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Inhibition of Receptor Binding Stabilizes Newcastle Disease Virus HN and F Protein-Containing Complexes

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Receptor binding of paramyxovirus attachment proteins and the interactions between attachment and fusion (F) proteins are thought to be central to activation of the F protein activity; however, mechanisms involved are unclear. To explore the relationships between Newcastle disease virus (NDV) HN and F protein interactions and HN protein attachment to sialic acid receptors, HN and F protein-containing complexes were detected and quantified by reciprocal coimmunoprecipitation from extracts of transfected avian cells. To inhibit HN protein receptor binding, cells transfected with HN and F protein cDNAs were incubated with neuraminidase from the start of transfection. Under these conditions, no fusion was observed, but amounts of HN and F protein complexes increased twofold over amounts detected in extracts of untreated cells. Stimulation of attachment by incubation of untransfected target cells with neuraminidase-treated HN and F protein-expressing cells resulted in a twofold decrease in amounts of HN and F protein complexes. In contrast, high levels of complexes containing HN protein and an uncleaved F protein (F-K115Q) were detected, and those levels were unaffected by neuraminidase treatment of cell monolayers or by incubation with target cells. These results suggest that HN and F proteins reside in a complex in the absence of receptor binding. Furthermore, the results show that not only receptor binding but also F protein cleavage are necessary for disassociation of the HN and F protein-containing complexes.

Paramyxoviruses, such as Newcastle disease virus (NDV), initiate infection by the combined action of the two viral glycoproteins, the hemagglutinin-neuraminidase (HN) protein and the fusion (F) protein. The HN protein, the virus attachment protein, binds to sialic acid-containing receptors, and the F protein directs the fusion of the virus membrane with the cell membrane (reviewed in reference 15). Paramyxovirus F proteins do not require the acid pH of endosomes to activate fusion activity (7, 15). Because of this acid-independent fusion, cells expressing the HN and F proteins can fuse with adjacent cells to form multinuclear cells or syncytia in a process that is often assumed to mimic virus-cell fusion (7, 15).

Viral fusion proteins have been classified into two groups based on their structure and mechanisms for mediating fusion (9, 17). Class 1 fusion proteins, which are trimers, include paramyxovirus F proteins as well as the influenza hemagglutinin protein and retrovirus env proteins (7). These fusion proteins are synthesized as an inactive precursor, and proteolytic cleavage generates two subunits, F₁ and F₂, in the case of paramyxoviruses (7, 15). The new amino terminus, generated by cleavage of the precursor, is the fusion peptide (FP). Class 1 fusion proteins also contain two important heptad repeat (HR) domains (reviewed in reference 3). The F protein HR domains are located just carboxyl terminal to the fusion peptide (HR1 or HRA) and adjacent to the transmembrane (TM) domain (HR2 or HRB). HR1 and HR2 peptides have a strong affinity, forming a very stable, six-stranded, coil-coil, with the HR1 forming an interior trimer and the HR2 binding in the

grooves of the trimer in an antiparallel orientation (1, 20, 51). Based on the inhibitory effect of HR1 and HR2 peptides on virus fusion (13, 16, 37, 38, 46, 48, 49), it is thought that the HR1 and HR2 domains are not associated prior to F protein activation while the two domains are complexed in the post-fusion F protein (1, 38).

Current models for F protein-directed fusion propose that the F protein, prior to the onset of fusion, is in a metastable conformation and that activation of fusion induces conformational changes in the F protein (1, 3, 38). These changes are proposed to expose the fusion peptide for insertion into a target membrane, anchoring the F protein in that membrane (1, 3, 45). The protein is thought to refold, forming the very stable HR1-HR2 complex, a change that pulls the target and the effector membranes together (1, 3, 45). Fusion of the two membranes then proceeds.

The structure of the intact, prefusion, cleaved, metastable F protein as it sits in membranes is not clear (2, 47). Furthermore, the mechanisms involved in the activation of paramyxovirus F proteins are poorly understood. The activation of acid pH-independent fusion proteins is usually linked to receptor binding (reviewed in reference 7). In the case of most paramyxovirus F proteins, it is clear that HN protein receptor binding is essential for F protein activity (30–32, 36). However, HN protein provides more than a docking function, since some mutations in the HN protein eliminated its fusion promotion activity without affecting attachment and neuraminidase activity (4, 8, 39, 40, 42). Furthermore, the requirement for paramyxovirus HN proteins in fusion is virus specific (10), providing strong support for the idea that there is a specific and necessary interaction between the two proteins required for fusion activation.

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How an HN-F protein interaction serves to activate the F protein is unknown. One model proposes that attachment of HN protein to sialic acid receptors stimulates an interaction of HN protein with F protein, and that interaction leads to F protein refolding into its postfusion conformation (3, 14, 15). A second model proposes that HN and F proteins form a complex prior to HN protein attachment and that HN protein has a role in the maintenance of the metastable conformation of the F protein. In this model, HN protein receptor binding stimulates a conformational change in the HN protein, releasing the F protein to refold into a more stable conformation, mediating membrane fusion in the process (23, 42, 50). A determination of which proposal is correct would be an important step in clarifying the molecular mechanisms involved in F protein activation. The first model predicts that an inhibition of HN protein attachment would block any association of the two proteins. The second model predicts that an inhibition of attachment should increase amounts of HN-F protein complexes in cells, while stimulation of attachment should result in a disassociation of the complexes. To address this question, we quantified HN-F protein complexes under conditions that inhibited HN protein attachment to sialic acid and conditions that promoted HN protein attachment. We found that amounts of HN-F protein complexes, detected by coimmunoprecipitation, were maximal under conditions that inhibited HN protein attachment, while stimulation of attachment and fusion resulted in a significant drop in the amounts of these complexes. Furthermore, we found that disassociation of the complex required not only HN protein attachment but also F protein cleavage. Our results support the proposal that HN and F proteins reside in a complex prior to attachment of HN protein to its receptors, a complex that disassociates with attachment and fusion.

MATERIALS AND METHODS

Cells. A spontaneously transformed fibroblast cell line derived from the East Lansing strain (ELL-0) of chicken embryos (UMNSAH/DF-1) was obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin-streptomycin and 10% fetal calf serum (FCS).

Plasmids. NDV cDNA sequences encoding HN, F wild type (Fwt), and cleaved F (F-K115Q) proteins were subcloned into the expression vector pCAGGS (28, 33) to generate pCAGGS-HN, pCAGGS-Fwt, and pCAGGS-F-K115Q, respectively. The F-K115Q protein cDNA contained a point mutation in the cleavage site sequence at residue 115 (K115Q) which eliminated the furin recognition site. Some experiments required the use of an F protein with the sequence tag, FLAG, added to the carboxyl terminus of Fwt protein, Fwt-FLAG, or the F-K115Q protein, F-K115Q-FLAG. The FLAG sequence, added by standard PCR protocols, did not interfere with surface expression of either protein, and in the case of Fwt protein, the tag did not interfere with cleavage or fusion activity of the protein (data not shown). Plasmids pTA and pB1-G were obtained from Clontech.

Antibodies. Anti-Fu1a is a mouse monoclonal antibody specific for NDV F protein and was obtained from M. Peebles (29). Anti-HR1, anti-HR2, and anti-Ftal antibodies were raised against peptides with sequences from the NDV F protein HR1, HR2, and cytoplasmic domains, respectively, and have been previously described (23, 26). Anti-F2-96 antibody was raised against a peptide corresponding to amino acids 96 to 116 of the NDV F protein. Anti-HN monoclonal antibodies, anti-1c, anti-2b, anti-3a, and anti14c, were obtained from R. Iorio (11, 12). Anti-AS antibody was raised against a peptide with a sequence from the NDV HN protein and has been previously described (21, 25, 41). Anti-A antibody was raised against a peptide with the sequence corresponding to amino acid 49 to amino acid 117 of the NDV HN protein. Anti-H antibody was raised against a peptide corresponding to amino acid 515 to amino acid 571 of

the NDV HN protein. Anti-FLAG monoclonal antibody was obtained from Sigma Corp.

Transfection. Transfections of ELL-0 cells were accomplished using Lipofectamine (Invitrogen) as recommended by the manufacturer. For each transfection, a mixture of DNA (0.5 μ g/35-mm plate) and 7 μ l of Lipofectamine in OptiMEM media (Gibco/Invitrogen) was incubated at room temperature for 45 min and added to cells previously washed with OptiMEM. The cells were incubated for 5 h, the Lipofectamine-DNA complexes were removed, and 2 ml of supplemented DMEM was added. Cells were incubated overnight at 37°C.

Coimmunoprecipitation. Transfected cells were washed with ice-cold phosphate-buffered saline (PBS) and then lysed in TNE buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100, 2.5 mg/ml *N*-ethylmaleimide, and 2 mg/ml DNase. Lysates were incubated on ice for 30 min and then sheared by passing through a 25-gauge needle five times and then vigorously vortexed. Excess primary antibody was added, and extracts were incubated on ice for at least 2 h. Saturating amounts of all antibodies (amounts of antibodies that resulted in maximal precipitation) were determined in preliminary experiments. Pansorbin cells, blocked overnight in TNE buffer containing 1% Triton X-100 and 5 mg/ml bovine serum albumin (BSA) and then prewashed in TNE containing 1% Triton X-100 and 1 mg/ml bovine serum albumen, were added in excess as determined in preliminary experiments, and incubation was continued at 4°C with constant mixing for at least 2 h. Immune complexes were collected by centrifugation (10,000 rpm for 30 s in a microcentrifuge) and washed three times in ice-cold TNE containing 0.5% Triton X-100. The pelleted complexes were resuspended in gel sample buffer.

Polyacrylamide gel electrophoresis and Western blot analysis. Total cell extracts or immunoprecipitates, diluted in gel sample buffer (125 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol) with or without 0.7 M β -mercaptoethanol, were loaded onto 8% polyacrylamide gels. After electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris, pH 8.2, 192 mM glycine, 15% methanol) and transferred to Immobilon-P (Millipore Corp.) membranes. The membranes were blocked overnight at 4°C in PBS containing 0.5% Tween 20 and 10% nonfat milk, washed with PBS-Tween 20, and incubated for 1 h at room temperature with primary antibody diluted to 1:1,000 in PBS-Tween 20. Membranes were then washed, incubated for 1 h at room temperature with secondary antibody and goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase (Amersham Biosciences) diluted to 1:40,000 in PBS-Tween 20, and then washed extensively in PBS-Tween 20. Bound antibody was detected using the ECL Western blotting detection reagent system (Amersham Biosciences). Quantification of the signal was accomplished using a Fluor-S imager (Bio-Rad). That the signal was proportional to the amount of protein in the blot was determined in preliminary experiments. Quantification of the signal was accomplished in the linear range of the film and the imager detection.

Fusion assay. Content mixing due to fusion was measured using modifications of a protocol previously described (8, 22). Briefly, a plasmid encoding a tetracycline-responsive transcriptional activator, tTA (Clontech), was cotransfected (1 μ g/35-mm plate) with pCAGGS-HN (0.5 μ g/35-mm plate) and pCAGGS-F DNAs (0.5 μ g/35-mm plate). A separate population of avian cells was transfected with a plasmid encoding the β -galactosidase protein under the control of the tetracycline-responsive transcriptional activator pB1-G (Clontech) (1 μ g/35-mm plate). After mixing the two cell populations, incubation was continued for 6 h. Cells were washed two times in PBS and lysed (Promega reporter cell lysis buffer), and extracts were assayed for β -galactosidase activity (Promega protocols). Activity due to any background fusion was measured after mixing avian cells transfected with only pTA and cells transfected with pB1-G. Values obtained were subtracted from values obtained with cells expressing wild-type HN and wild-type F proteins. Background was minimal and was at least 10-fold less than activities obtained using cells expressing HN and Fwt protein.

Surface biotinylation. Monolayers of cells were washed three times in PBS-CM buffer (PBS with 0.1 mM CaCl₂ and 1 mM MgCl₂). PBS-CM buffer was added to monolayers, which were then incubated on ice for 10 min. The buffer was removed, PBS-CM containing 0.5 mg/ml sulfo-succinimidyl 2 (biotinamido)-ethyl-1,3-dithiopropionate (Pierce) was added, and the monolayers were incubated on ice for 30 min. The cross-linker was removed, and 2 ml of DMEM was added. Monolayers were incubated on ice for 5 min, washed three times in PBS, and lysed as described above. To precipitate biotinylated molecules in immunoprecipitates, the immunoprecipitates were resuspended in TNE containing 0.5% Triton X-100 and then incubated with neutravidin-agarose (Pierce) that had been blocked in TNE containing 0.5% Triton X-100 and 5 mg/ml BSA and then washed in TNE containing 0.5% Triton X-100 and 1 mg/ml BSA. The mix was incubated on ice overnight and washed three times with TNE buffer containing 0.5% Triton X-100, and the precipitate was resuspended in gel sample buffer.

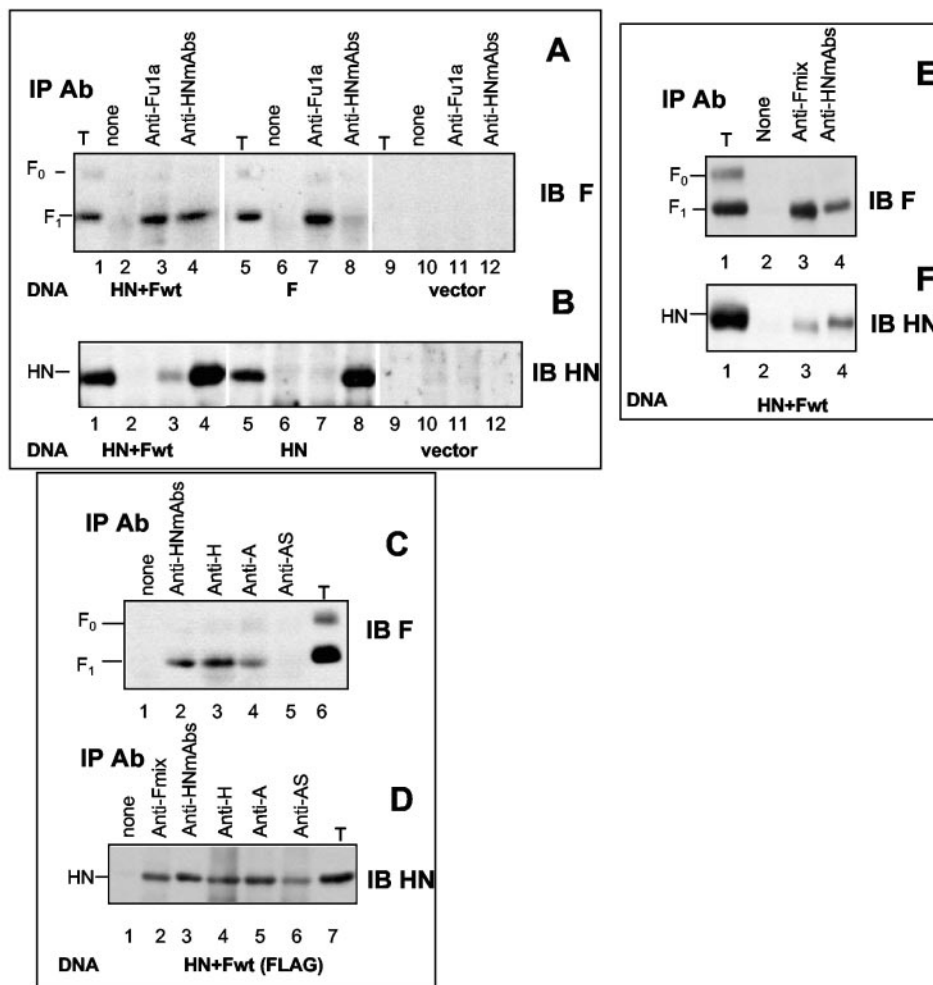


FIG. 1. Characterization of HN and Fwt protein complexes in avian cell extracts. (A and B) Cells were cotransfected with HN protein and F wild-type (Fwt) protein cDNAs (lanes 1 to 4), Fwt cDNA (lanes 5 to 8), or vector (lanes 9 to 12). Immune complexes were formed using antibodies (Ab) indicated at the top of panel A (IP Ab). IP, immunoprecipitation. (A) F protein detected in the complexes by Western blot analysis using anti-HR2 (IB-F). IB, immunoblot. (B) HN protein present in the complexes (detected using anti-AS) (IB HN). Anti-HNmAbs, a mix of anti-HN protein monoclonal antibodies. T, total extracts, representing 50% of that used for immunoprecipitation. (C and D) Cells were cotransfected with HN protein and Fwt-FLAG cDNAs. Immune complexes were formed with antibodies indicated at the top of panels C and D (IP Ab). Anti-Fmix, a mixture of anti-HR1, anti-HR2, anti-F2-96, anti-Ftail, and anti-Fu1a. F protein present in the immunoprecipitates was detected using anti-FLAG monoclonal antibody (IB F). HN protein present in the extracts was detected using anti-AS antibody (IB HN). (E and F) Surfaces of cells cotransfected with HN and Fwt protein cDNAs were biotinylated, and then cells were lysed. Immune complexes formed with the antibodies indicated at the top of panel E (IP Ab) were solubilized and then precipitated with neutravidin as described in Materials and Methods. The F protein in the resulting precipitates was detected by Western blot analysis with anti-HR2 (IB F), while the HN protein was detected using anti-AS (IB HN). All proteins were electrophoresed in the presence of reducing agent.

RESULTS

Detection of HN-F protein complexes in avian cells. To explore directly the relationship between HN and F protein-containing complexes and HN protein receptor binding, we first characterized HN-F protein complexes in extracts derived from transfected avian cells. While coimmunoprecipitation of NDV HN protein and F protein expressed in transfected mammalian cells has been demonstrated (6, 18), it was important to characterize these complexes in avian cell extracts since HN and F protein coexpression in these cells results in dramatically more fusion than in mammalian cells (unpublished observations). Proteins present in transfected cell extracts were pre-

cipitated with a mix of anti-HN protein-specific antibodies or anti-F protein-specific antibodies. The F protein present in the immunoprecipitates was detected by Western blot analysis using a rabbit anti-F protein peptide antibody, while the HN protein in the immunoprecipitates was detected in a separate Western blot using rabbit anti-HN protein-specific peptide antibody.

Figure 1A and B show that complexes containing both HN and F proteins could be detected by reciprocal coimmunoprecipitation from these avian cell extracts. F protein was precipitated with anti-HN protein monoclonal antibodies (Fig. 1A, lane 4). The F protein detected with anti-HN protein antibody

was not due to nonspecific aggregation, since omission of primary antibody in the precipitation protocol eliminated detection of F protein (lane 2). Furthermore, F protein in extracts derived from cells expressing only F protein was not precipitated with anti-HN protein antibodies (lane 8). Similarly, HN protein in these extracts was precipitated with an anti-F protein monoclonal antibody (Fig. 1B, lane 3) but not from extracts containing only HN protein (Fig. 1B, lane 7) and not when primary antibody was omitted during the precipitation protocol (Fig. 1B, lanes 2 and 6).

HN-F protein complexes contain primarily mature proteins.

It has been suggested that HN and F proteins may form nascent protein complexes (34, 43, 44), which could account for detection of HN-F protein complexes. However, complexes detected in protocols used here were composed primarily of mature proteins, folded proteins recognized by conformationally sensitive antibodies, and proteins that were posttranslationally modified. The F protein precipitated with the anti-HN protein monoclonal antibodies was primarily the cleaved form of the protein, F₁ (Fig. 1A). In addition, the anti-HN protein monoclonal antibodies used for precipitation recognize only mature, folded HN protein (24), suggesting that mature HN protein was complexed with F protein. However, two approaches were taken to verify this conclusion. First, anti-AS antibody, which recognizes only nascent HN protein (24, 25), did not precipitate F protein (Fig. 1C, lane 5), though it did precipitate significant amounts of HN protein (Fig. 1D, lane 6). In contrast, two other anti-HN protein peptide antibodies (anti-H and anti-A) did precipitate the F protein (Fig. 1C, lanes 3 and 4). Anti-A precipitates both nascent and mature protein, while anti-H precipitates only disulfide-linked HN protein (unpublished observations).

Second, Fig. 1E and F show that HN-F protein complexes included cell surface material. Surfaces of cells were biotinylated, and after cell lysis, proteins were precipitated with anti-HN protein or anti-F protein antibodies. The immune complexes were then precipitated with neutravidin in order to isolate only surface molecules. Clearly, F protein precipitated with anti-HN protein antibody included surface-biotinylated F protein (Fig. 1E, lane 4), and HN protein precipitated with anti-F protein antibodies included biotinylated HN protein (Fig. 1F, lane 3).

Figure 1D also shows that a mix of anti-F protein antibodies precipitated HN protein more efficiently than the anti-Fu1a monoclonal antibody (compare Fig. 1B, lanes 3 and 4, with D, lanes 2 and 3). Approximately 2.5 times more HN protein was reproducibly precipitated with the anti-F protein antibody mix than with the anti-Fu1a monoclonal antibody. In addition, under certain conditions, anti-HN protein monoclonal antibodies precipitated more F protein than the anti-Fu1a antibody (data not shown). Thus, the mix of F protein antibodies was used for subsequent experiments, since the monoclonal antibody likely does not detect all the HN and F protein complexes.

HN-Fwt protein complexes increase with attachment inhibition. In order to determine the effect of attachment on the levels of these HN-F protein-containing complexes, we incubated HN-F protein-expressing cells under conditions that minimized attachment. Neuraminidase removes the sialic acid receptors on surfaces of cells, inhibiting attachment and, therefore, fusion of these cells to adjacent untransfected cells (30).

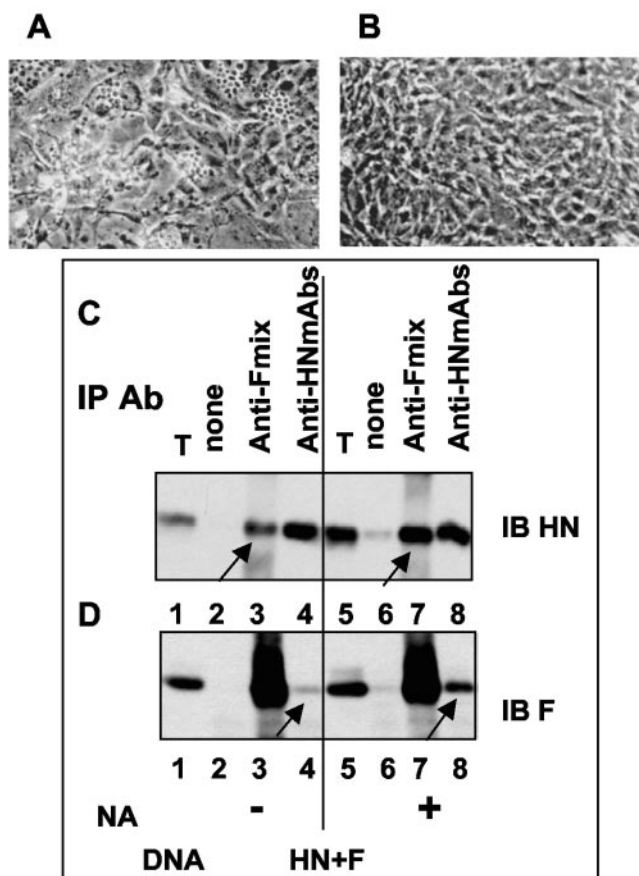


FIG. 2. Neuraminidase treatment of transfected cells increases detection of immune complexes. (A and B) Cells cotransfected with HN and Fwt protein cDNAs were untreated (A) or incubated with neuraminidase (0.5 U/ml, overnight incubation) (B). (C and D) Cells shown in panels A and B were lysed, and immune complexes were formed using antibodies (Ab) indicated at the top of panel C (IP Ab). IP, immunoprecipitation. The F protein in these complexes (electrophoresed in the absence of reducing agent) was detected using anti-HR2 (IB F) (C), and the HN protein in the complexes (electrophoresed in the presence of reducing agent) was detected using anti-AS (IB HN) (D). IB, immunoblot. Anti-HNmAbs, a mixture of monoclonal antibodies. Complexes derived from untreated cells (NA-) are shown in lanes 1 to 4. Complexes derived from neuraminidase-treated cells (NA+) are shown in lanes 5 to 8. T, total cell extract, which represents 25% (C, lane 1) or 50% (B, lane 5, and D, lanes 1 and 5) that were used for immunoprecipitation. (D) Signals in lanes 3 and 7 are a combination of comigrating F protein and precipitating rabbit antibody.

Indeed, cell-cell fusion normally seen in these monolayers (Fig. 2A) was completely inhibited by neuraminidase (Fig. 2B), indicating the effectiveness of the digestion. After neuraminidase digestion of cell monolayers, the amounts of HN protein precipitated with anti-F protein antibody mix were significantly increased over amounts detected in extracts from untreated cells (Fig. 2C, compare lanes 3 and 7, indicated by arrows). Similarly, neuraminidase treatment significantly increased amounts of F protein precipitated with anti-HN antibody (Fig. 2D, compare lanes 4 and 8). These results are consistent with the idea that HN and F protein-containing complexes form in the absence of HN protein receptor binding.

HN and F protein complexes decrease with attachment.

Detection of larger amounts of HN-F protein-containing complexes in the absence of attachment suggested that attachment may result in the disassociation of these complexes. To test this idea, we asked if the levels of these complexes, detected in extracts from neuraminidase-treated cells, could be reduced if these cells were mixed with untreated target cells to stimulate attachment. To monitor the effectiveness of neuraminidase digestion in eliminating surface receptors on target cells as well as HN and F protein-expressing cells (effector cells), fusion between effector and target cells, which requires HN protein attachment to the target cells, was quantitatively measured in a β -galactosidase reporter assay previously described (8, 22, 27). Figure 3A shows that while both untreated or neuraminidase-treated effector cells fused efficiently with untreated target cells, neuraminidase treatment of the target cells eliminated fusion, as did neuraminidase treatment of both target and HN-F protein-expressing cells.

Using these conditions, the HN-F protein complexes were isolated from extracts derived from mixtures of neuraminidase-treated effector cells and either untreated or neuraminidase treated target cells. The HN protein present in complexes is shown in Fig. 3B, and the F protein in the complexes is shown in Fig. 3C. The amounts of HN protein detected with anti-F protein antibody in the absence of attachment (lane 7) clearly decreased when attachment occurred between effector and target cells (lane 3). Similarly, the amounts of F protein detected with anti-HN protein antibodies in the absence of attachment (lane 8) decreased after attachment (lane 4).

Quantification of multiple experiments identical to those shown in Fig. 2 and 3 is presented in Fig. 4. Precipitation using extracts from cells not treated with neuraminidase and without an overlay of target cells (category 1) resulted in approximately 37% of mature HN protein (Fig. 4A) (and 27% of total HN protein; Fig. 4B) precipitated with anti-F protein antibody, while neuraminidase treatment of transfected cells resulted in approximately 80% of mature HN protein (43% of total HN protein) precipitated with anti-F protein antibody (Fig. 4, category 2). Mature HN protein is defined as the HN protein precipitated with the mix of anti-HN protein monoclonal antibodies (24). Total HN protein is that detected without prior immunoprecipitation. Furthermore, to eliminate effects of variations in expression between plates, the amount of HN precipitated with anti-F protein antibody was always compared to the amounts of HN protein in the same extract precipitated with anti-HN protein antibody.

In contrast to results with HN protein, it was possible to quantify F protein in complexes only relative to the total F protein in each extract, because anti-F protein rabbit antibodies used as precipitating antibody obscured the F protein (Fig. 2D, lanes 3 and 7, and Fig. 3C, lanes 3 and 7). Approximately 12% of the total F protein (that detected without immunoprecipitation) was precipitated with anti-HN protein antibody in the absence of neuraminidase treatment, while prior neuraminidase treatment resulted in approximately 28% of the total F protein precipitated with anti-HN protein antibody.

Quantification of results of multiple experiments using extracts prepared after the addition of overlay target cells is also shown in Fig. 4. The percent of mature HN protein precipitated with anti-F protein antibodies under conditions that in-

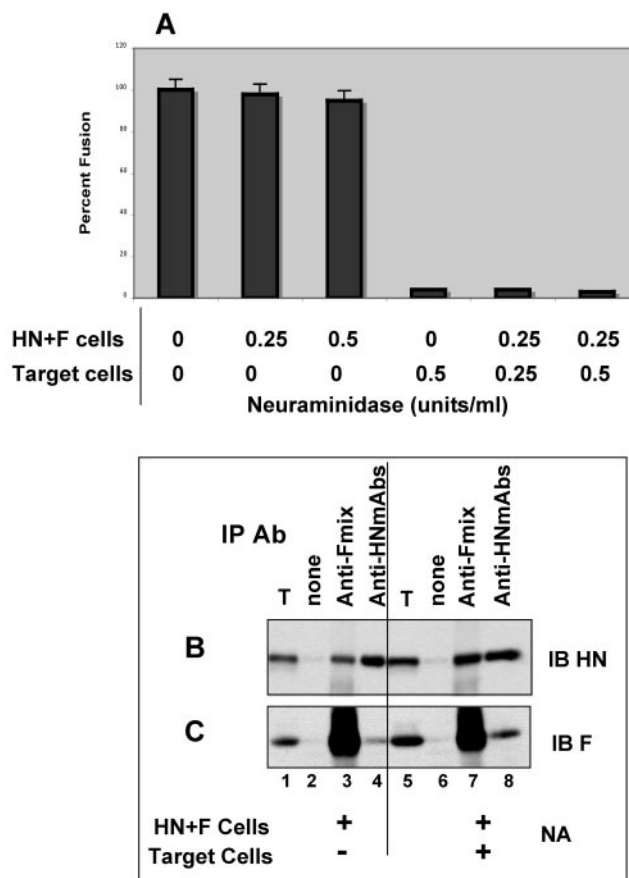


FIG. 3. HN protein receptor binding decreases amounts of HN and F protein complexes. (A) The fusion activities of mixtures of HN and F protein-expressing cells and target cells, either untreated or treated with neuraminidase (indicated at the bottom of the panel), were quantified using a β -galactosidase reporter assay. Cells cotransfected with HN and Fwt protein cDNAs as well as pTA were untreated or treated with the indicated concentrations of neuraminidase overnight. Target cells, transfected with pB1-G only, were untreated or incubated with the indicated concentrations of neuraminidase for 1 h prior to addition to HN and F protein-expressing cells. Fusion between the HN and F protein-expressing cells and target cells, quantified by measuring the β -galactosidase in cell extracts, is shown as the percent of activity detected in mixes of untreated cells. (B and C) Cells cotransfected with HN and F protein cDNAs were incubated overnight in 0.5 mg neuraminidase (+NA). Target cells were either untreated (-NA) (lanes 1 to 4) or treated with neuraminidase (+NA) for 1 h (lanes 5 to 8), washed, and then added on top of the HN and F protein-expressing cells, and the mix was incubated for 1 h at 37°C. Immune complexes were formed with antibodies (Ab) indicated at the top of panel B (IP Ab). IP, immunoprecipitation. The HN protein (electrophoresed in the presence of reducing agent) in the complexes was detected using anti-AS (IB HN), and the F protein (electrophoresed in the absence of reducing agent) was detected using anti-HR2 (IB F). IB, immunoblot. Anti-Fmix, a mixture of anti-HR1, anti-HR2, anti-F2-96, anti-Ftail, and anti-Fu1a. Anti-HNmAbs, a mixture of monoclonal antibodies. T, total cell extract, which represents 50% of the amount used for immunoprecipitation. (C) Bands in lanes 3 and 7 are a combination of comigrating F protein and precipitating rabbit antibody.

hibited attachment (category 5) was approximately 90% (44% of total HN protein). Attachment of untreated target cells to neuraminidase-treated HN and F protein-expressing cells resulted in detection of approximately 35% of mature HN pro-

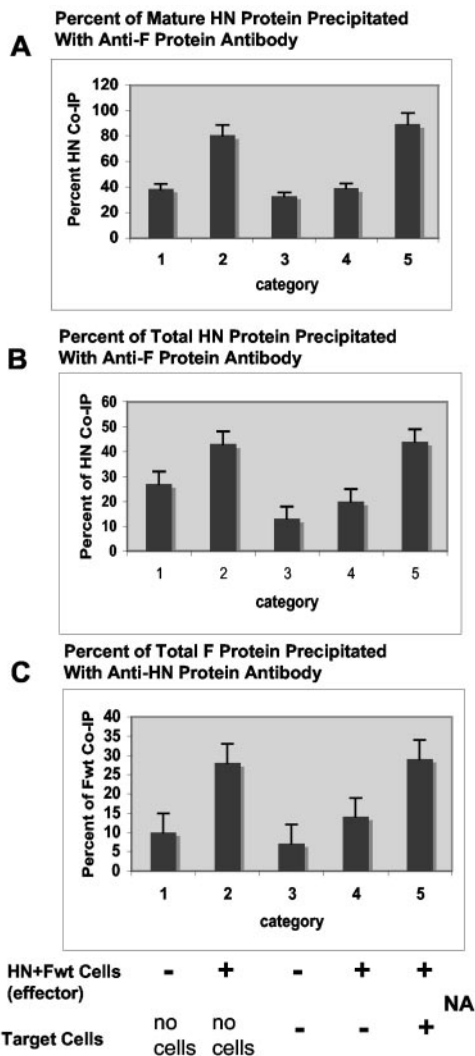


FIG. 4. Quantification of HN and Fwt protein complexes. The amounts of HN and Fwt protein detected in complexes in four separate experiments identical to those shown in Fig. 2 and 3 were averaged, and the averages and standard deviations are shown. The neuraminidase treatments of HN and F protein-expressing cells as well as target cells prior to lysis are shown at the bottom of the figure (+ or -NA). No cells, no target cells added to HN and F protein expressing cells. (A) Quantification of HN protein in the complexes as a percentage of the mature HN protein in each extract. (B) Quantification of HN protein in complexes as a percentage of total HN protein in each extract. (C) Quantification of F protein in the complexes as a percentage of the total F protein in each extract.

tein in the HN-F protein complexes (20% total HN protein) (category 4). A similar pattern of results was obtained for the F protein (Fig. 4C) by quantifying F protein in complexes relative to total F protein.

In addition, mixtures of untreated target and effector cells resulted in very low levels of detection of HN-F protein-containing complexes (Fig. 4A, B, and C, category 3).

F protein cleavage and HN-F protein complexes. To determine if F protein cleavage influenced the detection of HN-F protein complexes, extracts were prepared from cells transfected with HN protein cDNA and F-K115Q cDNA. F-K115Q

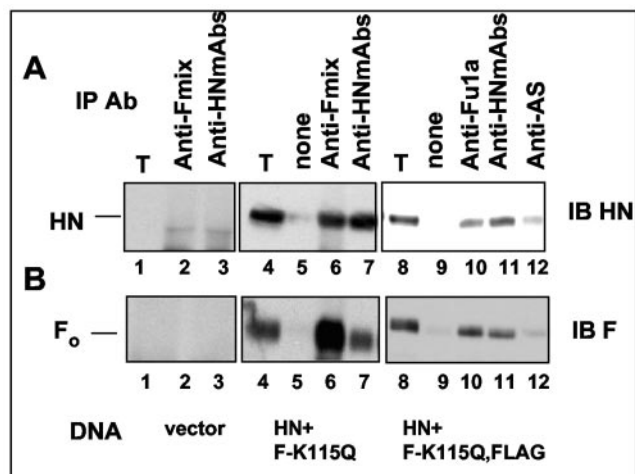


FIG. 5. Characterization of HN and F-K115Q protein complexes. Cells were transfected with vector (lanes 1 to 3), cotransfected with HN protein and F-K115Q protein cDNAs (lanes 4 to 7), or cotransfected with HN protein and F-K115AQ-FLAG protein cDNAs (lanes 8 to 12). Immune complexes were formed using antibodies (Ab) indicated at the top of panel A (IP Ab). IP, immunoprecipitation. (A) HN protein in the complexes were detected using anti-AS (IB-HN). (B) F protein present in the complexes was detected using anti-HR2 (lanes 1 to 7) or anti-FLAG monoclonal antibody (lanes 8 to 12) (IB F). IB, immunoblot. Anti-Fmix, a mixture of anti-HR1, anti-HR2, anti-F2-96, anti-Ftail, and anti-Fu1a. Anti-HNmAbs, a mixture of monoclonal antibodies. T, total extracts, which represents 50% of that used for immunoprecipitation. All proteins were electrophoresed in the presence of reducing agent.

cDNA contains a mutation in the F protein cleavage site sequence and, therefore, results in the expression of an un-cleaved F protein (19). Complexes of HN and F proteins in these extracts were detected as described above for Fig. 1. The anti-HN protein antibodies precipitated F-K115Q protein (Fig. 5B, lane 7) and the anti-F protein antibody mix precipitated HN protein (Fig. 5A, lane 6). The anti-Fu1a monoclonal antibody also precipitated HN protein (Fig. 5A, lane 10). Furthermore, the anti-AS antibody did not precipitate significant amounts of F-K115Q protein (Fig. 5B, lane 12), indicating that most of the detected F-K115Q protein was not in a complex with immature HN protein.

The HN-F-K115Q complexes were characterized from extracts untreated and treated with neuraminidase as described in Fig. 2. Furthermore, complexes were isolated from extracts after incubation of the transfected cells with an overlay of target cells, either untreated or treated with neuraminidase, as described in Fig. 3. The results of a typical experiment are shown in Fig. 6, and the quantification of the results of three identical experiments is shown in Fig. 7.

First, the amount of HN and F-K115Q protein complex detected in extracts derived from untreated cells was significantly higher than the amount of HN and Fwt protein complex (85% versus 37%, respectively, of mature HN protein was precipitated with anti-F protein antibodies). Furthermore, the amount of HN-F-K115Q protein complex was unchanged by neuraminidase treatment of these cells. These results suggest that attachment did not decrease the amounts of these complexes. In support of this conclusion, incubation with target

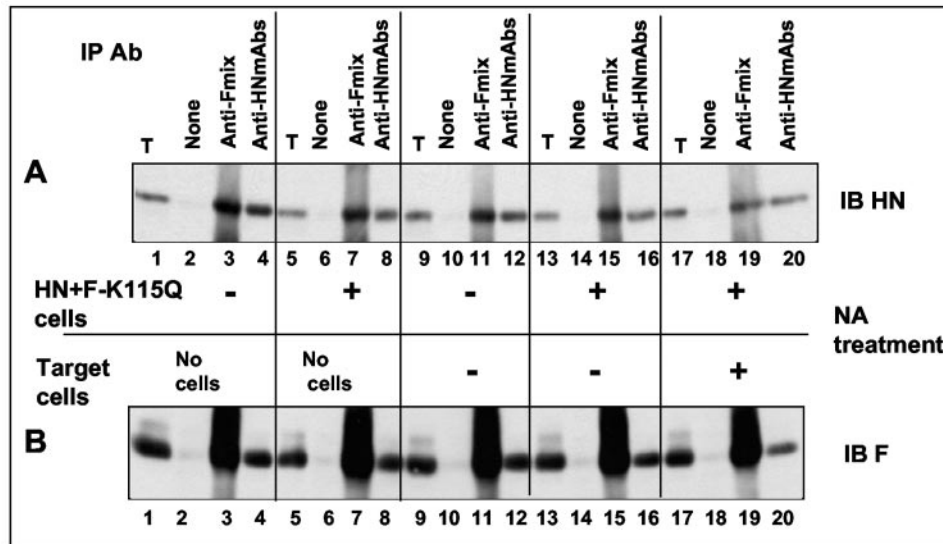


FIG. 6. Levels of attachment do not affect levels of HN and F-K115Q protein complexes. Cells cotransfected with HN and F-K115Q protein cDNAs were untreated (–NA, lanes 1 to 4) or incubated overnight in 0.5 U neuraminidase (+NA, lanes 5 to 8). Immune complexes were formed with antibodies (Ab) shown at the top (IP Ab). IP, immunoprecipitation. Alternatively, HN and F-K115Q cells were either untreated (lanes 9 to 12) or treated with neuraminidase overnight (lanes 13 to 20) and incubated with target cells that were either untreated (lanes 9 to 16) or treated with neuraminidase for 1 h (lanes 17 to 20), washed, and then added on top of the HN and F protein-expressing cells. Incubation of target cells with HN and F protein-expressing cells was for 1 h at 37°C. Immune complexes were formed with antibodies indicated at the top of panel A (IP Ab). The HN protein in the complexes was detected using anti-AS (IB HN), and the F-K115Q protein was detected using anti-HR2 (IB F). Anti-Fmix, a mixture of anti-HR1, anti-HR2, anti-F2-96, anti-Ftail, and anti-Fu1a. Anti-HNmAbs, a mixture of monoclonal antibodies. T, total cell extract, which represents 50% of the amount used for immunoprecipitation. All proteins were electrophoresed in the presence of reducing agent.

cells, either neuraminidase treated or untreated, did not significantly change the amounts of the complexes detected. This result cannot be due to a decrease in attachment activity of HN protein in the presence of the uncleaved F protein. Attachment, as measured by binding of red blood cells, was identical in cells coexpressing HN and Fwt protein or F-K115Q protein (data not shown). Thus, these results show that an uncleaved F protein also complexed with HN protein. They further suggest that disassociation of HN and F protein complexes required not only HN protein attachment but also F protein cleavage.

DISCUSSION

While paramyxovirus F proteins are directly responsible for membrane fusion, the viral attachment proteins are required for fusion mediated by most F proteins. HN protein receptor binding and the interactions between HN and F proteins are thought to be central to activation of the fusion protein activity; however, the molecular mechanism involved is one of the major unsolved problems in paramyxovirus entry. Initially it was proposed that attachment of HN protein to sialic acid receptors stimulates an interaction of HN protein with F protein, which leads to F protein activation, a model still favored by some (3, 14). In contrast, Takimoto et al. (42), Zaitsev et al. (50), and McGinnes et al. (23) have proposed that HN and F proteins form a complex prior to attachment and that HN protein receptor binding stimulates a conformational change in the HN protein, which activates the F protein. Takimoto et al. and Zaitsev et al. based their model on their analyses of the structure of the NDV HN protein and the phenotype of NDV HN mutants. These groups crystallized two forms of

the HN protein, one without bound ligand and one with neu5Ac2en, an inhibitor that can be generated by the catalysis of sialic acid from sialic acid-containing receptors (5, 50). They have proposed that HN protein without bound ligand is in the preattachment state, while HN protein with bound neu5Ac2en represents the postattachment, catalytic form of the protein. Comparisons of the two forms of HN protein showed that there were significant differences in the positions of the residues in the catalytic site as well as loops that form the HN protein dimer interface. Mutation of residues at the dimer interface resulted in an HN protein that could bind to sialic acid but that could not promote fusion (4, 42). These findings led these groups to propose that conformational changes in the HN protein dimer interface upon sialic acid binding transmit a signal to an associated F protein which activates its activity.

McGinnes et al. (23) also published evidence indicating that NDV HN and F proteins form complexes prior to HN protein receptor binding. A polyclonal antibody specific for the HR1 domain of the F protein bound to F protein on cell surfaces only when F protein was coexpressed with HN protein. Furthermore, the anti-HR1 antibody blocked the fusion of red blood cells to the HN-F protein-expressing cells when bound before HN protein engaged its receptors, but not after receptor binding. These combined results suggested that the HR1 domain was accessible to anti-HR1 antibody binding only in the presence of HN protein and only before engagement of HN protein with receptors. These conclusions are consistent with the idea that HN protein binds to the F protein prior to HN protein receptor binding.

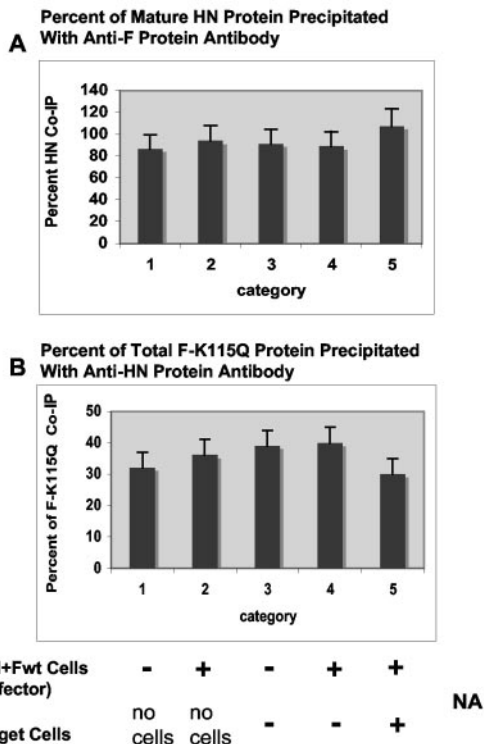


FIG. 7. Quantification of HN and F-K115Q protein complexes. The amounts of HN and F-K115Q proteins detected in complexes in three separate experiments identical to those shown in Fig. 6 were averaged, and the averages and standard deviations are shown. The treatments of HN and F protein-expressing cells, as well as target cells, prior to lysis are shown at the bottom of the figure (+ or -NA). No cells, no target cells added. (A) Quantification of HN protein in the complexes as a percentage of the total mature HN protein in each extract. (B) Quantification of F-K115Q protein in the complexes as a percentage of the total F-K115Q protein in each extract.

Results presented here provide more direct evidence in support of this proposal. If HN and F proteins form complexes only after attachment, then inhibition of attachment by neuraminidase digestion of cell surfaces should result in a significant decrease in these complexes. However, we have found that removal of surface sialic acid significantly increased amounts of HN and F protein-containing complexes. These results strongly support the idea that complexes form in the absence of HN protein attachment.

Finding increased amounts of HN and F protein complexes after neuraminidase digestion of cell surfaces suggested that HN protein receptor binding may cause disassociation of these complexes. In support of this idea, we found that stimulation of attachment and fusion by the addition of target cells to neuraminidase-treated HN and F protein-expressing cells resulted in a significant decrease in detection of HN and F protein complexes. This result is consistent with the idea that attachment is associated with release of the F protein from the complex.

Yin et al. (47) have recently described the crystal structure of the uncleaved, soluble form of the parainfluenza 3 F protein. While the uncleaved molecule should be in a prefusion conformation, the HR1 and HR2 domains were complexed in this structure, a conformation thought to be characteristic of the

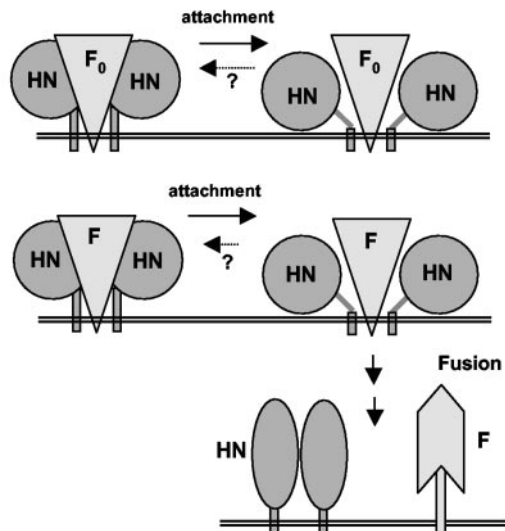


FIG. 8. Potential model for HN and F protein associations. (A) F-K115Q protein associates with both pre- and postattachment forms of the HN protein. Perhaps the two forms are in equilibrium due to HN protein neuraminidase. (B) Fwt protein associates with both forms of the HN protein but can proceed with fusion and disassociation from HN protein.

postfusion conformation. While Yin et al. proposed that the missing transmembrane and cytoplasmic domains of the soluble F protein may be important for maintaining the prefusion conformation, it is also possible the HN protein participates in the maintenance of the prefusion conformation of the F protein and that most paramyxovirus F proteins are expressed in a prefusion conformation only in the presence of the HN protein. Our previous findings and those presented here are consistent with this idea. That the HR1 domain is accessible to antibody binding only when expressed with HN protein is consistent with the idea that the HR1-HR2 complex forms in the absence of HN protein masking the HR1 domain from antibody binding (23). We have previously reported that the F protein HR2 domain can interact with a sequence in the HN protein membrane-proximal region (8). Such an interaction provides support for the idea that HN protein may prevent HR1-HR2 complex formation by binding to the HR2 domain. Furthermore, our results reported here, which indicate that HN and F proteins form a complex prior to receptor binding, are consistent with the idea that HN protein may facilitate the prefusion conformation of the F protein. In addition, our results that show disassociation of these complexes upon HN protein attachment and fusion are consistent with the idea that this disassociation allows the formation of the HR1-HR2 complex and, therefore, fusion.

Attachment of HN protein to receptors is not, however, sufficient for disassociation of HN and F protein-containing complexes. Amounts of complexes detected between HN and the uncleaved F-K115Q protein were unaffected by neuraminidase treatment of cell monolayers, and the complexes did not disassociate upon addition of target cells. It is possible that the interactions of the uncleaved and cleaved F proteins with the HN protein are different. Alternatively, additional conforma-

tional changes in cleaved F protein or the actual fusion process itself may result in dissociation of the two proteins.

Results presented here with the F-K115Q protein, coupled with structural studies of the NDV HN protein (5, 50), suggest that the uncleaved F protein may associate with both the preattachment form and the postattachment form of HN protein (Fig. 8A). The finding, described above for Fig. 1, that the anti-F protein monoclonal antibody did not precipitate all the HN and F protein complexes suggests that HN and Fwt protein complexes may also exist in at least two forms (Fig. 8B). Some additional conformational change possible only in the cleaved F protein may be required for complex disassociation, as illustrated in Fig. 8B. Alternatively, the intermediate complex (shown in Fig. 8B) containing a cleaved F protein may, with a certain frequency, proceed to full fusion, which results in complex disassociation.

Results reported here using transfected cells are consistent with our previous results using infected cells (41). We have previously shown that HN and F proteins on surfaces of infected cells could be cross-linked and that this cross-linking could occur after elimination of cell surface sialic acid with neuraminidase. Furthermore, we saw an increase in cross-linked complexes after neuraminidase treatment of infected cells consistent with the increase, reported here, of HN and F protein-containing complexes after neuraminidase digestion. In our previous studies, we also added overlay cells to infected cells, but we did not detect a decrease in levels of the HN and F protein cross-linked complexes. In these experiments, however, the overlay cells were incubated with infected cells at 4°C. Thus, it is likely that disassociation of the complexes did not occur, because fusion did not proceed at the lower temperature. This result is consistent with our observations, reported here, that attachment alone, which can occur at 4°C, is not sufficient for disassociation of the HN and F protein-containing complexes.

Plempner et al. (35) have reported that complexes between measles virus hemagglutinin and F proteins vary inversely with the levels of fusion. This finding is very consistent with the results presented here. Increased fusion should result in more disassociation of HN and F protein complexes, while less fusion would favor increased detection of complexes. Perhaps, as suggested by Plempner et al., a tighter interaction between the two proteins would decrease disassociation and, therefore, fusion. It is also possible that any change that results in an F protein with lowered fusion activity would, secondarily, decrease the disassociation of the complex.

Results reported here are also consistent with the report that an attachment-defective HN protein can still complex with the NDV F protein (18). On the other hand, Li et al. (18) and Deng et al. (6) reported that other attachment-negative HN protein mutants did not coimmunoprecipitate with F protein. However, reciprocal coimmunoprecipitation with the mutant protein was not demonstrated in these reports, and only one anti-F protein monoclonal antibody was used to precipitate HN protein. It is possible that some mutant HN proteins block the binding site for this F protein antibody. Indeed, as noted above, we have found that a single anti-F protein monoclonal antibody did not precipitate all the HN protein in a complex with F protein. If there are two forms of an HN-F protein complex, as we have proposed in Fig. 8, then it is possible that

one form cannot be detected by a single monoclonal antibody. Alternatively, these mutant HN proteins may have conformational abnormalities that result in defects in association with F protein.

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