% at 74 kDa. Thus, F_0 and F_1 proteins were associated with both intermediate-size as well as large, cross-linked complexes containing HN protein. Surprisingly, a 66-kDa species containing primarily F_0 and F_1 proteins was precipitated with HN antiserum.

A similar analysis of cross-linked complexes immunoprecipitated with F antiserum (Fig. 5C) showed that material in the stacker gel as well as the complexes ranging in size from 140 to 300 kDa contained HN, F_0, and F_1 proteins. The 120-kDa species as well as the 66-kDa species contained almost exclusively F_0 and F_1 proteins. The distributions of total F_0 and F_1 were 5 and 6% in the stacker gel, 23 and 21% in the 160- to 300-kDa region, 5 and 22% at 120 kDa, and 67 and 51% at 66 kDa. Thus, HN and F precipitated by heterologous antisera were associated with 160-kDa and larger complexes. A small amount of non-cross-linked HN protein was also coprecipitated with F protein after cross-linking.

Cross-linking at various times of infection. To explore the appearance of cross-linked complexes with time of infection, infected cells were incubated with DTSSP at various time points postinfection (Fig. 6). As early as 4.25 h postinfection, 10% of the total HN and F_1 proteins and 5% of the total F_0 protein could be precipitated with heterologous antisera. For the HN and F_0 proteins, these percentages increased with time postinfection until 5.75 h and then dropped, while for the F_1 protein, the percentage increased until 5.25 h before decreasing. These results support the idea that complexes observed later in infection (7.5 h [Fig. 2 to 5]) cannot be due exclusively to close packing of the molecules which might accompany high levels of expression of viral proteins at the cell surface.

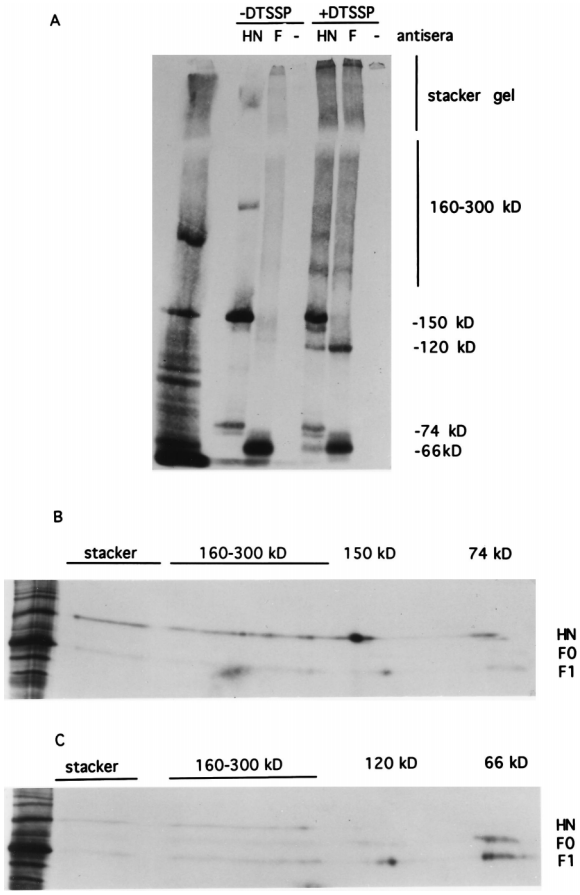

FIG. 5. Two-dimensional SDS-PAGE analysis of cross-linked proteins from AV-infected cells. AV-infected cells were radiolabeled, cross-linked with 0.5 mM DTSSP, lysed, and immunoprecipitated as for Fig. 2. (A) Precipitated proteins which were not cross-linked (−DTSSP) or were incubated in the presence of cross-linker (+DTSSP) were analyzed by SDS-PAGE under nonreducing conditions as described for Fig. 3 except that the complexes were resolved on a lower-percentage polyacrylamide gel. (B) A lane identical to the HN antisera +DTSSP lane was excised from the gel, reduced in βME, and analyzed by SDS-PAGE under reducing conditions. The top portion of the gel in panel A is observed on the left of panel B. (C) A lane identical to the F antiserum +DTSSP lane was excised from the gel, reduced in βME, and analyzed by SDS-PAGE under reducing conditions. Leftmost lane, virus-infected cell extract not immunoprecipitated.

Effect of F_0 cleavage on detection of HN and F cross-linked species. While F_0 protein was detected in cross-linked complexes obtained with HN antisera, the predominant species detected was F_1 protein. Furthermore, it has been reported that in measles virus-infected cells, only the F_1 protein, not the F_0 protein, can be precipitated in a cross-linked complex with HA (13). To determine the importance of cleavage of the F_0 protein in the detection of HN-F protein complexes, we used the avirulent B1 strain of NDV. When Cos cells are infected

TABLE 1. Distribution of HN and F after cross-linking as determined by two-dimensional SDS-PAGE

Protein	Distribution (%)[a]		
	AV	B1	
		−Trypsin	+Trypsin
HN			
Stacker	15	34	9
160–300 kDa	32	5	19
150 kDa	42	3	9
74 kDa	11	58	63
F_0			
Stacker	5	51	9
160–300 kDa	23	20	25
120 kDa	5	9	14
66 kDa	67	20	51
F_1			
Stacker	6	ND	14
160–300 kDa	21	ND	37
120 kDa	22	ND	17
66 kDa	51	ND	31

[a] Determined from densitometer scans of Fig. 4, 8, and 9. HN values were obtained from proteins precipitated with HN antisera (Fig. 4B, 8B, and 9B), while F_0 and F_1 values were obtained from proteins precipitated with F antiserum (Fig. 4C, 8C, and 9C). ND, no data.

with NDV strain B1, the F_0 protein generated by the virus is not cleaved into F_1 and F_2 proteins due to the absence of a furin recognition site in the F protein sequence (1, 5, 22, 29). Cleavage does occur, however, upon addition of exogenous trypsin (reviewed in reference 3).

Cos cells infected with NDV strain B1 or AV were labeled and cross-linked as described above except that the monolayers were mock treated or incubated with exogenous trypsin before cross-linking. F antiserum precipitated HN protein equally from B1-infected cell lysates derived from either trypsin-treated or untreated cells (Fig. 7). HN antisera precipitated the F protein whether or not it was cleaved. These results indicate that cleavage of the F_0 protein is not required for the formation of a cross-linked complex with HN. In contrast to AV-infected cells, however, total HN protein precipitated by F antiserum and total F protein precipitated with HN antisera were considerably reduced.

Cross-linked, precipitated proteins were characterized by two dimensional SDS-PAGE as described for Fig. 4. In the first dimension, in the absence of trypsin treatment under nonreducing conditions, proteins precipitated with HN antisera (Fig. 8A, HN antisera plus DTSSP; Table 1) yielded HN monomer (74 kDa), a small amount of 160- to 300-kDa species, and a large amount of protein in the stacker gel, as well as heterogeneous material with sizes greater than 160 kDa. Analysis of these complexes in the second dimension under reducing conditions (Fig. 8B) showed that the material in the stacker gel and at 74 kDa contained most of the HN protein (Table 1), a result very different from that for AV-infected cells. Electrophoresis of precipitates obtained with F antiserum in the first dimension (Fig. 8A, F antiserum plus DTSSP) resulted in F_{nr} (66 kDa), a 120-kDa species, 160- to 300-kDa species, a large amount of protein in the stacker gel, and heterogeneous material throughout the gel. Electrophoresis in the second dimension under reducing conditions (Fig. 8C) showed that as in assays using the HN antisera, most of the cross-linked F protein was observed migrating in the stacker gel.

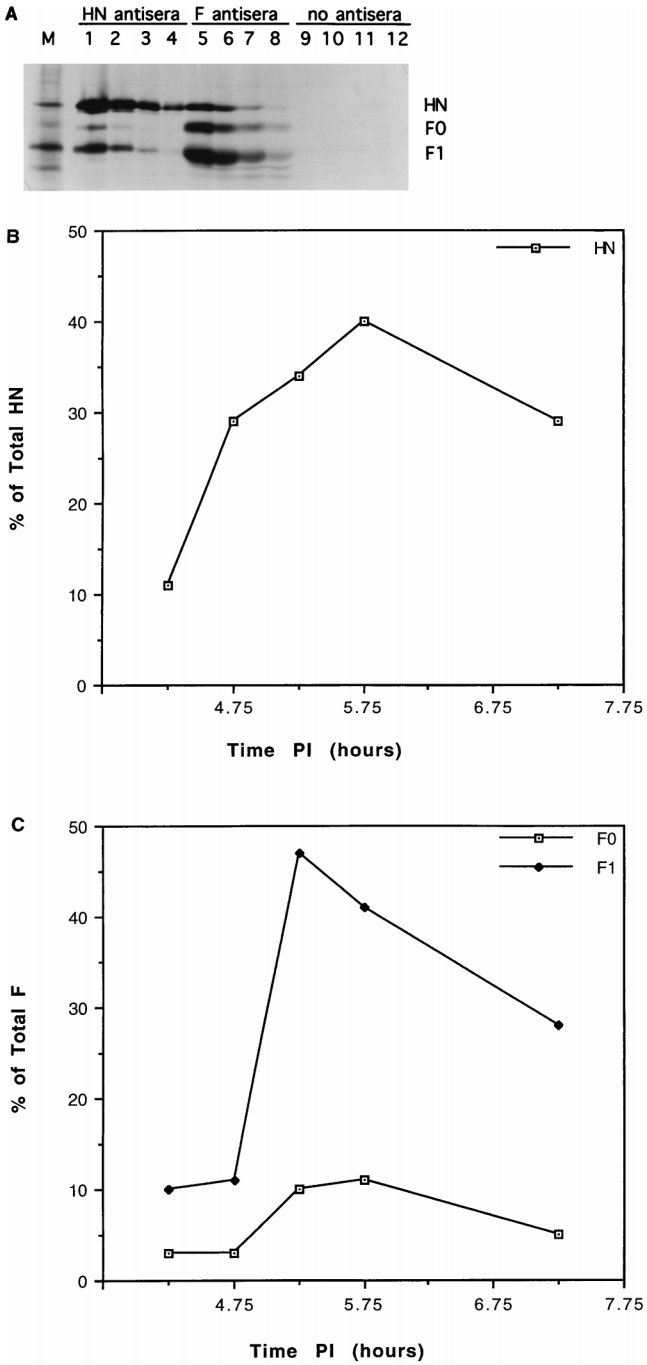

FIG. 6. Cross-linking of proteins from AV-infected cells at various times postinfection. At various times postinfection, AV infected Cos cells were pulse-labeled with [^{35}S]methionine-cysteine for 15 min and then chased in nonradioactive medium for 2 h. Cross-linking with 0.5 mM DTSSP was performed at 4.25 (lanes 4, 8, and 12), 4.75 (lanes 3, 7, and 11), 5.25 (lanes 2, 6, and 10), and 5.75 (lanes 1, 5, and 9) h postinfection. Cells were then lysed and immunoprecipitated as for Fig. 2, and the precipitates were analyzed by SDS-PAGE under reducing conditions (A). Lane M, virus-infected cell extract not immunoprecipitated. (B) Three separate experiments were scanned on a Molecular Dynamics densitometer, and densitometer units of HN protein precipitated with F antiserum were plotted against time postinfection (PI). (C) Densitometer units of F proteins precipitated with HN antisera were plotted against time postinfection.

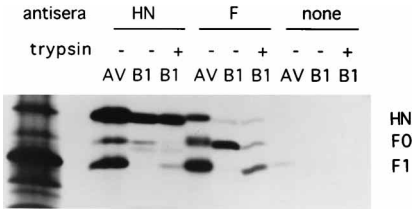

FIG. 7. Cleavage of B1 fusion protein at the cell surface. At 5 h postinfection, AV- or B1-infected Cos cells were pulse-labeled with [^{35}S]methionine-cysteine for 15 min and then chased in nonradioactive medium for 2 h. The cells were treated (+) or mock treated (−) with trypsin for 10 min, washed with soybean trypsin inhibitor, cross-linked with 0.5 mM DTSSP, and lysed in Triton X-100 buffer containing soybean trypsin inhibitor as described for Fig. 2. Precipitates were analyzed by SDS-PAGE under reducing conditions. Leftmost lane, virus-infected cell extract not immunoprecipitated.

After trypsin treatment, analysis of precipitates obtained with both HN and F antisera in the first dimension (Fig. 9A, HN antisera plus DTSSP and F antiserum plus DTSSP) showed an increase in heterogeneous cross-linked complexes which electrophoresed with molecular masses of between 160 and 300 kDa, similar to that observed in AV-infected cells. Cross-linked material precipitated with F antiserum, however, contained a higher percentage of F_1 protein in the stacker region and from 160 to 300 kDa than was observed in AV-infected cells. Thus, in the absence of trypsin digestion, the majority of cross-linked material precipitated was very large. Trypsin treatment resulted in an increase of cross-linked species of between 160 and 300 kDa, similar in size to the cross-linked material observed from AV-infected cells. Therefore, while cleavage of the F protein did not increase the amount of cross-linked complex, the size of the complex changed upon cleavage.

Effect of attachment on the HN-F interaction. HN protein binds to uninfected cells via sialic acid residues on its receptor, and when the sialic acid residues are cleaved with neuraminidase, the HN protein can no longer bind. To determine the effect of HN protein binding on cross-linking of HN and F proteins, cross-linking was accomplished under conditions which minimize attachment as well as conditions which maximize attachment. To minimize attachment, infected cells were incubated in neuraminidase, a treatment which has been shown to block attachment and subsequent fusion (20). To maximize attachment, an overlay of uninfected cells was added. Infected cells treated with neuraminidase were also incubated with an overlay of uninfected cells.

Four monolayers of cells were infected with NDV strain AV. One monolayer (+N+OL) was incubated with neuraminidase and pulse-labeled, and an uninfected cell overlay was added. Another monolayer (+N−OL) was incubated with neuraminidase and pulse-labeled, and no overlay was added. A third monolayer (−N+OL) was mock treated with neuraminidase and pulse labeled, and an uninfected cell overlay was added. The last monolayer (−N−OL) was mock treated with neuraminidase and pulse-labeled, and no overlay was added. The cell surface proteins of all four plates were then subjected to cross-linking as described in Materials and Methods. Proteins precipitated with HN as well as F antisera are shown in Fig. 10. Incubating infected cells in neuraminidase resulted in HN, F_0, and F_1 proteins migrating faster due to the cleavage of sialic acid residues from the proteins. The amount of HN protein precipitated with F antiserum was averaged from three separate experiments. Approximately 33% (±16%) of total precipitated HN protein under conditions which minimize attachment (+N−OL), while conditions which maximize attachment (−N+OL) resulted in the precipitation of 30% (±8%) of total precipitable HN protein. Precipitation of F_0 and F_1 proteins with HN antisera yielded 4% (±2%) and 27% (±17%) of total F proteins, respectively, under attachment-minimizing conditions (+N−OL), while 3% (±0.5%) and 18% (±2.7%) were precipitated under attachment-maximizing conditions (−N+OL). This result is consistent with the formation of a cross-linked species before attachment. Furthermore, binding of the HN protein to sialic acid residues of uninfected cells resulted in a slight decrease in the ability of the F_1 protein to be cross-linked.

DISCUSSION

One interpretation of the finding that syncytium formation requires both an HN and an F protein derived from the same paramyxovirus is that there is a physical interaction between the two proteins. We have been able to precipitate a potential complex of mature HN and F proteins in infected cells by using monospecific antisera against either protein, although the

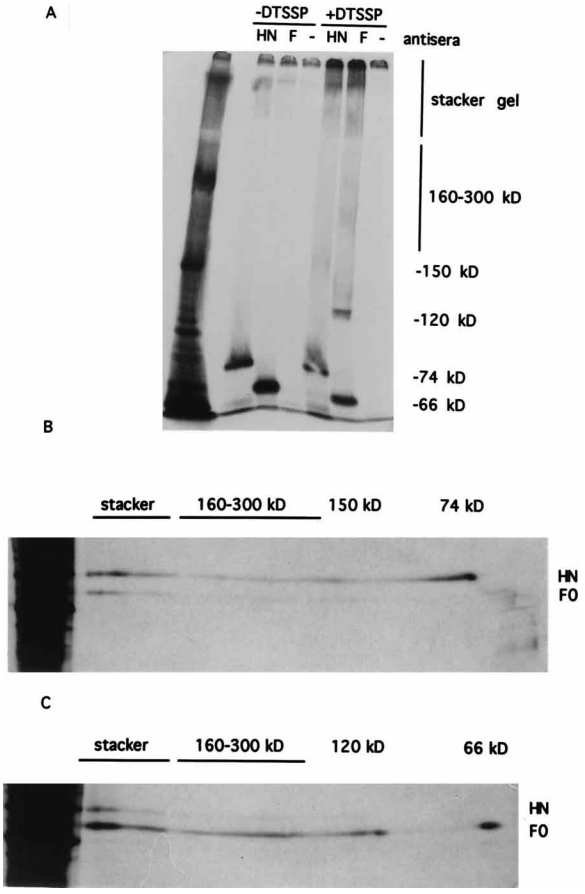

FIG. 8. Two-dimensional SDS-PAGE analysis of cross-linked proteins from B1-infected cells expressing an uncleaved F protein. B1-infected cells were radiolabeled, cross-linked with 0.5 mM DTSSP, lysed, and immunoprecipitated as for Fig. 7 (in the absence of trypsin treatment). (A) Precipitated proteins which were not cross-linked (−DTSSP) or were cross-linked (+DTSSP) were analyzed by SDS-PAGE under nonreducing conditions. (B) A lane identical to the HN antisera +DTSSP lane was excised from the gel, reduced in βME, and analyzed by SDS-PAGE under reducing conditions. The top portion of the gel in panel A is observed on the left of panel B. (C) A lane identical to the F antiserum +DTSSP lane was excised from the gel, reduced in βME, and analyzed by SDS-PAGE under reducing conditions. Leftmost lane, virus-infected cell extract not immunoprecipitated.

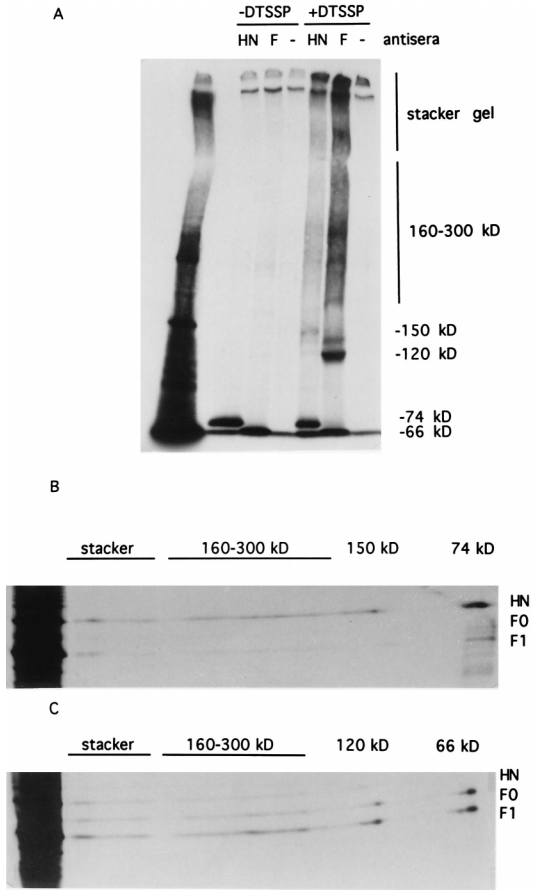

FIG. 9. Two-dimensional SDS-PAGE analysis of cross-linked proteins from B1-infected cells expressing a cleaved F protein. B1-infected cells were radiolabeled, incubated with trypsin, cross-linked with 0.5 mM DTSSP, lysed, and immunoprecipitated as for Fig. 7. (A) Precipitated proteins which were not cross-linked (−DTSSP) or were incubated in the presence of cross-linker (+DTSSP) were analyzed by SDS-PAGE under nonreducing conditions. (B) A lane identical to the HN antisera +DTSSP lane was excised from the gel, reduced in βME, and analyzed by SDS-PAGE under reducing conditions. The top portion of the gel in panel A is observed on the left of panel B. (C) A lane identical to the F antiserum +DTSSP lane was excised from the gel, reduced in βME, and analyzed by SDS-PAGE under reducing conditions. Leftmost lane, virus-infected cell extract not immunoprecipitated.

amount of coprecipitation detected was very small. Approximately 5% of the total HN protein was precipitable with F antiserum, and 2 to 3 and 1 to 4% of F_0 and F_1 proteins, respectively, were precipitable with HN antisera. These amounts could not be increased by using various cell lysis and immunoprecipitation conditions (unpublished observations). Furthermore, coprecipitation was detected as early as after a 5-min radioactive pulse-label. While these results may indicate that only a small percentage of the two proteins actually physically interact, it remained possible that conditions required for cell lysis and/or immunoprecipitation could destabilize any complexes containing HN and F proteins. To stabilize these potential complexes at the cell surface, we utilized the membrane-impermeable protein cross-linker DTSSP. Cross-linking resulted in an increased detection of complexes containing HN and F proteins. At a chase time when maximal levels of proteins are at the cell surface and at optimal cross-linker concentrations, approximately 28% of the total HN protein and 22% of the total F_1 protein could be precipitated with heterologous antisera. These percentages were not increased with higher cross-linker concentrations. Such increased levels of coprecipitation of HN and F proteins after cross-linking are consistent with the idea that HN-F protein complexes may not be very stable after cell lysis and immunoprecipitation.

It has been reported that measles HA and F proteins can be cross-linked on the surfaces of cells expressing these two proteins from vaccinia virus vectors (12), although another study of surface cross-linking of simian virus 5-infected cells as well as NDV-infected cells reported no cross-linking of HN and F proteins (21). Russell et al., however, used several conditions that differed significantly from ours and may account for our contrasting results (21). For example, Russell et al. cross-linked infected cells in suspension after removing them from a monolayer, while we cross-linked infected cell monolayers. Detachment of cells from surfaces may result in the rearrangement of surface proteins. Another study, by Markwell and Fox (13), found no evidence of cross-linking of HN and F proteins in both Sendai virus and NDV virions. This study may differ from ours in that virions instead of infected cells, and different cross-linkers, were used.

Interestingly, after cross-linking, HN antisera precipitated only 5% of total F_0 protein, an amount not very different from that observed by coimmunoprecipitation. It is possible that only a minor population of F_0 proteins are in a complex with HN proteins and that all of these molecules can be precipitated with antisera against HN. Alternatively, DTSSP may not efficiently cross-link F_0 to HN. Malvoisin and Wild (12) reported chemical cross-linking of HA and F_1 proteins of measles virus at the cell surface but were unable to detect any F_0 protein in the cross-linked complexes. They argued that only the cleaved form of the protein can interact with the HA protein. Using the lysis conditions reported by Malvoisin and Wild, we were unable to precipitate any cross-linked material containing F_0 protein (unpublished observations). Thus, different lysis conditions may be responsible for these different results.

We explored the requirement for F protein cleavage in the formation of cross-linked complexes by using NDV strain B1, which has an uncleaved F_0 protein. The amount of cross-linking between the fusion protein of this virus and the HN protein did not change, regardless of whether the F protein was uncleaved or cleaved by the addition of exogenous trypsin. This result argues that cleavage per se is not required for the formation of the complex. However, for unknown reasons, the

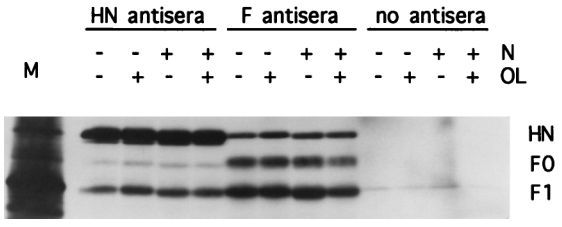

FIG. 10. Effect of attachment on HN and F cross-linking. Four cell monolayers were infected with AV. At 2.5 h postinfection, two plates were treated and two plates were mock treated with neuraminidase (0.2 U/ml) and incubated at 37°C for an additional 2.5 h. Cells were radiolabeled with [^{35}S]methionine-cysteine for 15 min in the presence or absence of neuraminidase and incubated in DMEM for 2 h in the presence or absence of neuraminidase. After the chase, an overlay of uninfected cells was added to one plate in the presence and to one plate in the absence of neuraminidase. The other two plates did not receive an overlay. The surface proteins were immediately cross-linked in 0.5 mM DTSSP, and the cells were lysed and immunoprecipitated as for Fig. 2. The precipitates were analyzed by SDS-PAGE under reducing conditions. −N, cells mock-treated with neuraminidase; +N, cells treated with neuraminidase; −OL, no uninfected cell overlay added; +OL, uninfected cell overlay added; lane M, virus-infected cell extract not immunoprecipitated.

amount of cross-linking of HN and F proteins after infection with this strain of virus is considerably less than the amount observed with strain AV.

Results with strain B1 showed that there was no difference in cross-linking upon cleavage of the F protein. However, the sizes of the cross-linked complexes were significantly different upon cleavage. The HN-F_0 protein complexes were resolved primarily in the stacker region of the gel before cleavage, while after cleavage, the majority of the HN-F_1 protein complexes were in the size range of 160 to 300 kDa. It has been previously shown that cleavage alters the conformation of the fusion protein (7). Perhaps cleavage results in less close packing of the HN and F molecules and, therefore, smaller cross-linked complexes.

It has been proposed that attachment of HN protein to its receptor stimulates the interaction of HN and F proteins (10). However, attempts to increase the participation of HN proteins in attachment by adding an uninfected Cos cell overlay did not alter HN-F protein cross-linking. Incubating infected cells in neuraminidase, which should block attachment, also failed to significantly alter the amount of HN and F_0 in cross-linked complexes, although the amount of F_1 cross-linked was slightly increased under these conditions.

In an attempt to characterize cross-linked species derived from individual proteins, we transfected cells with the HN gene alone or the F gene alone, using a simian virus 40-based vector. We were not able to cross-link HN proteins but were able to cross-link F proteins, indicating that F-F cross-linking is possible. These cross-linked complexes electrophoresed with sizes of 200 to 300 kDa (unpublished observations). Characterization of the cross-linked complexes formed between strain AV HN and F proteins showed that while some complexes were very large and resolved in the stacker gel, most were in three to four relatively discrete species ranging in size from 160 to 300 kDa. If there is no cross-linking of the HN protein except for the naturally occurring intermolecular disulfide bonding, then the smaller (160- to 300-kDa) complexes precipitated with HN antisera should have one to four HN proteins and one to four F proteins, which may be a mixture of both HN-F proteins as well as F-F protein complexes.

In contrast to the results obtained from infected cells, DTSSP cross-linking of transfected cells coexpressing HN and F proteins did not result in the detection of any HN-F protein complexes (unpublished observations). Given the failure to detect HN-F protein complexes in transfected cells, it seemed possible that their detection in infected cells could be due to close packing of molecules as a result of high levels of viral glycoprotein expression in infected cells at 7.25 h postinfection. However, these cross-linked complexes were readily detected as early as 4.25 h postinfection, when the concentrations of viral proteins at the cell surface should be considerably reduced (16). Thus, the surfaces of infected cells may differ from those of transfected cells. In this regard, it is interesting that the only studies of cross-linking of HN and F proteins in cells expressing these proteins from a vector were accomplished in the context of vaccinia virus infection (12, 32).

After cross-linking, HN antisera precipitated a 120-kDa complex which, when reduced, contained primarily F_1 protein. In addition, the precipitates contained a 66-kDa polypeptide which comigrated with the nonreduced form of the fusion protein. This result suggests that after cross-linking, F protein which is not cross-linked to HN protein can be precipitated with HN antiserum. The most likely explanation for this observation is that cross-linking stabilizes a complex containing HN and F proteins which is able to interact noncovalently with F molecules which are then coprecipitated with the cross-linked complex. Indeed, it has been reported that the fusion protein of paramyxoviruses forms SDS-resistant oligomeric structures (28). Such structures would be resistant to the precipitation conditions used here.

The current model for an HN-F protein interaction in paramyxoviruses which require both glycoproteins for fusion promotion is that F protein is synthesized as a metastable prefusion form that is activated by a conformational change in HN protein caused by binding to its receptor (10, 11, 24). Whether this HN-F protein interaction occurs before or after HN protein attachment is not predicted in this model. Our results suggest that HN and F proteins interact prior to HN protein attachment in a subcellular compartment preceding the trans-Golgi in the trafficking pathway to the plasma membrane. Indeed, work by Tanaka et al. (27) suggests that human parainfluenza virus type 3 HN and F proteins interact in the endoplasmic reticulum. This idea is consistent with our results, as an interaction was detected during a 5-min pulse-label, prior to proteolytic cleavage of F_0. By analogy with influenza HA1 and HA2 and the data presented here, perhaps HN and F proteins interact in the rough endoplasmic reticulum and hold each other in a prefusion conformation. Upon attachment of the HN protein to its receptor, HN protein undergoes a conformational change which causes a conformational change in the associated F protein. The conformational change in F protein thus allows the release of the hydrophobic fusion peptide into the target membrane and permits fusion to take place.

ACKNOWLEDGMENT

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REFERENCES

1. **Barr, P. J.** 1991. Mammalian subtilisins: the long-sought dibasic processing endoproteases. Cell **66:**1–3.
2. **Citovski, V., P. Yanai, and A. Loyter.** 1986. The use of circular dichroism to study conformational changes induced in Sendai virus envelope glycoproteins. J. Biol. Chem. **261:**2235–2239.
3. **Collins, P. L., R. M. Chanock, and K. McIntosh.** 1996. Parainfluenza viruses, p. 1205–1242. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
4. **Deng, R., Z. Wang, A. Mirza, and R. Iorio.** 1995. Localization of a domain on the paramyxovirus attachment protein required for the promotion of cellular fusion by its homologous fusion protein spike. Virology **209:**457–469.
5. **Glickman, R. L., R. J. Syddall, R. M. Iorio, J. P. Sheehan, and M. A. Bratt.** 1988. Quantitative basic residue requirements in the cleavage-activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. J. Virol. **62:**354–356.
6. **Hightower, L. E., and M. A. Bratt.** 1975. Protein metabolism during the steady state of Newcastle disease virus infection. J. Virol. **15:**696–706.
7. **Hsu, M.-C., A. Scheid, and P. W. Choppin.** 1981. Activation of the Sendai virus protein (F) involves a conformational change with exposure of a new hydrophobic region. J. Biol. Chem. **256:**3557–3563.
8. **Hu, X., R. Ray, and R. W. Compans.** 1992. Functional interactions between the fusion protein and hemagglutinin-neuraminidase of human parainfluenza viruses. J. Virol. **66:**1528–1534.
9. **Iorio, R. M., R. L. Glickman, A. M. Riel, J. P. Sheehan, and M. A. Bratt.** 1989. Functional and neutralization profile of seven overlapping antigenic sites on the HN glycoprotein of Newcastle disease virus: monoclonal antibodies to some sites prevent attachment. Virus Res. **13:**245–262.
10. **Lamb, R. A.** 1993. Paramyxovirus fusion: a hypothesis for changes. Virology **197:**1–11.
11. **Lamb, R. A., and D. Kolakofsky.** 1996. *Paramyxoviridae*: the viruses and their replication, p. 1177–1204. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
12. **Malvoisin, E., and T. F. Wild.** 1993. Measles virus glycoproteins: studies on the structure and interaction of the haemagglutinin and fusion proteins. J. Gen. Virol. **74:**2365–2372.
13. **Markwell, M. A., and C. F. Fox.** 1980. Protein-protein interactions within paramyxoviruses identified by native disulfide bonding or reversible chemical cross-linking. J. Virol. **33:**152–166.
14. **McGinnes, L. W., A. Semerjian, and T. G. Morrison.** 1985. Conformational changes in Newcastle disease virus fusion glycoprotein during intracellular transport. J. Virol. **56:**341–348.

15. **Morrison, T., C. McQuain, and L. McGinnes.** 1991. Complementation between avirulent Newcastle disease virus and a fusion protein gene expressed from a retrovirus vector: requirements for membrane fusion. J. Virol. **65:** 813–822.
16. **Morrison, T. G., and L. W. McGinnes.** 1989. Avian cells expressing the Newcastle disease virus hemagglutinin-neuraminidase protein are resistant to Newcastle disease virus infection. Virology **171:**10–17.
17. **Morrison, T. G., C. McQuain, K. F. O'Connell, and L. W. McGinnes.** 1990. Mature, cell-associated HN protein of Newcastle disease virus exists in two forms differentiated by posttranslational modifications. Virus Res. **15:**113–134.
18. **Morrison, T. G., and L. J. Ward.** 1984. Intracellular processing of the vesicular stomatitis virus glycoprotein and the Newcastle disease virus hemagglutinin-neuraminidase glycoprotein. Virus Res. **1:**225–239.
19. **Morrison, T. G., L. Ward, and A. Semerjian.** 1985. Intracellular processing of the Newcastle disease virus fusion glycoprotein. J. Virol. **53:**851–857.
20. **Moscona, A., and R. W. Peluso.** 1992. Fusion properties of cells infected with human parainfluenza virus type 3: receptor requirements for viral spread and virus-mediated membrane fusion. J. Virol. **66:**6280–6287.
21. **Russell, R., R. G. Paterson, and R. A. Lamb.** 1994. Studies with cross-linking on the oligomeric form of the paramyxovirus fusion protein. Virology **199:** 160–168.
22. **Sakaguchi, T., Y. Matsuda, R. Kiyokage, N. Kawahara, K. Kiyotani, N. Katunuma, Y. Nagai, and T. Yoshida.** 1991. Identification of endoprotease activity in the *trans* Golgi membranes of rat liver cells that specifically processes *in vitro* the fusion glycoprotein precursor of virulent Newcastle disease virus. Virology **184:**504–512.
23. **Sergel, T., L. W. McGinnes, and T. G. Morrison.** 1993. The fusion promotion activity of the NDV HN protein does not correlate with neuraminidase activity. Virology **196:**831–834.
24. **Sergel, T., L. W. McGinnes, M. E. Peeples, and T. G. Morrison.** 1993. The attachment function of the Newcastle disease virus hemagglutinin-neuraminidase protein can be separated from fusion promotion by mutation. Virology **193:**717–726.
25. **Staros, J. V.** 1982. N-hydroxysulfosuccinimide active esters: bis(N-hydroxysuccinimide) esters of two dicarboxylic acids are hydrophilic, membrane impermeant, protein crosslinkers. Biochemistry **21:**3950–3955.
26. **Tanabayashi, K., and R. W. Compans.** 1996. Functional interaction of paramyxovirus glycoproteins: identification of a domain in Sendai virus HN which promotes cell fusion. J. Virol. **70:**6112–6118.
27. **Tanaka, Y., B. R. Heminway, and M. S. Galinski.** 1996. Down-regulation of paramyxovirus hemagglutinin-neuraminidase glycoprotein surface expression by a mutant fusion protein containing a retention signal for the endoplasmic reticulum. J. Virol. **70:**5005–5015.
28. **Taylor, S. S., B. Newton, and M. E. Peeples.** 1993. Newcastle disease virus fusion proteins are homooligomeric, abstr. PA102. *In* Abstracts of the 12th Annual Meeting of the American Society of Virology.
29. **Toyoda, T., T. Sakaguchi, K. Imai, N. M. Inocencio, B. Gotoh, M. Hamaguchi, and Y. Nagai.** 1987. Structural comparison of the cleavage-activation site of the fusion glycoprotein between virulent and avirulent strains of Newcastle disease virus. Virology **158:**242–247.
30. **Tsurudome, M., M. Kawano, T. Yuasa, N. Tabata, M. Nishio, H. Komada, and Y. Ito.** 1995. Identification of regions on the hemagglutinin-neuraminidase protein of human parainfluenza virus type 2 important for promoting cell fusion. Virology **213:**190–203.
31. **Wang, C., G. Raghu, T. Morrison, and M. E. Peeples.** 1992. Intracellular processing of the paramyxovirus F protein: critical role of the predicted amphipathic alpha helix adjacent to the fusion domain. J. Virol. **66:**4161–4169.
32. **Yao, Q., X. Hu, and R. W. Compans.** 1997. Association of the parainfluenza virus fusion and hemagglutinin-neuraminidase glycoproteins on cell surfaces. J. Virol. **71:**650–656.