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

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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 F0. Furthermore, under conditions that maximized HN protein binding to its receptor, there was no change in the percentages of HN and F0 proteins precipitated with heterologous antisera, but a decrease in F1 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 F1 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 F0 (uncleaved fusion protein) into F1 and F2 (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.

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)

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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 × 10⁴ 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 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 70% of the sequence of the carboxy terminus of the F protein (24). Polyclonal antisera for NDV-NH 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 NH 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 μCi of [35S]methionine-cysteine (EXPRE 35S35S; New England Nuclear) per ml. The cells were then chased in nonradioactive medium for 2 h.

The virus was adsorbed for 30 min at 37°C before DMEM supplemented with nonessential amino acids, vitamins, penicillin-streptomycin, and 10% fetal calf serum.

**Antibodies.** Monoclonal antibodies specific for NDV-HN were a generous gift of Ron Iorio. Antibodies were used anti-1c, anti-2b, anti-3a, and anti-4a (9).

Polyclonal antisera for NDV F was against a portion of 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 NH protein (24).

**Chemical cross-linking.** For coimmunoprecipitation, 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 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.

**Attachment assay.** At 5 h postinfection, AV-infected Cos cells were pulse-labeled with [35S]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 for NDV F were visualized by autoradiography. 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 antisera lanes), or no antisera (−) 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.

**RESULTS**

**Commmunoprecipitation of HN and F.** To investigate a potential interaction between the HN and F proteins of NDV (strain AV)-infected cells, communoprecipitation 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 communoprecipitated F protein, but a mixture was used to ensure that all populations of HN protein were immunoprecipitated. Concentrations of F ranging from 0.15 to 0.3% were used in nonreducing 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 F₁ and F₂ proteins could be precipitated with HN antisera. In addition, a protein that comigrated with HN protein could be precipitated with F antisera. Very little nonspecific precipitation, particularly at SDS concentrations of 0.25% and higher, was found. The amount of HN, F₁, and F₂ proteins coprecipitated with heterologous antisera decreased with increasing SDS concentrations. In 0.25% SDS, immunoprecipitation with F antisera resulted in the coprecipitation of approximately 6% of total HN protein, and immunoprecipitation with HN antisera resulted in the coprecipitation of F₁ and 1% of total F₁ and F₂ proteins, respectively. Various lysins and immunoprecipitation conditions were investigated, without a significant change in the results.

**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...
antisera contained uncleaved F₀ 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 F₀ 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 antisera 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 F1-size molecule at all concentrations of cross-linker. This protein is likely F₁ 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, F₁ protein was precipitated in increasing amounts, while a decrease was observed at 1.0 mM DTSSP. F₀ 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 antiserum (not shown) showed that approximately 28% of total labeled HN protein could be precipitated with F antiserum, while 5% of total labeled F₀ protein and 22% of total labeled F₁ 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-
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 antisera precipitated only a 66-kDa protein, the nonreduced, monomeric form of F protein (F\textsubscript{n}) (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\textsubscript{0} and F\textsubscript{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\textsubscript{1} protein, with minimal amounts of HN protein and F\textsubscript{0} protein present. The distribution of HN protein (Table 1) among these species varied, with minimal percentages of HN protein and F\textsubscript{0} protein, and a substantial amount of F\textsubscript{1} protein. The distribution of F\textsubscript{0} and F\textsubscript{1} proteins was similar to that observed in the absence of cross-linker.

A similar analysis of cross-linked complexes immunoprecipitated with F antisera (Fig. 5C) showed that material precipitated in the stacker gel as well as the complexes ranging in size from 140 to 300 kDa contained F\textsubscript{0} and F\textsubscript{1} proteins. The 120-kDa species as well as the 66-kDa species contained almost exclusively F\textsubscript{0} and F\textsubscript{1} proteins. The distributions of total F\textsubscript{0} and F\textsubscript{1} proteins 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\textsubscript{1} proteins and 5% of the total F\textsubscript{0} protein could be precipitated with heterologous antisera. For the HN and F\textsubscript{1} proteins, these percentages increased with time postinfection until 5.75 h and then dropped, while for the F\textsubscript{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.

**Effect of F\textsubscript{0} cleavage on detection of HN and F cross-linked species.** While F\textsubscript{0} protein was detected in cross-linked complexes obtained with HN antisera, the predominant species detected was F\textsubscript{1} protein. Furthermore, it has been reported that in measles virus-infected cells, only the F\textsubscript{1} protein, not the F\textsubscript{0} protein, can be precipitated in a cross-linked complex with HA (13). To determine the importance of cleavage of the F\textsubscript{0} protein in the detection of HN-F protein complexes, we used the avirulent B1 strain of NDV. When Cos cells are infected

![Figure 5](https://example.com/figure5.png)
with NDV strain B1, the F₀ protein generated by the virus is not cleaved into F₁ and F₂ 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 F₀ 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. 4B, 8B, and 9B), while F₀ and F₁ values were obtained from proteins precipitated with F antiserum (Fig. 4C, 8C, and 9C). ND, no data.

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<td>-Trypsin</td>
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* 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 F₀ and F₁ values were obtained from proteins precipitated with F antiserum (Fig. 4C, 8C, and 9C). ND, no data.

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 [35S]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) Densitometer units of HN protein precipitated with F antiserum (Fig. 4C, 8C, and 9C) were plotted against time postinfection (PI). (C) Densitometer units of F₀ proteins precipitated with HN antiserum were plotted against time postinfection.
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 F₁ 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 in 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, F₀, and F₁ 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 maximize attachment (+N−OL), while conditions which minimize attachment (−N+OL) resulted in the precipitation of 30% (±8%) of total precipitable HN protein. Precipitation of F₀ and F₁ 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 F₁ 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
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 F₀ protein, an amount not very different from that observed by communoprecipitation. It is possible that only a minor population of F₀ 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 F₀ to HN. Malvoisin and Wild (12) reported chemical cross-linking of HA and F₁ proteins of measles virus at the cell surface but were unable to detect any F₀ 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 F₀ 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 F₀ 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 amount of coprecipitation detected was very small. Approximately 5% of the total HN protein was precipitable with F antisera, and 2 to 3 and 1 to 4% of F₀ and F₁ 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 F₁ protein could be precipitated with heterologous antisera. These percentages were not increased with

![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 antisera + 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. 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 [35S]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 infected cell overlay added; +OL, infected cell overlay added; lane M, virus-infected cell extract not immunoprecipitated.](https://example.com/figure-10)
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-F1 protein complexes were resolved primarily in the stacker region of the gel before cleavage, while after cleavage, the majority of the HN-F1 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, in cross-linked complexes, although the amount of F1 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 antiserum 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 antiserum precipitated a 120-kDa complex which, when reduced, contained primarily F1 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 F0. 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.

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REFERENCES


