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MECHANISMS OF NEWCASTLE DISEASE VIRUS-MEDIATED MEMBRANE

FUSION

A Dissertation Presented

By

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Submitted to the Faculty of the

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Molecular Genetics and Microbiology

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                                        Molecular Genetics and Microbiology

                                        November, 1999

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This work is dedicated to my

husband, Rodney.

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I would like to thank my mentor, Trudy Morrison, for her patience in teaching me how to be a good scientist and her good humor while reading many drafts of manuscripts. I would like to thank the members of my research advisory committee, Tony, Duane, Reid, and Maria, for all their efforts on my behalf during the last several years. Next, I would like to thank members of the Morrison lab, past and present. I would like to thank Lori for teaching me many techniques and helping me troubleshoot, as well as for being my running partner, lunch partner and partner in crime. I would like to thank Tia for all the excitement she brought to lab—never a dull moment. Thanks also to Kathy and Suzanne who make me laugh and bring light into the lab. I would also like to thank Petra, who was always there with an ear and a diversion. Finally, I would like to thank my family, Mom, Sy, Andrea, Jason, and especially Rodney, for all their love and support these many years.

ABSTRACT

For many paramyxoviruses, including Newcastle disease virus (NDV), syncytia 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 (23, 73). Because a potential interaction in paramyxovirus infected cells has never been clearly demonstrated, such an interaction was explored in Chapter 2 using coimmunoprecipitation and crosslinking. Both HN and F proteins could be precipitated with heterologous antisera after a five minute radioactive pulse as well as after a two hour chase in non-radioactive media, but at low levels. Chemical crosslinking increased detection of complexes containing HN and F proteins at the cell surface. After crosslinking, intermediate as well as high molecular weight species containing both proteins were precipitated with monospecific antisera. Precipitation of proteins with anti-HN after crosslinking resulted in the detection of complexes which electrophoresed in the stacker region of the gel, from 160-300 kD, at 150 kD and at 74 kD. Precipitates obtained with anti-F after crosslinking contained species which migrated in the stacker region of the gel, between 160-300 kD, at 120 kD and at 66 kD. The 3-4 discrete complexes ranging in size from 160-300 kD contained both HN and F proteins when precipitated with either HN or F antisera. That crosslinking 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 crosslinking at early time points post infection, when levels of viral surface glycoproteins are low. Use of cells infected with an avirulent strain of NDV showed that chemically crosslinked 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 RER. Upon attachment of the HN protein to its receptor, the HN protein undergoes a conformational change which causes a subsequent change in the associated F protein, releasing the hydrophobic fusion peptide into the target membrane and initiating fusion.

Chapter 3 explores the stalk region of the NDV HN protein, which has been implicated in both fusion promotion and virus specificity of that activity. The NDV F protein contains two heptad repeat motifs which have been shown by site-directed mutagenesis to be critical for fusion (7, 51, 57). Heptad repeat motifs mediate protein-protein interactions by enabling the formation of coiled-coils. Upon analysis of the stalk region of the NDV HN protein, we identified two heptad repeats. Secondary structure analysis of these repeats suggested the potential for these regions to form alpha-helices. To investigate the

importance of this sequence motif for fusion promotion, we mutated the hydrophobic "a" position amino acids of each heptad repeat to alanine or methionine. In addition, hydrophobic amino acids in other positions were also changed to alanine. Every mutant protein retained levels of attachment activity that was greater than or equal to the wild-type protein and bound to conformation-specific monoclonal as well as polyclonal antisera. Neuraminidase activity was variably affected. Every mutation, however, showed a dramatic decrease in fusion promotion activity. The phenotypes of these mutant proteins indicate that individual amino acids within the heptad repeat region of the stalk domain of the HN protein are important for the fusion promotion activity of the protein. These data are consistent with the idea that the HN protein associates with the F protein via specific interactions between the heptad repeat regions of both proteins.

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CHAPTER 1

Introduction

The process by which enveloped viruses are able to mediate membrane

fusion has long been a question in virology. The purpose of this thesis is to

explore the mechanisms governing this event using the paramyxovirus

Newcastle disease virus (NDV) as a model system. The viral hemagglutinin-

neuraminidase (HN) protein and the fusion (F) protein were investigated in

order to elucidate their roles in the fusion event.

<u>Molecular biology.</u> Paramyxoviruses are enveloped, negative stranded RNA

viruses that are spherical or pleiomorphic in shape (31). The paramyxovirus

family consists of 4 genera: paramyxovirus (Sendai virus [SeV], human

parainfluenza viruses [hPIV] 1 and 3), rubulavirus (simian virus 5 [SV5], mumps

virus [MuV], NDV, and hPIV 2), morbillivirus (measles [MV]), and pneumovirus

(respiratory syncytial virus). Most rubulavirus genomes encode 7 proteins: the

nucleocapsid (NP) protein, the phosphoprotein (P), the large (L) protein, the

matrix (M) protein, the small hydrophobic protein (SH), the HN protein, and the F

protein. The NP protein encapsidates the RNA genome, associates with the

viral polymerase during replication, and may interact with the M protein during

assembly. The L protein, along with the P protein, is thought to form the viral

polymerase. The P protein also forms a complex with the NP protein, which is

likely active in genomic encapsidation. The M protein lies on the inner leaflet of the virion phospholipid bilayer. This protein can self-associate and may be the driving force in the budding of progeny virions from the plasma membrane (50). The SH protein is an integral membrane protein of unknown function. This protein is present in MV and MuV but has not been identified in NDV. The virion lipid bilayer contains two glycoprotein spikes—the F protein and the HN protein. The F protein serves to mediate viral fusion, while the HN protein has three activities: neuraminidase (NA) activity, attachment (HA) activity, and an undefined role in fusion termed fusion promotion.

Glycoprotein structure. The F protein is a type I integral membrane protein with a carboxy terminal cytoplasmic tail, a transmembrane domain, and an ectodomain (Fig.1A) (reviewed in (31)). It is synthesized as an inactive precursor termed F_0 which is cleaved in the *trans* Golgi in virulent virus strains such as NDV AV to form an active fusogenic protein containing disulfide-linked F_1 and F_2 subunits. Cleavage results in the exposure of a hydrophobic peptide at the new amino terminus termed the fusion peptide. Immediately carboxy terminal to the fusion peptide is a heptad repeat motif termed heptad repeat 1 (HR1). A second heptad repeat region (HR2) is located just amino terminal to the putative transmembrane domain. The oligomeric structure of the F protein has been shown to be a homotrimer.

The HN protein is a type II integral membrane glycoprotein with an amino terminal cytoplasmic tail, a transmembrane domain and a large

ectodomain (Fig.1B) (reviewed in (31)). It contains a signal sequence for translocation into the ER which also functions as a transmembrane domain. The HN protein of NDV strain AV is a homooligomer made up of two disulfide linked monomers which interact noncovalently to form a tetramer. The HN protein of the avirulent B1 strain does not contain disulfide linked monomers, although a tetramer is formed. The three dimensional structures of the HN and F proteins have not been solved, but the tertiary structure of the HN protein is thought to resemble the influenza NA protein (15, 34).

Membrane fusion. Enveloped viruses enter cells via two different mechanisms—acid pH-dependent and acid pH-independent fusion. During acid pH-dependent fusion, the virus is endocytosed and membrane fusion occurs between the virion membrane and the endosomal membrane upon exposure of the virion to acid pH (Fig. 2, Part 1). Viruses such as influenza viruses, flaviviruses, rhabdoviruses, and alphaviruses enter the cell in an acid-dependent manner. Viruses such as retroviruses and paramyxoviruses enter cells via an acid pH-independent pathway whereby the virion fuses with the lipid bilayer of the target cell at the plasma membrane (Fig. 2, Part 2). Viruses that infect cells in a pH-independent manner may also infect cells by fusion between the infected cell and a neighboring uninfected cell (Fig. 2, Part 3). This fusion event results in the formation of multinucleated cells or syncytium, and is a major cytopathic effect mediated by paramyxoviruses (30).

Viral fusion is thought to occur in several steps (reviewed in (41)). The first step is binding of the attack cell membrane to the target cell membrane via a protein scaffold (Fig. 3A). This step is mediated by the binding of the viral attachment protein to its receptor. This is followed by a close approach or dimpling of the target and attack membranes which is thought to be mediated by the viral fusion protein (Fig. 3B). The third step, hemifusion, occurs when the outer leaflets of the phospholipid bilayers from the target and attack membranes are mixed (Fig. 3C). Hemifusion is thought to take place spontaneously due to local tensions and attractive forces between the membranes. The next step is the formation of an early fusion pore (Fig. 3D) which can rapidly open and close (flicker) or expand into a late fusion pore which can no longer flicker and close (Fig. 3E). This is followed by the last step in the process, complete fusion (Fig. 3F). This final step involves a complete expansion of the fusion pore and results in the release of the attack cell or virion contents into the target cell.

Influenza virus-mediated membrane fusion. Influenza virus is an enveloped virus that infects cells by an acid pH-dependent membrane fusion event. Influenza-mediated membrane fusion is directed by its surface glycoprotein, hemagglutinin (HA) (59, 60, 69). The HA protein, like the F protein, is a homotrimer which consists of two subunits, HA1 and HA2, and is synthesized as a fusion-inactive precursor termed HA0. In the first step of fusion, the HA1 subunit binds to its receptor at the plasma membrane at a neutral pH

(Influenza-mediated fusion reviewed in reference (32)). The bound virion is then endocytosed, and, upon exposure to acid pH, conformational changes are triggered in both the HA1 and HA2 subunits of the protein. The HA2 subunit rearranges into a long, triple-stranded coiled-coil which repositions the hydrophobic fusion peptide toward the membrane of the target cell. The insertion of the fusion peptide into the target cell enables the second step of fusion, close approach, to occur. It has been proposed that after HA2 binding to the attack membrane, a simultaneous bending of several trimers may pull the attack and target membranes together, resulting in close approach (69). A lag phase ensues at this point which is dependent upon time, temperature, and the density of HA proteins in the attack membrane. It is thought that this lag is caused by the need to recruit a minimum number of HA proteins to the fusion complex. After the lag phase, the next step of fusion, hemifusion, ensues. After hemifusion, the formation of the early fusion pore occurs. This pore may rapidly open and close and has been observed by the detection of flickering of pore conductances. The pore may then widen into a narrow pore followed by an expansion into a dilated pore (late fusion pore). Studies with recombinant HA proteins containing a glycophosphatidylinositol anchor instead of a cytoplasmic tail have shown that only mixing of the outer membranes of the bilayers occurred and no mixing of the inner membranes or aqueous contents was detected, implying that the cytoplasmic tail is involved in formation of the fusion pore (28). It has been hypothesized that the expansion into a dilated

pore may be facilitated by bending of the HA trimers in order to relax the extreme curvature of the membranes. The role of influenza proteins in complete fusion, the final step of the process, is unknown.

HIV-1-mediated membrane fusion. HIV-1 is a virus that infects cells in a pH independent manner at the plasma membrane. The HIV surface glycoprotein contains two components, the membrane bound gp41, which is responsible for membrane fusion, and a non-covalently associated gp120, which serves as the attachment protein (11, 29). Like influenza HA and paramyxovirus F proteins, the HIV-1 glycoprotein is synthesized as an inactive precursor, gp160, which must be proteolytically processed to be fusogenic. gp120 initiates fusion by binding to CD4. This causes a conformational change in the gp 120 subunit which exposes or creates a binding site for specific chemokine receptors. Binding to CD4 also causes conformational changes in gp41 which promotes the formation of a coiled-coil. The formation of this presumably fusion active coiled-coil conformation is thought to occur via an interaction between two alpha-helical heptad repeat regions, resulting in the formation of a six helical bundle (11). The fusion peptide, located at one end of this coiled-coil structure, is then able to insert into the target membrane, leading to the close approach of the virion and target membranes. The role of gp120 and gp41 in other stages of membrane fusion is unclear.

Paramyxovirus-mediated membrane fusion. NDV-mediated membrane fusion is a leading example of a viral two component system whereby two separate

proteins are thought to cooperate in fusion promotion. The paramyxovirus HN protein has been shown to bind a sialic acid containing receptor (31). After binding, one model postulates that the HN protein triggers a conformational change in the F protein causing its hydrophobic fusion peptide to be released into the target cell and promoting close approach (30). Tight regulation of the release of the fusion peptide is thought to be a critical step in fusion because premature release has been hypothesized to result in the aggregation and therefore inactivation of the fusion protein.

After release of the fusion peptide into the target membrane, the next step in fusion is hemifusion. Although the mechanism of hemifusion in paramyxovirus-mediated fusion is unclear, it appears that the heptad repeat regions of the F protein may play a role. Peptide inhibition studies from SV5 show that synthetic peptides with sequences from the HR2 region of the F protein block this lipid mixing step. Because HR2 peptides have been found to self associate (unpublished observations), these results imply a role for HR2 in hemifusion (26). The HR1 region may also be involved in hemifusion as recent reports using both NDV and SV5 systems have shown that peptides corresponding to HR1 and HR2 functionally interact (3, 26, 75).

The involvement of paramyxovirus glycoproteins in the formation of early and late fusion pores is not well understood. While the exact mechanism of complete fusion is unclear, SV5 F proteins containing truncated cytoplasmic tails were reported to be deficient in cytoplasmic content mixing, suggesting

that this region of the F protein is necessary for the latter steps of fusion to occur (2). Complete fusion may also be quantitated in paramyxoviruses by observing syncytia formation. Mutations in the cytoplasmic tail region of the NDV F protein were found to negatively affect syncytia formation, supporting the SV5 results (57). Whether other proteins are involved in paramyxovirus-mediated membrane fusion is unknown (30).

Regions of the F protein that are critical for fusion. Mutational analyses of the NDV F protein have shown that many regions of the protein are critical for fusion. Mutations in the amino terminus of the F_1 subunit affected cleavage of the F_0 protein and thus inhibited fusion (43). Mutational analyses of the highly conserved heptad repeat regions HR1 and HR2 were found to abolish fusion as well (7, 52, 58). The cytoplasmic tail region was also determined by mutagenesis to be critical for fusion (2, 57). Mutagenic studies of the fusion peptide have shown that this region is important for fusion as proteins containing mutations in this region fuse better than the wild-type protein (22), while other mutations block fusion (58).

Evidence for HN involvement in membrane fusion. Early research with SeV gave the first clues to the mechanism of paramyxovirus-mediated membrane fusion. At this time, it was known that the paramyxovirus F protein promoted viral fusion and that the HN protein served to bind the virion to its receptor. It was suspected, however, that the HN protein might play a role in membrane fusion along with the F protein. Circular dichroism studies of reconstituted

membrane vesicles containing HN and F proteins showed that the spectra of vesicles containing the HN protein alone or the F protein alone differed from the spectra obtained when the HN and F proteins were present in the same vesicles (13). These data suggested that a conformational change occurred in the HN and/or F proteins when they were present together in the same vesicle, implying a possible interaction between the proteins.

For NDV, it was discovered that the HN and F proteins were necessary and sufficient for syncytia formation to occur (42). This was found to be the case with other paramyxoviruses as well (with the exception of SV5 and perhaps RSV) (20, 27, 30). It was determined that another viral attachment protein could not complement the NDV F protein in order to permit fusion (42). Furthermore, Hu et al. (24) found that the HN and F proteins from the paramyxoviruses hPIV2 and hPIV3 must be from the same virus to cause syncytium formation. Such evidence for a virus-type specificity implied that direct interactions may occur between the HN and F proteins from the same virus.

Recent work from several laboratories has also supported the notion that the virus specificity of the HN protein is mediated by the stalk region. Deng et al. constructed chimeric HN proteins where regions of hPIV3 and NDV were interchanged (17). They found that the transmembrane domain as well as a portion of the presumed stalk region were necessary determinants in F specificity for fusion. Another study of chimeras between PIV2 and SV41

reported that both the presumed stalk domain as well as the globular head region of the HN protein conferred F specificity (67). A third study with SeV-hPIV3 chimeric proteins also determined that the presumed stalk region was necessary for F protein specificity leading to fusion (62) .

The initial interpretation of virus specificity has been questioned by several reports. Experiments performed using cotransfected cells expressing the hPIV3 F protein and either SV5 HN, SeV HN, or MV H proteins showed a downregulation of attachment protein expression (63). Downregulation was not observed when the homologous hPIV3 proteins were expressed, however. Further, when the attachment proteins were coexpressed with an hPIV3 F protein containing an ER retention signal, there was a downregulation of surface expression for the heterologous attachment proteins that was even more pronounced than was observed in the presence of the wild-type hPIV3 F protein, as well as a downregulation of surface expression of the homologous HN protein. These results suggested that heterotypic HN/H and F proteins may interact in the ER and that this interaction leads to the downregulation of the heterotypic attachment protein.

Another report also suggested that an early interaction between heterologous HN and F proteins leads to a downregulation of the HN protein. Bousse et al. noted that when hPIV1 F was overexpressed in transfected cells expressing SeV or hPIV1 HN proteins, there was a downregulation of both the homologous and heterologous HN proteins at a time point consistent with an

effect on translation or an early stage of protein folding (5). The SeV F protein did not downregulate the hPIV1 HN protein, suggesting that downregulation is not mediated by all paramyxovirus F proteins in the presence of a heterologous HN protein. This report differs from the report by Tanaka et al. because of the observation of a homologous downregulation.

A third group investigated heterologous downregulation by coexpressing an hPIV3 F protein containing an ER retention signal with an hPIV2 HN protein in transfected cells (65). This group observed a downregulation of the heterologous HN protein which was consistent with an early block in protein synthesis or protein misfolding.

Shortly after these results were published other reports came to very different conclusions. Work from Yao et al. demonstrated that hPIV2 HN could coimmunoprecipitate hPIV2 F protein at the plasma membrane, but not SeV F or SV5 F proteins (73). Furthermore, hPIV3 HN protein could coimmunoprecipitate hPIV3 F protein but not hPIV2 protein although all three proteins were expressed at the plasma membrane. Antibody cocapping experiments supported the coimmunoprecipitation results. The authors concluded that there was evidence only for a homologous interaction between the HN and F proteins at the cell surface. Another group, Paterson et al., found that SV5 and HPIV3 F and HN proteins with an ER retention signal could not retain the respective homotypic HN or F protein in the ER (49). The authors concluded that there was no intracellular interaction between the HN and F

proteins and speculated that the association may occur instead at the plasma membrane.

Why there are so many conflicting reports about potential HN/H-F protein-protein interactions is not clear. The reports from Deng et al. (17), Tsurudome et al. (67), Tanabayashi et al. (62), Yao et al. (74), and Paterson et al. (49) did not communicate a downregulation of the attachment protein caused by a heterologous F protein. Whether this phenomena was not observed or simply was not reported is unknown. Bousse et al. (5) reported that no downregulation was observed for the hPIV1 HN protein when it was coexpressed with the SeV F protein. This is interesting because it suggests that the downregulation phenomena may not be a mechanism utilized by all paramyxoviruses to prevent heterologous fusion.

The first research which provided evidence for a potential HN/HA-F protein-protein interaction was conducted with MV (38). MV proteins expressed at the plasma membranes of transfected cells under the control of a vaccinia virus vector were chemically crosslinked. It was found that MV HA and F_1 proteins could be crosslinked, but that the F_0 protein was not a component of this crosslinked complex. Similar experiments using SV5 infected cells were reported with no detection of an HN-F protein interaction (53). The first account of an HN-F protein interaction in the absence of chemical crosslinkers was reported by Yao et al. (74) who utilized a vaccinia driven transfection system to coimmunoprecipitate PIV2 HN and F proteins at the plasma membrane. We

addressed the question of an HN-F protein interaction using NDV-infected cells. This work is presented in Chapter 2. Recently, Deng et al. also used a vaccinia driven transfection system to study a potential NDV HN-F protein interaction (16).

Motifs in fusion proteins. Various proteins from diverse systems are involved in membrane fusion such as vesicle trafficking, neurotransmitter release, egg fertilization, and viral fusion (41). Fusion proteins involved in membrane trafficking and viral fusion have been well studied and, while they differ greatly, the proteins involved share two common features—they contain a hydrophobic fusion peptide, and this peptide is located at the end of a long bundle of alpha helices (60). As mentioned above, HIV-1 and influenza undergo structural rearrangements upon triggering of fusion which convert non-helical regions of the proteins into alpha helical coiled-coils. In the well characterized vesicle trafficking system, three cellular proteins make up a minimum fusion complex, N-ethylmaleimide sensitive factor (NSF), soluble NSF attachment protein (SNAP), and the SNAP receptor (SNARE) (60). Upon forming a core complex, regions of the SNAP and SNARE proteins convert from being relatively unstructured to containing an alpha helical structure that is very stable. The conservation of the coiled-coil motif in fusion proteins from systems as diverse as viral fusion and membrane trafficking argues that coiled-coils are critical for fusion.

The formation of coiled-coils is mediated by regions of proteins containing heptad repeats (10, 37). A heptad repeat is a motif in which a hydrophobic amino acid is repeated every seven residues designated a through g (37). Heptad repeat regions containing hydrophobic or neutral residues in the a- and d-positions may impart an alpha helical structure in that portion of the protein and thus enable protein-protein interactions via the formation of coiled-coils (37). In recent years, mutagenesis as well as peptide inhibition studies of many viral fusion proteins have shown that heptad repeats are critical for fusion (7, 31, 51, 70, 71, 73, 75), and indeed, are important motifs in many diverse proteins (37). Interestingly, the HN protein of NDV contains several heptad repeat regions. One such repeat spans the presumed transmembrane region of the protein and was determined by mutagenesis to be critical in fusion promotion as well as oligomerization of the protein (40). Chapter 3 of this thesis investigates the importance of another heptad repeat region in the presumed stalk domain of the HN protein for fusion promotion.

A

F_2 F_1

ss

signal
sequence   CS   HR1   HR2   TM   tail

FP

F_0

B

tail

TM   HRB

HRA
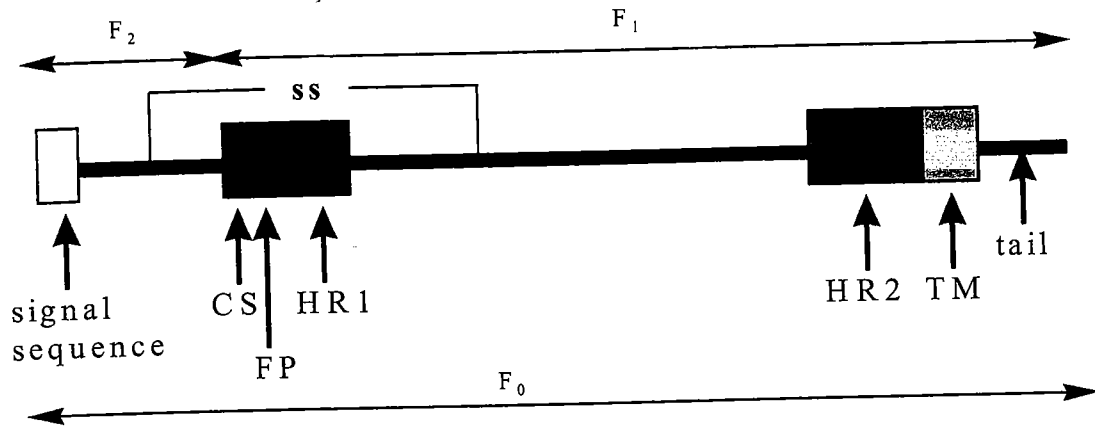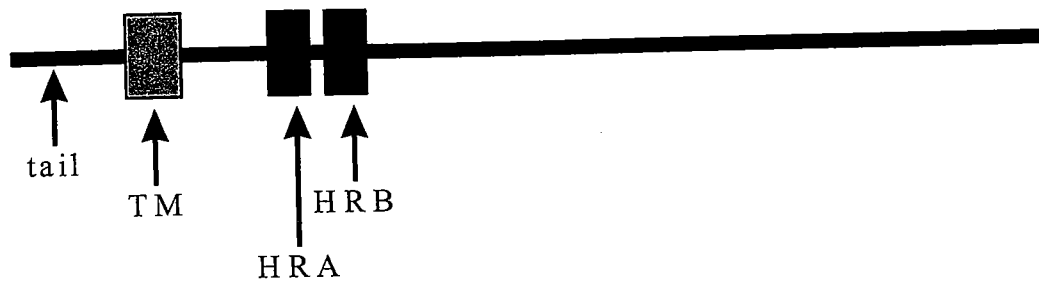
Legend to Figure 1. Schematic representation of the primary amino acid
sequence of the F and HN proteins.
A. The NDV F protein. B. The NDV HN protein. The amino termini are to the
left and the carboxy termini are to the right. SS, disulfide bond; HR, heptad
repeat domain; TM, transmembrane domain; FP, fusion peptide; cs, cleavage
site; tail, cytoplasmic tail domain.

attack cell   target cell
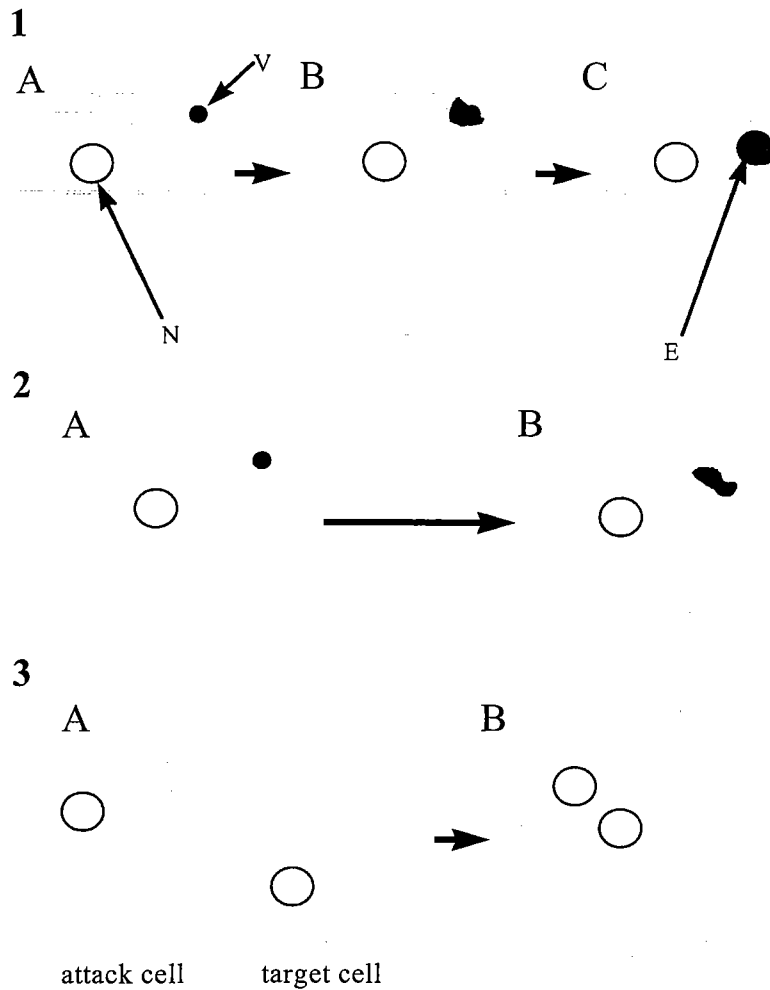
Legend to Figure 2. Acid-dependent and independent membrane fusion. 1. (A) The first step of acid-dependent fusion is binding of the virus to its receptor on the host cell. (B) The next step is endocytosis. (C) The final step is membrane fusion which occurs in an endosomal compartment upon exposure to acid pH and results in the release of genetic material from the virus into the cytoplasm of the host cell. 2. (A) The first step of acid-independent fusion is binding of the virus to its receptor on the host cell. (B) The next step is membrane fusion between the virus and the host cell at the plasma membrane resulting in the release of viral genetic material into the cytoplasm. 3. A cell infected with a virus that is capable of pH-independent membrane fusion may fuse with uninfected neighboring cells. (A) Attachment of an infected (attack) cell to an uninfected (target) cell. (B) Fusion of attack and target cells to form a syncytium. N, nucleus; V, virion; E, endosome.
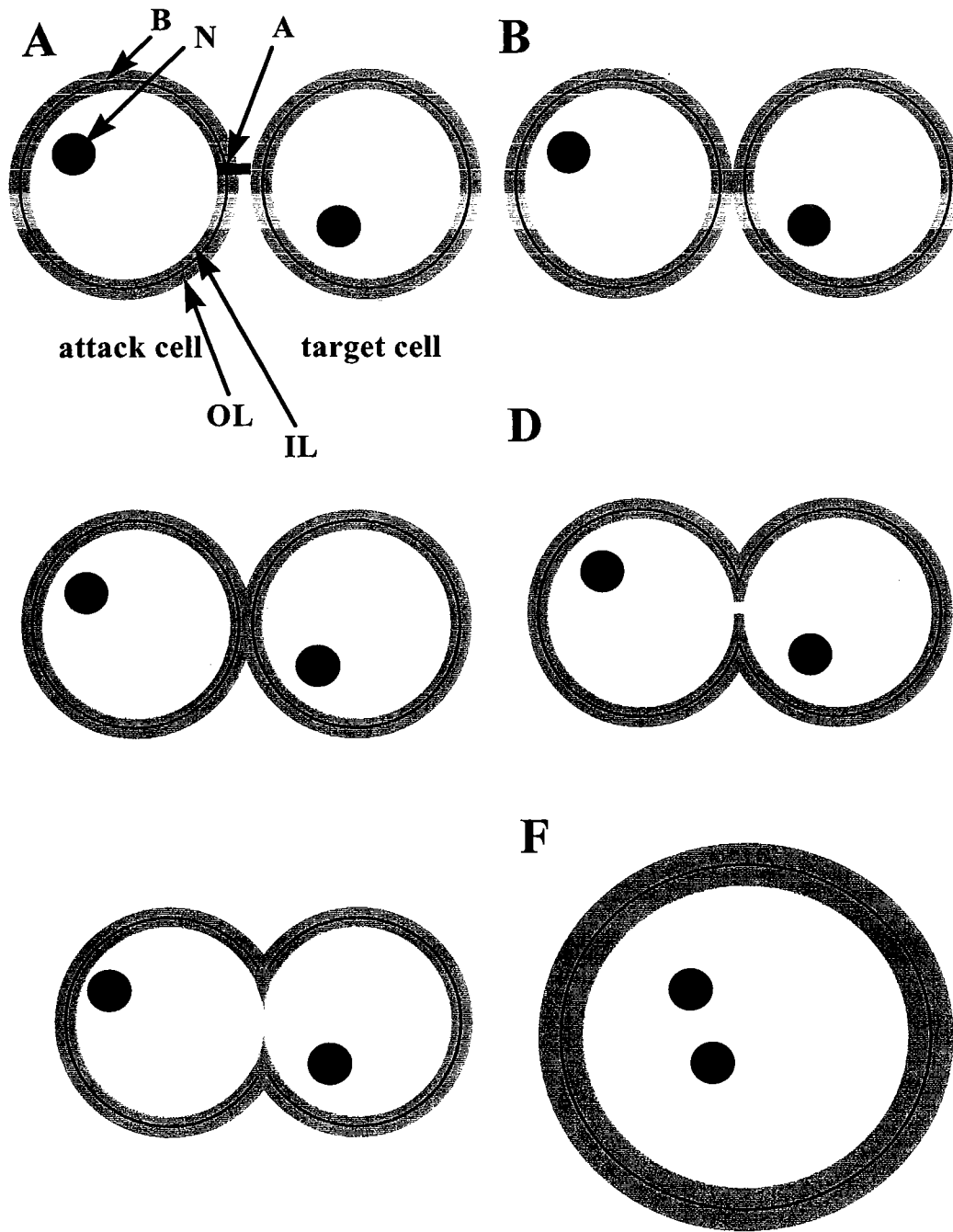
Legend to Figure 3. Steps of viral fusion. (A) Binding of attack to target cell. (B) Close approach of outer leaflets. (C) Hemifusion. (D) Formation of an early fusion pore. (E) Formation of a late fusion pore. (F) Complete fusion. B, lipid bilayer; A, attachment protein; IL, inner leaflet of plasma membrane; OL, outer leaflet of plasma membrane; N, nucleus.

CHAPTER 2

DETECTION OF AN INTERACTION BETWEEN THE HN AND F PROTEINS IN

NEWCASTLE DISEASE VIRUS-INFECTED CELLS

This chapter was published in Journal of Virology in Sept., 1997 essentially as

presented here, accounting for some duplication in background and

discussion presented in Chapter 1 and Chapter 4.

Introduction

The paramyxovirus Newcastle disease virus (NDV) encodes two surface

glycoproteins, the hemagglutinin-neuraminidase or HN protein and the fusion

or 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 (30)), 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 HA

cannot complement the NDV F protein to permit fusion (42). 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 (13).

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 (24). Recent work from several laboratories supports the idea that the stalk domain of the HN protein determines F protein specificity for fusion (17, 62, 67). The first direct demonstration of an interaction was in the measles system where Malvoisin and Wild (38) used a vaccinia expression system to assay for possible HA-F protein interactions. Using this system, Malvoisin and Wild chemically crosslinked 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 proteins using vaccinia virus vectors as well (74).

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 using coimmunoprecipitation and chemical crosslinking. 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 crosslinking. Precipitation of this complex was not dependent upon cleavage of F_0 into F_1 and F_2, the complex did not dissociate immediately after HN protein attachment to uninfected cells, nor was it dependent upon attachment of the HN protein to its receptor.

Materials and Methods

<u>Cells and viruses.</u> 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 (21).

<u>Infections.</u> 5×10^5 Cos-7 cells were plated on 35 mm tissue culture plates in

DMEM. 16-20 hours after plating, the cells were infected with either the AV or

B1 strain of NDV at a multiplicity of infection of 10. The virus was adsorbed for

30 minutes at 37° before additional DMEM supplemented with $CaCl_2$ was

added. The infection was allowed to proceed for an additional 4.5 hours at

37°.

<u>Antibodies.</u> Monoclonal antibodies specific for NDV HN were a generous gift of

Dr. Ron Iorio. Antibodies used were anti-1c, anti-2b, anti-3a and anti-4a (25).

Polyclonal antisera specific for NDV F was raised against a peptide with the

sequence of the carboxy terminus of the F protein (56). 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 (56).

<u>Radiolabeling, lysis and immunoprecipitation of protein.</u> At five hours post-infection, cells were radiolabeled for fifteen minutes at 37° in DMEM containing 70% of the cysteine of standard media and lacking methionine. The labeling media contained 11 mCi/mL ^{35}S-methionine ^{35}S-cysteine (EXPRE^{35}S^{35}S, New England Nuclear). The cells were then chased in non-radioactive media for two hours. For five minute pulse-chase experiments, the cells were incubated in media lacking methionine for twenty minutes prior to the label. These cells were then chased in non-radioactive media containing 0.1 mg/mL cycloheximide and 2 mM non-radioactive 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 crosslinking experiments. For coimmunoprecipitation, the monolayer was lysed in RSB buffer (0.01 M Tris-HCl, pH7.4, 0.01 M NaCl) containing 0.5% sodium deoxycholate, 2.5 mg/mL N-ethylmaleimide (NEM), 2 mg/mL iodoacetamide, and 13 mM Chaps on ice. After crosslinking, the monolayer was lysed in RSB buffer containing 0.5% sodium deoxycholate, 2.5 mg/mL NEM, 2 mg/mL iodoacetamide and 1% Triton. After lysis, the nuclei were removed by centrifugation.

For immunoprecipitation of NDV proteins, infected cell lysates were incubated with antisera for one hour at room temperature. Fixed, killed staphylococcus aureus (SA) cells (Boehringer Mannheim) resuspended in

PBS, 0.5% polyoxyethylenesorbitan monolaurate and 1 mg/mL bovine serum albumin were added to the lysate in the presence of 0.15-0.4% sodium dodecyl sulfate (SDS) and incubated at room temperature with agitation for thirty minutes. The SA cells were pelleted and the supernatants were removed. The pellets were washed three times with PBS, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS. The SA cells were then resuspended in sample buffer and stored at -20° until analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

Chemical crosslinking. Crosslinking was accomplished by a modification of the procedure described by Russell et al (53). 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 one hour at 4°. The crosslinker was quenched at 4° with 74 mM glycine for five minutes and the cells were washed with PBS, pH 8.5, and lysed with Triton X-100 containing lysis buffer at 4°. Extracts were immunoprecipitated as described above and analyzed by SDS-PAGE. For analysis of large, crosslinked species, two dimensional SDS-PAGE was utilized. Immunoprecipitates were electrophoresed under non-reducing conditions and individual lanes were cut from the gel and incubated in run buffer containing 10% β-mercaptoethanol (βME) for five minutes. The proteins in the gel slice were then electrophoresed under reducing conditions.

<u>Cleavage of cell surface fusion protein.</u> 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 ten minutes at 37°. 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.

<u>Attachment Assay.</u> At 150 minutes post infection, the infected monolayer was treated or mock-treated with neuraminidase (Sigma) (0.2 units in 1 mL in DMEM) and incubated at 37° for an additional 150 minutes. During the pulse-label and the chase, the cells were treated or mock-treated with neuraminidase. After the chase, approximately 1×10^6 uninfected Cos cells were added to half of the plates at 4°.

Results

<u>Coimmunoprecipitation of HN and F.</u> 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 five hours post-

infection, a time at which there is significant synthesis of both proteins (45, 46),

cells were subjected to a fifteen minute pulse-label and a two hour chase in

non-radioactive media. Proteins present in infected cell lysates were

immunoprecipitated using 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. 4). 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 antisera. 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 antisera 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.

AV infected cells were also subjected to a five minute pulse and chased in non-radioactive media for various lengths of time (Fig. 5). Coprecipitation with heterologous antisera was observed at all time points. Indeed, even after a five minute pulse, a time at which the F protein should still be localized in the rough endoplasmic reticulum, extracts precipitated with HN antisera contained uncleaved F_0 protein, while F antisera precipitated HN protein. Because the F protein is proteolytically cleaved in the trans Golgi (45), 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 crosslinking of HN and F proteins. To stabilize the potential association of HN and F proteins on infected cell plasma membranes, use of the chemical crosslinker DTSSP was investigated. DTSSP is a membrane impermeable crosslinker (61), therefore incubation of intact cells with this

crosslinker will link only proteins expressed on the cell surface. Furthermore, DTSSP is cleavable with reducing agents, thus individual proteins in a crosslinked complex can be resolved on polyacrylamide gels after reduction in β-mercaptoethanol (βME).

After a pulse-chase labeling protocol, monolayers were incubated with different concentrations of crosslinker ranging from 0.05 to 1.0 mM. After lysis, proteins in the cytoplasmic extracts were precipitated with either HN antisera, F antisera, or no antisera under conditions more stringent then those reported in Fig. 4 (see Materials and Methods). The precipitates were analyzed by SDS-PAGE under reducing conditions (Fig. 6A). All concentrations of crosslinker resulted in the precipitation of a crosslinked complex which contained proteins that comigrate with HN and F proteins. The amount of HN protein precipitated by F antisera increased with increasing crosslinker concentrations up to maximal levels at 0.5 and 1.0 mM DTSSP (Fig. 6B). The ratio of HN:F protein also increased with increasing crosslinker concentration up to 0.5 mM DTSSP (fig. 6B). HN antisera precipitated an F_1 sized molecule at all concentrations of crosslinker. 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 crosslinker. Furthermore, this material is in a crosslinked complex and not nonspecific aggregates of NPs, since no NP sized molecules were resolved under non-reducing 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 crosslinker

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 antisera, while 5% of total labeled F_0

protein and 22% of total labeled F_1 protein were precipitated with HN antisera.

Thus, crosslinking 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. 4).

To determine sizes of the crosslinked species, immunoprecipitates

were analyzed by SDS-PAGE under non-reducing conditions (Fig. 7). In the

absence of crosslinker, HN antisera precipitated HN monomer (74 kD), HN

dimer (150 kD) and HN tetramer (~300 kD) (25). As crosslinker concentration

increased, the HN dimer migrated more slowly. Importantly, at all

concentrations of DTSSP, HN antisera precipitated a 66 kD species, a species

with a molecular weight of approximately 150 kD, four high molecular weight

species ranging from approximately 160 to 300 kD and heterogeneous

material with sizes of 120 kD and larger. HN monomer was resolved as two

species, and the levels observed remained unchanged with increasing crosslinker.

In the absence of crosslinking, F antisera precipitated only a 66 kD protein, the non-reduced, monomeric form of F protein (F_{nr}) (68). With increasing crosslinker concentrations, there was a gradual decrease of the F monomer. In addition, there was detection of a species with an apparent molecular weight of approximately 120 kD that comigrated with material precipitated with HN antisera. The amount of this species increased at 0.5 mM DTSSP, but decreased when higher crosslinker concentrations were used, presumably because increasing crosslinker concentrations resulted in the formation of higher molecular weight complexes. Three larger complexes which migrated between 160 and 300 kD as well as heterogeneous material 120 kD and larger were observed at all concentrations of crosslinker 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 crosslinked species, precipitated proteins were characterized by two dimensional polyacrylamide gel electrophoresis. The first dimension separated crosslinked complexes under non-reducing conditions as described in Fig. 7 (Fig. 8A) and the second dimension separated these complexes under reducing conditions. Such analysis of crosslinked complexes immunoprecipitated with HN antisera (Fig.

8B) showed that material present in the stacker gel as well as intermediate sized complexes with molecular weights of 160-300 kD 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 kD resolved primarily into F_1 protein with minimal amounts of HN protein and F_0 protein present. The distribution of HN protein (Table1) among these species was 15% in the stacker gel, 32% from 160-300 kD, 42% at 150 kD and 11% at 74 kD. Thus, F_0 and F_1 proteins were associated with both intermediate sized as well as large, crosslinked complexes containing HN protein. Surprisingly, a 66 kD species containing primarily F_0 and F_1 proteins was precipitated with HN antisera.

A similar analysis of crosslinked complexes immunoprecipitated with F antisera (Fig. 8C) showed that material in the stacker gel as well as the complexes ranging in size from 140 to 300 kD contained HN, F_0 and F_1 proteins. The 120 kD species as well as the 66 kD species contained almost exclusively F_0 and F_1 proteins. The distribution of total F_0 and F_1 was 5% and 6% in the stacker gel, 23% and 21% in the 160-300 kD region, 5% and 22% at 120 kD, and 67% and 51% at 66 kD. Thus, HN and F precipitated by heterologous antisera were associated with complexes 160 kD and larger. A small amount of uncrosslinked HN protein was also coprecipitated with F protein after crosslinking.

<u>Crosslinking at various times of infection.</u> To explore the appearance of crosslinked complexes with time of infection, infected cells were incubated with DTSSP at various time points post-infection (PI) (Fig. 9). As early as 4.25 hours PI, 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 post-infection until 5.75 hours and then dropped, while for the F_1 protein, the percentage increased until 5.25 hours before decreasing. These results support the idea that complexes observed later in infection (7.5 hours, Fig. 5-8) cannot be due exclusively to close packing of the molecules which might accompany high levels of expression of viral proteins at the cell surface.

<u>The Effect of F_0 Cleavage on the Detection of HN and F Crosslinked Species.</u>

While F_0 protein was detected in crosslinked 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 and not the F_0 protein can be precipitated in a crosslinked complex with HA (39).

To determine the importance of cleavage of the F_0 protein in the detection of HN-F protein complexes, the avirulent B1 strain of NDV was utilized. When Cos cells are infected with NDV-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 (4, 19, 54, 66). Cleavage does occur, however, upon addition of exogenous trypsin (reviewed in (14)).

Cos cells, infected with NDV strain B1 or AV were labeled and crosslinked as described above, except that the monolayers were mock treated or incubated with exogenous trypsin before crosslinking. F antisera precipitated HN protein equally from B1 infected cell lysates derived from either trypsin treated (+trypsin) or untreated cells (-trypsin) (Fig. 10). 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 crosslinked complex with HN. In contrast to AV infected cells, however, total HN protein precipitated by F antisera as well as total F protein precipitated with HN antisera was considerably reduced.

Crosslinked, precipitated proteins were characterized by two dimensional SDS-PAGE as described in Fig. 8. In the first dimension, in the absence of trypsin treatment under non-reducing conditions, proteins precipitated with HN antisera (Fig. 11A, HN antisera +DTSSP; Table 1) yielded HN monomer (74 kD), a small amount of 160-300 kD species and a large amount of protein in the stacker gel, as well as heterogeneous material with sizes greater than 160 kD. Analysis of these complexes in the second dimension under reducing conditions (Fig. 11B) showed that the material in the stacker gel and at 74 kD contained most of the HN protein (see Table 1), a result very different than that observed in AV infected cells. Electrophoresis of

precipitates obtained with F antisera in the first dimension (Fig. 11A, F antisera +DTSSP) resulted in F_{nr} (66 kD), a 120 kD species, 160-300 kD species, a large amount of protein in the stacker gel, as well as heterogeneous material throughout the gel. Electrophoresis in the second dimension under reducing conditions (Fig. 11C) showed that like the HN antisera results, most of the crosslinked F protein was observed migrating in the stacker gel.

After trypsin treatment, analysis of precipitates obtained with both HN and F antisera in the first dimension (Figure 12A, HN antisera +DTSSP, F antisera +DTSSP; showed an increase in heterogeneous crosslinked complexes which electrophoresed with molecular weights between 160 and 300 kD, similar to that observed in AV infected cells. Crosslinked material precipitated with F antisera, however, contained a higher percentage of F_1 protein in the stacker region and from 160-300 kD than was observed in AV infected cells. Thus, in the absence of trypsin digestion, the majority of crosslinked material precipitated was very large. Trypsin treatment resulted in an increase of crosslinked species between 160-300 kD, similar in size to the crosslinked material observed from AV infected cells. Therefore, while cleavage of the F protein did not increase the amount of crosslinked complex, the size of the complex changed upon cleavage.

<u>The effect of attachment on the HN and F interaction.</u> 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 crosslinking of HN and F proteins, crosslinking was accomplished under conditions which minimize attachment as well as conditions which maximize attachment. To minimize attachment, infected cells were incubated in neuraminidase (N), a treatment which has been shown to block attachment and subsequent fusion (47). 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, pulse-labeled and an uninfected cell overlay was added. Another monolayer (+N-OL) was incubated with neuraminidase, pulse-labeled and no overlay was added. A third monolayer (-N+OL) was mock-treated with neuraminidase, pulse labeled and an uninfected cell overlay was added. The last monolayer (-N-OL) was mock-treated with neuraminidase, pulse-labeled and no overlay was added. The cell surface proteins of all four plates were then subjected to crosslinking as described in Materials and Methods. Proteins precipitated with HN as well as F antisera are shown in Fig. 13. Incubating infected cells in neuraminidase (+N) resulted in HN, F_0 and F_1 proteins migrating with a faster mobility due to the cleavage of sialic acid residues from the proteins. The amount of HN protein precipitated with F antisera 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 crosslinked 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 crosslinked.

Discussion

One interpretation of the finding that syncytia 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 using monospecific antisera against either protein, although the amount of coprecipitation detected was very small. Approximately 5% of the total HN protein was precipitable with F antisera and 2-3% and 1-4% of F_0 and F_1 proteins, respectively, were precipitable with HN antisera. These amounts could not be increased using various cell lysis and immunoprecipitation conditions (unpublished observations). Furthermore, coprecipitation was detected as early as after a five minute 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 crosslinker DTSSP. Crosslinking 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 crosslinker 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 crosslinker concentrations. Such increased levels of coprecipitation of HN and F proteins after crosslinking 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 crosslinked on the surfaces of cells expressing these two proteins from vaccinia vectors (38), although another study of surface crosslinking of SV5 infected cells as well as NDV infected cells reported no crosslinking of HN and F proteins (53). Russell et al, however, used several conditions that differed significantly from ours and may account for our contrasting results. For example, Russell et al crosslinked infected cells in suspension after removing them from a monolayer, while we crosslinked infected cell monolayers. Detachment of cells from surfaces may result in the rearrangement of surface proteins. Another study by Markwell and Fox (39) found no evidence of crosslinking of HN and F proteins in both Sendai virus and Newcastle disease virus virions. This report may differ from ours both because virions were used instead of infected cells, and different crosslinkers were utilized.

Interestingly, HN antisera precipitated only 5% of total F_0 protein after crosslinking, 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 crosslink F_0 to HN. Malvoisin and Wild (38) reported chemical crosslinking of HA and F_1 proteins of measles virus at the cell surface, but were unable to detect any F_0 protein in the crosslinked complexes. They argued that only the cleaved form of the protein can interact with the HA protein. Using the same lysis conditions reported by Malvoisin and Wild, we were unable to precipitate any crosslinked 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 crosslinked complexes using NDV strain B1, which has an uncleaved F_0 protein. The amount of crosslinking between the fusion protein of this virus and the HN protein did not change, independent 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 crosslinking 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 crosslinking upon cleavage of the F protein. However, the sizes of the

crosslinked 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 160-300 kD size range. It has been previously shown that cleavage alters the conformation of the fusion protein (23). Perhaps cleavage results in less close packing of the HN and F molecules and, therefore, smaller crosslinked complexes.

It has been proposed that attachment of HN protein to its receptor stimulates the interaction of HN and F proteins (30). However, attempts to increase the participation of HN proteins in attachment by adding an uninfected Cos cell overlay did not alter HN-F protein crosslinking. Incubating infected cells in neuraminidase, which should block attachment, also failed to significantly alter the amount of HN and F_0 in crosslinked complexes, although the amount of F_1 crosslinked was slightly increased under these conditions.

In an attempt to characterize crosslinked species derived from individual proteins, we transfected cells with the HN gene alone or the F gene alone using an SV40 based vector. We were not able to crosslink HN proteins, but were able to crosslink F proteins, indicating that F-F crosslinking is possible. These crosslinked complexes electrophoresed with sizes of 200-300 kD (unpublished observations). Characterization of the crosslinked 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 kD. If there is no crosslinking of the HN protein except for the naturally occurring intermolecular disulfide bonding, then the smaller (160-300 kD) complexes precipitated with HN antisera should have 1-4 HN proteins and 1-4 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 crosslinking 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 hours post infection. However, these crosslinked complexes were readily detected as early as 4.25 hours post infection, when the concentrations of viral proteins at the cell surface should be considerably reduced (42). Thus, the surfaces of infected cells may differ from transfected cells. In this regard, it is interesting that the only reports of crosslinking of HN and F proteins in cells expressing these proteins from a vector were accomplished in the context of vaccinia virus infection (38, 74).

After crosslinking, HN antisera precipitated a 120 kD complex which, when reduced, contained primarily F_1 protein. In addition, the precipitates contained a 66 kD polypeptide which comigrated with the non-reduced form of

the fusion protein. This result suggests that after crosslinking, F protein which is not crosslinked to HN protein can be precipitated with HN antisera. The most likely explanation for this observation is that crosslinking stabilizes a complex containing HN and F proteins which is able to interact noncovalently with F molecules which are then coprecipitated with the crosslinked complex. Indeed, it has been reported that the fusion protein of paramyxoviruses forms SDS resistant oligomeric structures (64). Such structures would be resistant to the precipitation conditions utilized 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 pre-fusion form that is activated by a conformational change in HN protein caused by binding to its receptor (30, 31, 56). 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. (63) 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 five minute 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 RER and hold each other in a pre-fusion 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.

TABLE 1. Distribution of HN and F after crosslinking 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. 7, 11, and 12. HN values were obtained from proteins precipitated with HN antisera (Fig. 7B, 11B, and 12B), while F_0 and F_1 values were obtained from proteins precipitated with F antiserum (Fig. 7C, 11C, and 12C). ND, no data.

		0.15			0.2			0.25			0.3			% SDS
M		HN	F	-	HN	F	-	HN	F	-	HN	F	-	antisera
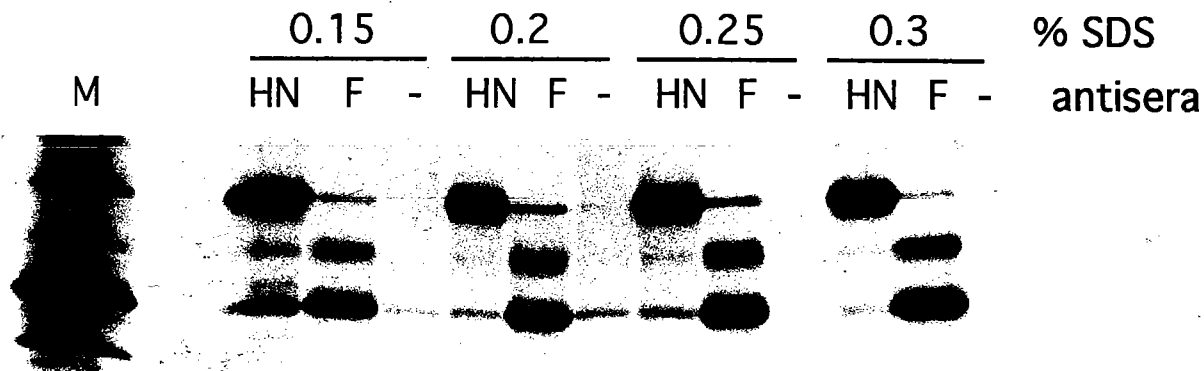
Legend to Figure 4: Coimmunoprecipitation of HN and F from AV infected cells. At 5 hours post-infection, AV infected Cos cells were pulse-labeled with ^{35}S methionine-cysteine for 15 minutes and then chased in non-radioactive media for two hours. The cells were lysed in Chaps buffer as described in Materials and Methods. Proteins present in extracts from $2x10^5$ cells were precipitated with a mixture of monoclonal antibodies a-1c, a-2b, a-3a and a-4a (HN antisera lanes), polyclonal antisera against the cytoplasmic tail of F (F antisera lanes), or no antisera (-antisera lanes), 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. HN, hemagglutinin-neuraminidase protein; F_0, uncleaved fusion protein; F_1, cleaved fusion protein; M, virus infected cell extract not immunoprecipitated.

anti-HN (poly)   anti-F (poly)   anti-HN (mono)   no antiserum

C   0 5 15 30 0 5 15 30 0 5 15 30 0 5 15 30

M   1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

HN
FO
F1
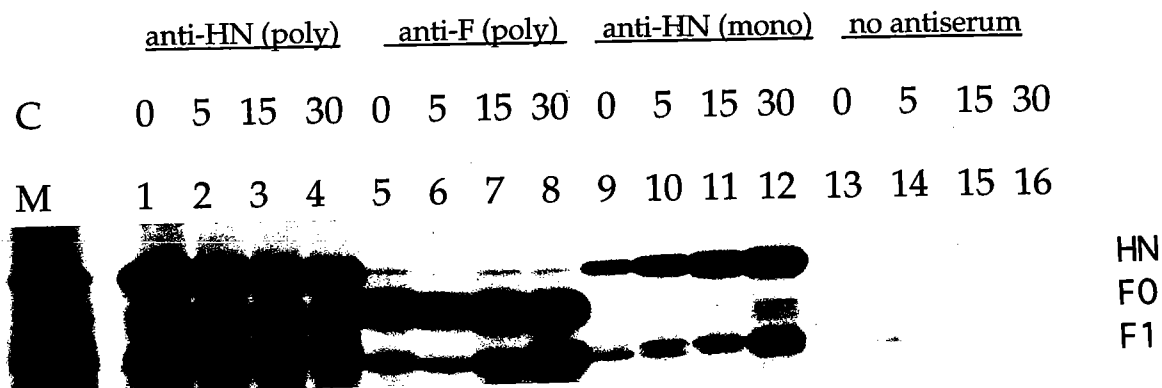
Legend to Figure 5: Coimmunoprecipitation of HN and F after a five minute pulse-label. At 5 hours post-infection, AV infected Cos cells were washed and incubated in media lacking methionine for 20 minutes. Cells were pulse-labeled with ^{35}S methionine-cysteine for 5 minutes and then chased in non-radioactive media containing cycloheximide and excess methionine for 0 min (lanes 1, 5, 9, 13), 5 min (lanes 2, 6, 10, 14), 15 min (lanes 3, 7, 11, 15) or 30 min (lanes 4, 8, 12, 16) as described in Materials and Methods. The cells were lysed in Triton buffer and precipitated with polyclonal antisera against amino acids 117-515 and 117-268 of the HN protein (lanes 1-4), polyclonal antisera against the cytoplasmic tail of F protein (lanes 5-8), a mixture of conformation-specific monoclonal antibodies against HN protein (lanes 9-12), or no antisera (lanes 13-16) in the presence of 0.25% SDS. The precipitated proteins were analyzed by SDS-PAGE in the presence of reducing agent. The lower band in lanes 9-12 is non-specific. In M lane, NP and P proteins comigrate with the F_1 protein. C, chase time (min); poly, polyclonal antiserum; mono, mixture of monoclonal antisera; HN, hemagglutinin-neuraminidase protein; F_0, uncleaved fusion protein; F_1, cleaved fusion protein; M, virus infected cell extract not immunoprecipitated.

A

	HN antisera	F antisera	no antisera	
	0 .05 .1 .25 .5 1	0 .05 .1 .25 .5 1	0 .05 .1 .25 .5 1	mM DTSSP

HN
FO
F1

B

DTSSP Titration

Densitometer Units

mM DTSSP
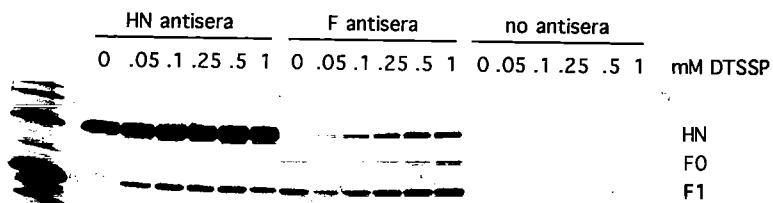
C

DTSSP (mM)	Ratio HN: F
0	0.08:1.6
0.05	0.8:1.7
0.1	0.96:1.63
0.25	1.0:1.58
0.5	1.0:1.48
1.0	0.82:1.66

Legend to Figure 6: Titration of the chemical crosslinker DTSSP. AV infected cells were pulse labeled as in Fig. 4. After the two hour chase in DMEM, surface proteins were crosslinked 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 (a mixture of monoclonal antibodies a-1c, a-2b, a-3a and a-4a), F antisera at subsaturating levels or no antisera in the presence of 0.4% SDS and analyzed by SDS-PAGE under reducing conditions (Panel A). Panel 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. HN, hemagglutinin-neuraminidase protein; F_0, uncleaved fusion protein, F_1, cleaved fusion protein; extreme l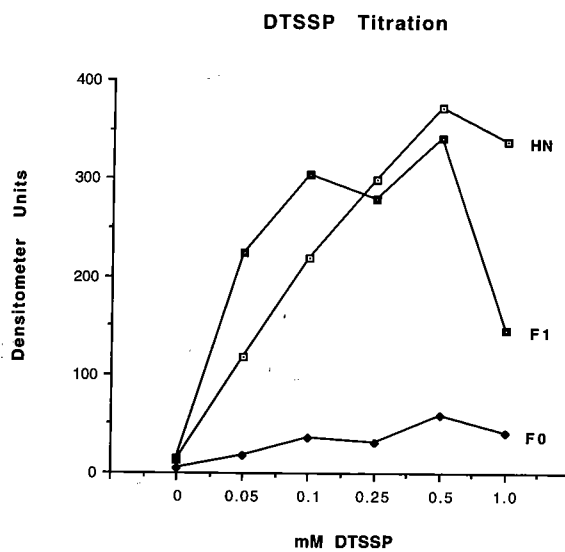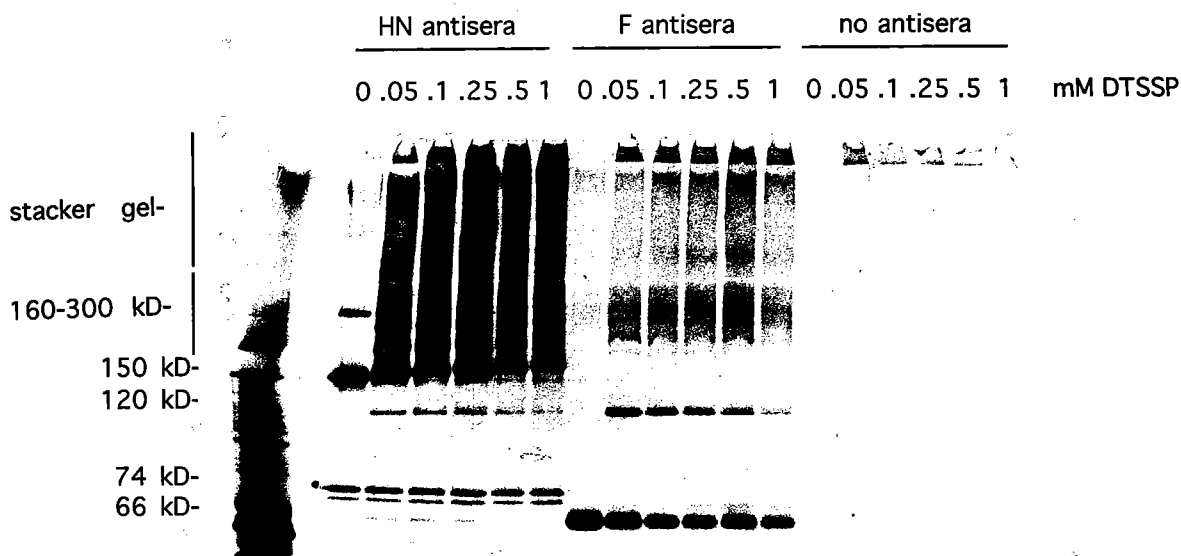eft lane, virus infected cell extract not immunoprecipitated. Panel C: Panel A was scanned on a Molecular Dynamics densitometer and the densitometer units of HN, F_0 and F_1 precipitated with F antisera were listed as a ratio of HN:F.

	HN antisera	F antisera	no antisera	
	0 .05 .1 .25 .5 1	0 .05 .1 .25 .5 1	0 .05 .1 .25 .5 1	mM DTSSP

stacker gel-

160-300 kD-

150 kD-
120 kD-

74 kD-
66 kD-

Legend to Figure 7: Non-reduced crosslinked proteins observed in the presence of increasing concentrations of DTSSP. AV infected cells were radiolabeled, crosslinked, and lysed as in Fig. 5. Proteins from the cytoplasmic extracts were precipitated with HN antisera (a mixture of monoclonal antibodies a-1c, a-2b, a-3a and a-4a), F antisera, or no antisera in the presence of 0.4% SDS. The precipitates were analyzed by SDS-PAGE under non-reducing conditions. Molecular weights were assigned using NDV, strain AV, infected cell extracts as markers; extreme left lane, virus infected cell extract not immunoprecipitated.
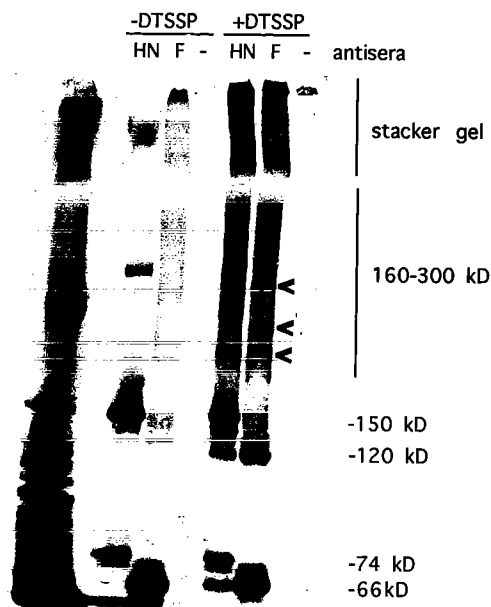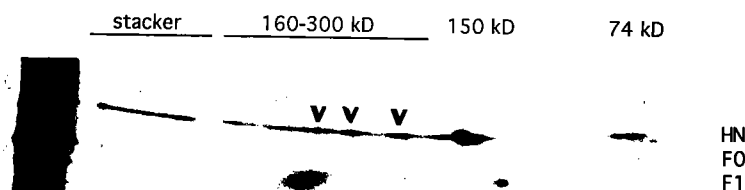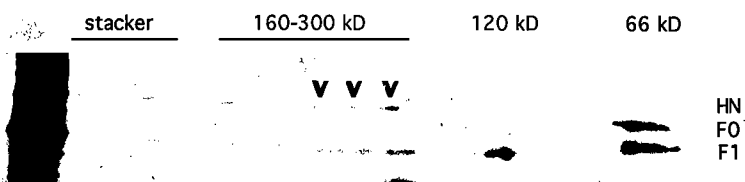
Legend to Figure 8: Two dimensional SDS-PAGE analysis of crosslinked proteins from AV infected cells. AV infected cells were radiolabeled, crosslinked with 0.5 mM DTSSP, and lysed as in Fig. 5. Proteins from the cytoplasmic extracts were precipitated with HN antisera (a mixture of monoclonal antibodies a-1c, a-2b, a-3a and a-4a), F antisera, or no antisera in the presence of 0.4% SDS. Panel A: Precipitated proteins which were not crosslinked (-DTSSP) or were incubated in the presence of crosslinker (+DTSSP) were analyzed by SDS-PAGE under non-reducing conditions as described in Fig. 6 except the complexes were resolved on a lower percentage polyacrylamide gel. Panel B: A lane identical to the HN antisera +DTSSP lane was excised from the gel, reduced in bME and analyzed by SDS-PAGE under reducing conditions. The top portion of the gel in Panel A is observed on the left side of panel B. Panel C: A lane identical to the F antisera +DTSSP lane was excised from the gel, reduced in bME and analyzed by SDS-PAGE under reducing conditions. v, complexes containing HN and F proteins; HN, hemagglutinin-neuraminidase protein; F_0, uncleaved fusion protein; F_1, cleaved fusion protein; extreme left lane, virus infected cell extract not immunoprecipitated.

A
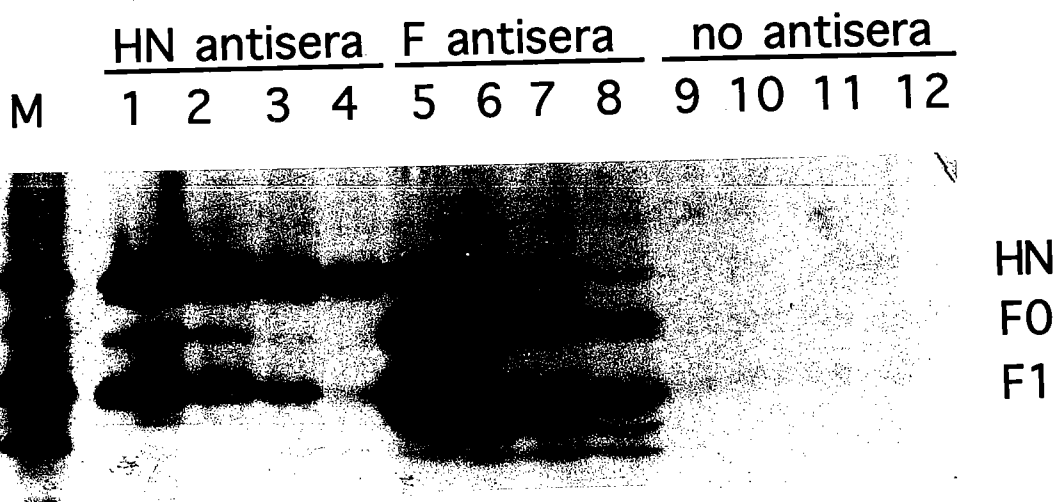
B
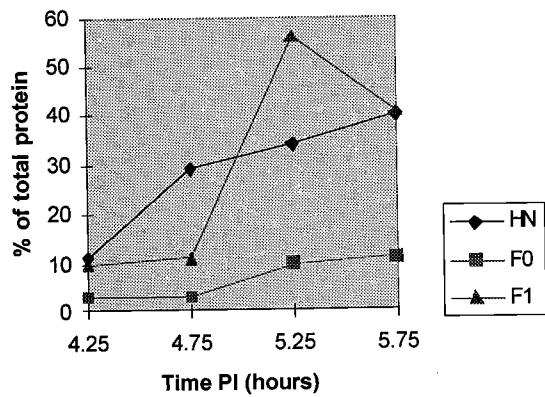
Legend to Figure 9: Crosslinking of proteins from AV infected cells at various times post-infection. At various times post-infection, AV infected Cos cells were pulse-labeled with ^{35}S methionine-cysteine for 15 minutes and then chased in non-radioactive media for two hours. Crosslinking with 0.5 mM DTSSP was performed at 4.25 (lanes 4, 8, 12), 4.75 (lanes 3, 7, 11), 5.25 (lanes 2, 6, 10) and 5.75 (lanes 1, 5, 9) hours post-infection. Cells were then lysed and as for Fig. 5. Proteins from the cytoplasmic extracts were precipitated with HN antisera (a mixture of monoclonal antibodies a-1c, a-2b, a-3a and a-4a) or F antisera in the presence of 0.4% SDS and the precipitates were analyzed by SDS-PAGE under reducing conditions (Panel A). Panel B: Three separate experiments were scanned on a Molecular Dynamics densitometer and densitometer units of HN protein precipitated with F antisera and F proteins precipitated with HN antisera were plotted against time PI. HN, hemagglutinin-neuraminidase protein; F_0, uncleaved fusion protein; F_1, cleaved fusion protein; M, virus infected cell extract not immunoprecipitated.

antisera   HN   F   none

trypsin   -   -   +   -   -   +   -   -   +

AV B1 B1   AV B1 B1   AV B1 B1

HN
FO
F1
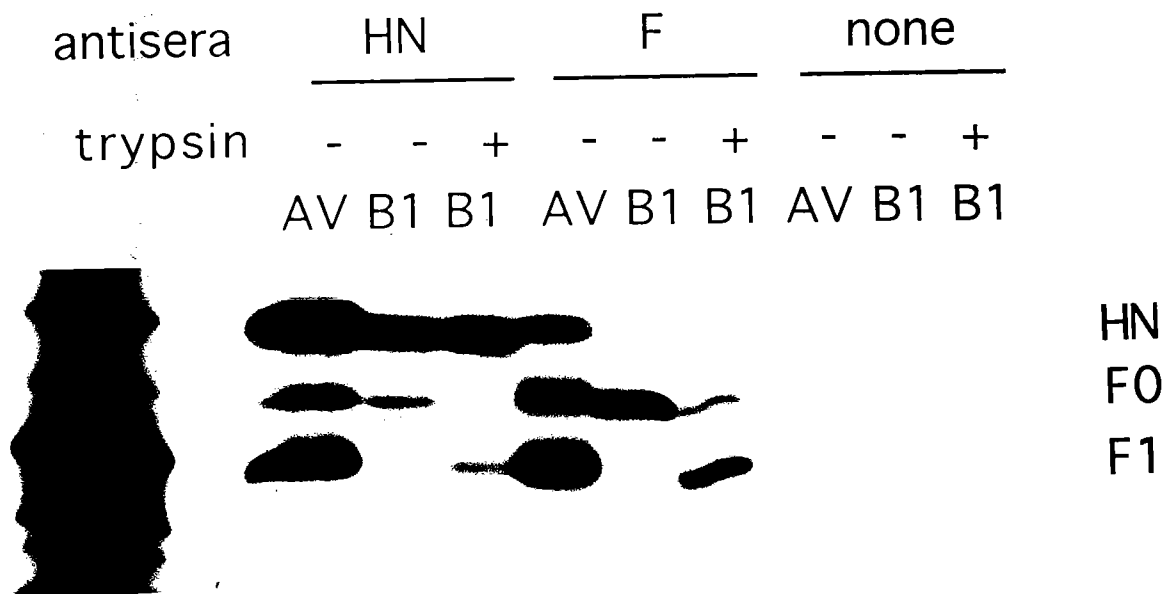
Legend to Figure 10: Cleavage of B1 fusion protein at the cell surface. At five hours post-infection, AV or B1 infected Cos cells were pulse-labeled with ^{35}S methionine-cysteine for 15 minutes and then chased in non-radioactive media for two hours. The cells were treated or mock-treated with trypsin for 10 minutes, washed with soybean trypsin inhibitor, crosslinked with 0.5 mM DTSSP and lysed in Triton X-100 buffer containing soybean trypsin inhibitor as described in Fig. 5. Proteins from the cytoplasmic extracts were precipitated with HN antisera (a mixture of monoclonal antibodies a-1c, a-2b, a-3a and a-4a), F antisera, or no antisera in the presence of 0.4% SDS. Precipitates were analyzed by SDS-PAGE under reducing conditions. AV, AV infected cell precipitates; B1, B1 infected cell precipitates; -trypsin, mock-treated with trypsin; +trypsin, incubated with trypsin; HN hemagglutinin-neuraminidase protein; F_0, uncleaved fusion protein; F_1, cleaved fusion protein; leftmost lane, virus infected cell extract not immunoprecipitated.

A

-DTSSP   +DTSSP

HN F -   HN F -   antisera

stacker gel

160-300 kD

-150 kD

-120 kD

-74 kD
-66 kD

B

stacker   160-300 kD   150 kD   74 kD

HN
F0

C

stacker   160-300 kD   120 kD   66 kD

HN
F0

Legend to Figure 11: Two dimensional SDS-PAGE analysis of crosslinked proteins from B1 infected cells expressing an uncleaved F protein. B1 infected cells were radiolabeled, crosslinked with 0.5 mM DTSSP, and lysed as in Fig. 10 (in the absence of trypsin treatment). Proteins from the cytoplasmic extracts were precipitated with HN antisera (a mixture of monoclonal antibodies a-1c, a-2b, a-3a and a-4a), F antisera, or no antisera in the presence of 0.4% SDS. Panel A: Precipitated proteins which were not crosslinked (-DTSSP) or were crosslinked (+DTSSP) were analyzed by SDS-PAGE under non-reducing conditions. Panel B: A lane identical to the HN antisera +DTSSP lane was excised from the gel, reduced in bME, and analyzed by SDS-PAGE under reducing conditions. The top portion of the gel in Panel A is observed on the left side of panel B. Panel C: A lane identical to the F antisera +DTSSP lane was excised from the gel, reduced in bME and analyzed by SDS-PAGE under reducing conditions. HN, hemagglutinin-neuraminidase protein; F_0, uncleaved fusion protein; F_1, cleaved fusion protein; extreme left lane, virus infected cell extract not immunoprecipitated.
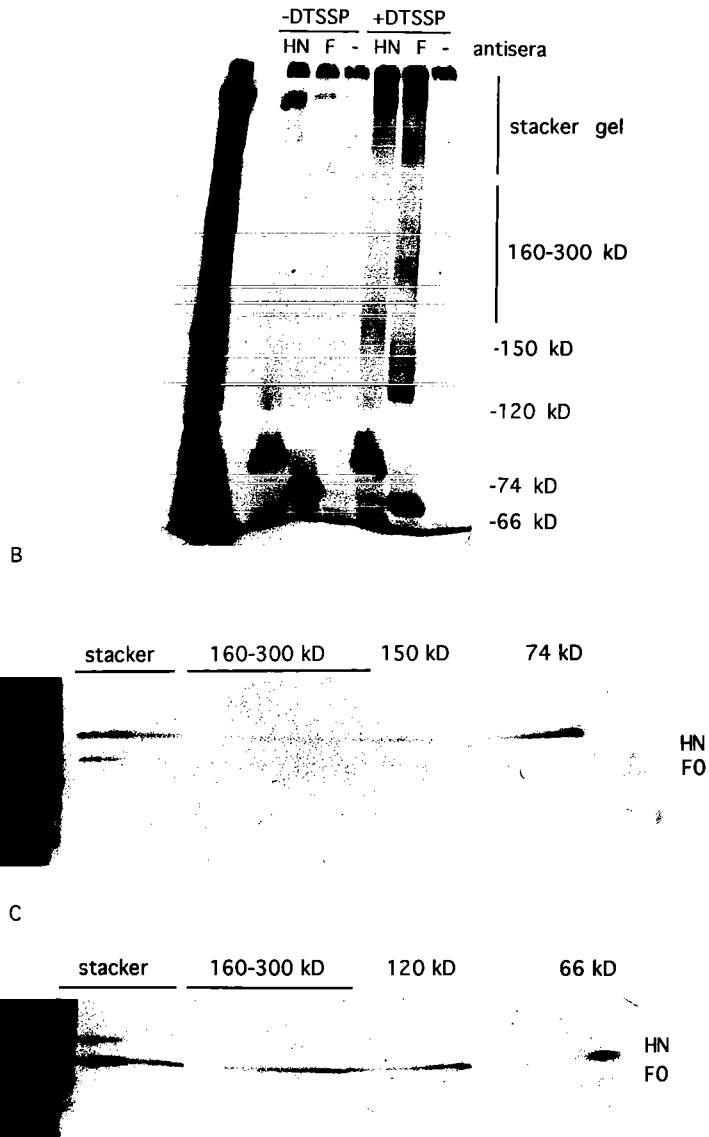
A

-DTSSP +DTSSP

HN F -   HN F -   antisera

stacker gel

160-300 kD

-150 kD
-120 kD

-74 kD
-66 kD

B

| stacker | 160-300 kD | 150 kD | 74 kD |

HN
F0
F1

C

| stacker | 160-300 kD | 120 kD | 66 kD |

HN
F0
F1

Legend to Figure 12: Two dimensional SDS-PAGE analysis of crosslinked proteins from B1 infected cells expressing a cleaved F protein. B1 infected cells were radiolabeled, incubated with trypsin, crosslinked with 0.5 mM DTSSP, and lysed as in Fig. 10. Proteins from the cytoplasmic extracts were precipitated with HN antisera (a mixture of monoclonal antibodies a-1c, a-2b, a-3a and a-4a), F antisera, or no antisera in the presence of 0.4% SDS. Panel A: Precipitated proteins which were not crosslinked (-DTSSP) or were incubated in the presence of crosslinker (+DTSSP) were analyzed by SDS-PAGE under non-reducing conditions. Panel B: A lane identical to the HN antisera +DTSSP lane was excised from the gel, reduced in bME, and analyzed by SDS-PAGE under reducing conditions. The top portion of the gel in Panel A is observed on the left side of panel B. Panel C: A lane identical to the F antisera +DTSSP lane was excised from the gel, reduced in bME and analyzed by SDS-PAGE under reducing conditions. HN, hemagglutinin-neuraminidase protein; F_0, uncleaved fusion protein; F_1, cleaved fusion protein; extreme left lane, virus infected cell extract not immunoprecipitated.
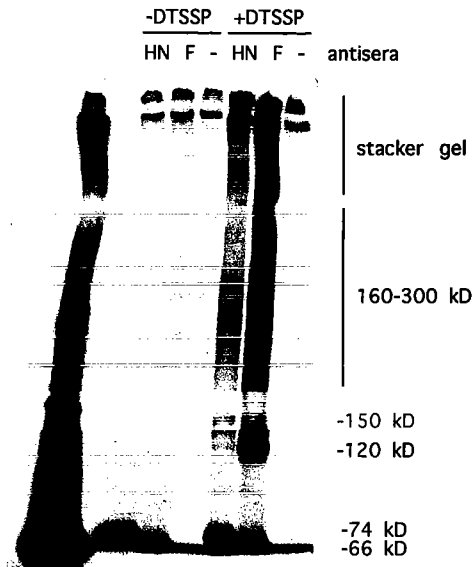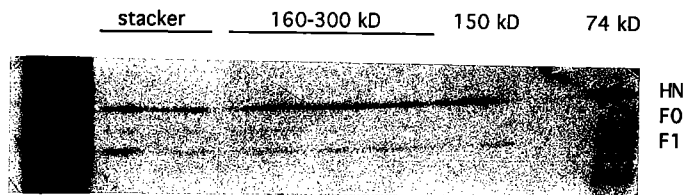
A

	HN antisera	F antisera	no antisera	
	− − + +	− − + +	− − + +	N
M	− + − +	− + − +	− + − +	OL

HN
F0
F1

B

N:	−	−	+	+
OL:	−	+	−	+
HN:	26	38	35	29
	16	22	17	18
	9	30	47	18
F_0:	nd	2	2	0.1
	2	3	5	7
	3	3	4	3
F_1:	27	15	17	3
	14	20	44	30
	13	18	19	13

Legend to Figure 13: The effect of attachment on HN and F crosslinking. Four cell monolayers were infected with AV. At 2.5 hours post-infection, two plates were treated and two plates were mock-treated with neuraminidase (0.2 units/mL) and incubated at 37° for an additional 2.5 hours. Cells were radiolabeled with ^{35}S methionine-cysteine for 15 minutes in the presence or absence of neuraminidase, and incubated in DMEM for 2 hours in the presence or absence of neuraminidase. After the chase, an overlay of uninfected cells was added to one plate in the presence and one plate in the absence of neuraminidase. The other two plates did not receive an overlay. The surface proteins were immediately crosslinked in 0.5 mM DTSSP, and the cells were lysed as in Fig. 5. Proteins from the cytoplasmic extracts were precipitated with HN antisera (a mixture of monoclonal antibodies a-1c, a-2b, a-3a and a-4a), F antisera, or no antisera in the presence of 0.4% SDS. The precipitates were analyzed by SDS-PAGE under reducing conditions. Panel A: Autoradiograph. Panel B: Coimmunoprecipitation of HN, F_0, and F_1 with heterologous antisera expressed as a percentage of the total protein immunoprecipitated with homologous antisera (taken from three separate experiments).   -N, cells mock-treated with neuraminidase; +N, cells treated with neuraminidase; -OL, no uninfected cell overlay added; +OL, uninfected cell overlay added; HN, hemagglutinin-neuraminidase protein; F_0, uncleaved F protein; F_1, cleaved F protein; extreme left lane, virus infected cell extract not immunoprecipitated.
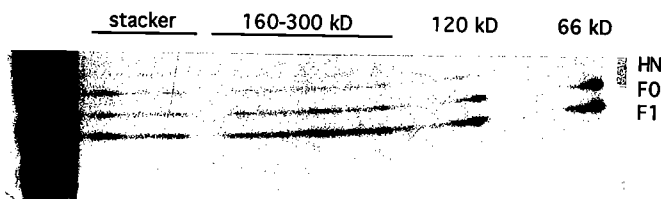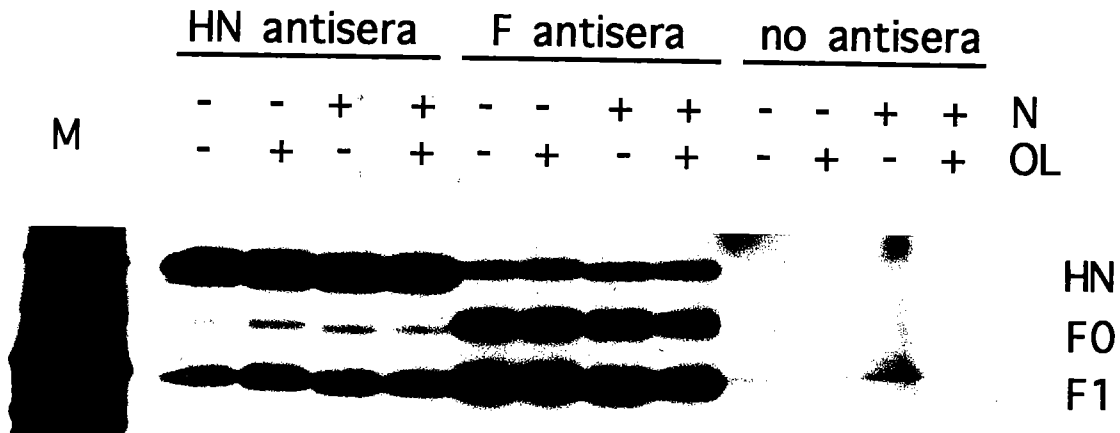
CHAPTER 3

MUTATIONAL ANALYSIS OF HEPTAD REPEATS IN THE MEMBRANE-

PROXIMAL REGION OF NEWCASTLE DISEASE VIRUS HN PROTEIN

This chapter was published in Journal of Virology in May, 1999 essentially as

presented here, accounting for some duplication in background and

discussion presented in Chapter 1 and Chapter 4.

Introduction

Newcastle disease virus (NDV) is one of many paramyxoviruses that

requires two surface glycoproteins in order fuse with uninfected cells. In

paramyxovirus mediated fusion, the fusion (F) protein is thought to directly

mediate the fusion event, and, with the exception of simian virus 5 (SV5), the

viral attachment protein is also necessary (30). Thus the hemagglutinin-

neuraminidase (HN) protein, which serves as the attachment protein for NDV,

has three functions; attachment, neuraminidase, and an undefined role in

fusion termed fusion promotion.

The requirement for the HN protein in fusion is virus specific and recent work from several laboratories suggests that the presumed stalk domain of various HN proteins confers this specificity. Deng et al. constructed chimeric HN proteins containing regions from human parainfluenza virus 3 (hPIV3) and NDV (17). Their results suggest that both the presumed transmembrane domain as well as a portion of the presumed stalk region of the HN protein confer F protein specificity for fusion. In a similar approach using parainfluenza virus 2 (PIV2) and simian virus 41 (SV41) chimeras, Tsurudome et al. also found that the presumed stalk region of the HN protein defines F protein specificity (67). Additionally, they reported that the globular head was necessary for maximal fusion promotion. However, they found that PIV2 and SV41 chimeras did not require a transmembrane sequence specific to either PIV2 or SV41 for fusion promotion. Tanabayashi et al also created chimeric HN proteins combining Sendai virus (SeV) and hPIV3 and found that only the stalk region of the HN protein was important for fusion specificity (62). Thus, while there is disagreement about the role of the transmembrane region and the globular head domain in virus specificity, it is clear that the stalk regions of HN proteins from various paramyxoviruses are crucial for F protein specificity. We have previously expressed HN proteins containing mutations in the stalk domain (56). These mutant proteins separated fusion promotion activity from attachment activity and led us to conclude that the stalk region of the NDV HN protein is critical for fusion promotion.

Virus specificity of the HN protein argues for an interaction between the HN and F proteins required for fusion (24), and, as described above, studies of chimeric HN proteins as well as point mutations suggest that it is the stalk domain that interacts with the F protein. While no clear studies of F protein chimeras have shown which domains of the F protein are important for an interaction with the HN protein, mutational analysis of the F protein has shown that several domains are important in fusion, including the fusion peptide as well as the heptad repeat regions HR1 and HR2 (7, 26, 30, 52, 58).

Heptad repeat regions are often involved in protein-protein interactions. Given the importance of heptad repeat domains in the F protein, the transmembrane-proximal location of one of them, as well as the apparent role of the transmembrane-proximal presumed stalk region of the HN protein in fusion promotion, we explored the potential for the presence of heptad repeats in this region of the HN protein. We found heptad repeat domains in all paramyxovirus and rubulavirus attachment proteins. Furthermore, use of secondary structure prediction software revealed that the heptad repeats from all the viruses analyzed showed a high probability of forming alpha helices.

We explored the importance of individual amino acids within these potential helices by mutation. The hydrophobic "a" position amino acids were the first residues chosen for mutagenesis because the "a" positions of heptad repeats are often important for mediating protein-protein interactions. Thus, we hypothesized that such mutations would have the potential to cause a more

deleterious effect on fusion promotion than mutations in other positions of the helices. Indeed, we found that all proteins altered in the "a" positions negatively effected fusion. However, mutations in other positions of the helix also negatively effected fusion. All mutant proteins had wild-type levels of hemagglutination and variable neuraminidase activity. These results argue that a specific amino acid sequence within the stalk is important for the fusion promotion activity of the HN protein, a result that would be expected if the region is involved in a specific interaction with the F protein.

Materials and Methods

Cells. 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, glutamine, penicillin/streptomycin, and 10% fetal calf serum.

Antibodies. Anti-NDV was raised against UV-inactivated NDV (strain AV) virions (56). Monoclonal antibodies specific for NDV HN protein were a generous gift of Dr. Ron Iorio. Antibodies used were anti-2b, anti-3a, anti-4a, anti-1,4c, and anti-2,3c (25).

Site-directed mutagenesis. Positive-sense oligonucleotides were synthesized by DNA International, Operon or Life Technologies. The oligonucleotides used for mutagenesis (written 5' to 3') were L74A (GGAAAGATTACATCTGCAGCCGGCTCCAATCAGGATGTAG),

V81A (GGTTCCAATCAGGATGTCGCGGATAGGATATACAAGC),

V88A (GGATATACAAGCAGGCAGCTCTTGAATCTCCG),

L96A (GGCAGCGCTAAACACCG),

I103A (GAATCTATAGCAATGAATGC),

L110A (CAATAACATCCGCCTCTTATC),

L74M (GGAAAGATTACATCTGCAATGGGTTCCAATCAGGATGTAG),

L96M (CTTGAATCTCCGTTGGCAATGCTAAACACCGAATCTATA),

L90A (GGATATACAAGCAGGTGGC<u>CGCG</u>GAATCTCCGTTGGC),

L97A (GAATCTCCGTTGGCATTG<u>GCC</u>AACACCGAATCTATAATT),

I102A (CTAAACACCGAATC<u>CGCG</u>ATTATGAATGCAATAACATCC). Bases which

were altered are underlined. Double mutants were made by sequential

mutagenesis. Oligonucleotide-directed mutagenesis of pSVL (Pharmacia)

containing the HN gene (42) was accomplished using the Morph Site-Specific

Plasmid DNA Mutagenesis Kit from 5 Prime 3 Prime, Inc. or the Chameleon

Double-Stranded, Site-Directed Mutagenesis Kit from Stratagene using the

methods and reagents supplied with each kit. Mutant pSVL-HN cDNAs were

identified by sequencing or by the introduction of a novel restriction site into the

mutant gene. Each HN mutant gene was then fully sequenced to ensure that

no extraneous mutations were generated in other parts of the gene.

<u>Transient gene expression.</u> Two methods were used to express HN cDNAs in

Cos-7 cells. DEAE-dextran transfection was performed by a modification of

Levesque et al as described previously (35, 52). Lipofectin (Gibco)

transfections were done essentially as suggested by the manufacturer and

were described previously (36) except cells were incubated with the Lipofectin-

OptiMem-DNA mixture at 37° for 20-24 h.

<u>Radiolabeling, lysis, and immunoprecipitation of protein.</u> At 48 h post-

transfection, cells were radiolabeled for 2 h at 37° in DMEM containing 70% of

the cysteine of standard media and lacking methionine. The labeling media

contained 11 mCi/mL ^{35}S-methionine and ^{35}S-cysteine (EXPRE^{35}S^{35}S, New

England Nuclear). The cells were chased in non-radioactive media for 2 h (4 h for cell surface assays).

At the end of the chase period, cells were washed once in phosphate buffered saline (PBS) and lysed in RSB buffer (0.01 M Tris-HCl, pH7.4, 0.01 M NaCl) containing 0.5% sodium deoxycholate, 2.5 mg/mL N-ethylmaleimide, 2 mg/mL iodoacetamide and 1% Triton. Lysates were homogenized by passage through a 21-gauge needle four times. After lysis, the nuclei were removed by centrifugation.

Cell lysates were incubated with antisera for one hour at room temperature. Fixed, killed staphylococcus aureus (SA) cells (Boehringer Mannheim) resuspended in PBS, 0.5% polyoxyethylenesorbitan monolaurate and 1 mg/mL bovine serum albumin were added to the lysate in the presence of 0.4% sodium dodecyl sulfate (SDS) and incubated at room temperature with agitation for thirty minutes. The SA cells were pelleted and the supernatants were removed. The pellets were washed three times with PBS, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS. The SA cells were then resuspended in sample buffer and stored at -20° until analysis by SDS-polyacrylamide gel electrophoresis (PAGE). Samples were incubated at 100° for 5 min prior to loading on 8% SDS-PAGE gels.

Fusion Assay. After a 48 h incubation in DMEM, twenty of the largest fusion areas were counted for each mutant and averaged as described previously

(56). Values obtained for fusion activities were taken from three separate experiments and averaged.

Cell Surface Assay. Transfected cells were radiolabeled as above and chased for four hours in non-radioactive media. Analysis of protein at the cell surface was done as described previously (56). Briefly, after a four hour chase, transfected monolayers were incubated on ice in 500 ul phosphate buffered saline, 20 ul complement inactivated antiserum (against UV inactivated NDV virions), and 1 ul 10% sodium azide, for 30 min with agitation. Unbound antiserum was removed from the monolayer, the cells were lysed and proteins expressed at the plasma membrane were immunoprecipitated. Proteins at the cell surface were quantitated from autoradiographs by densitometry and values obtained were taken from at least three separate experiments and averaged.

Attachment Assay. At 48 h post-transfection, attachment was assayed as described previously (44).

Neuraminidase Assay. At 48 h post-transfection, neuraminidase was assayed as described previously (44). Values obtained for neuraminidase activities were taken from three separate experiments and averaged.

Results

Mutagenesis of HN protein stalk domain. The stalk region of the NDV HN

protein has been defined as amino acids 49-146 (15, 34). Visual inspection of

this sequence showed that it contains two heptad repeats of hydrophobic

amino acids (leucine, valine, isoleucine) separated by a space of 7 amino

acids (Fig. 14). The heptad repeat motif is found in many proteins and is

thought to impart an alpha helical secondary structure (37). Secondary

structure prediction software (Baylor College of Medicine) predicts that the

heptad repeats in the stalk region of the HN protein do indeed have the

potential to form two alpha-helices with an intervening region of 7 amino acids

(Fig. 15).

The stalk region of other paramyxovirus HN proteins were similarly

analyzed for secondary structure. The attachment proteins of the

paramyxoviruses and rubulaviruses SV41, mumps virus (MuV), SV5, hPIV3,

SeV and avian parainfluenza 4 (avian para 4) also contain heptad repeats of

hydrophobic amino acids with the potential to form alpha-helices, although

morbilliviruses and pneumoviruses lacked predicted helical structure in this

region. Furthermore, most sequences were predicted to form two alpha

helices with an intervening space as observed in the NDV HN protein.

To investigate the importance of each of these NDV HN protein heptad

repeat regions for fusion as well as determine the relative importance of

specific amino acids and helical structure, conservative mutations of hydrophobic residues were made, changing heptad repeat "a" position residues to alanine. Alanine was chosen because it has a short side chain and should, therefore, not disrupt a potential helical structure (6). Mutations were made to generate the mutants L74A, V81A, V88A, L96A, I103A, and L110A (Fig. 14).

Expression of mutant proteins. To characterize the expression of these mutant proteins, Cos cells were transfected with either wild-type or mutant HN cDNA as described in Materials and Methods. HN proteins were immunoprecipitated with anti-NDV antisera and analyzed by SDS-PAGE under reducing (Fig. 16A) or non-reducing (Fig. 16B) conditions. Mutant HN proteins were immunoprecipitated in varying amounts, although the amounts precipitated were typically at least as high as the wild-type protein (Fig. 16A). Disulfide-linked dimers were observed for each of the mutant proteins suggesting that the conformation necessary for dimer formation was present (Fig. 16B). Cytoplasmic extracts containing each of the mutant proteins were immunoprecipitated with five different conformation-specific monoclonal antibodies (Fig. 16C, Materials and Methods). In every case, the mutant proteins were precipitated at levels as least as high as observed for the wild-type protein, supporting the idea that the proteins were folded correctly. Clearly, all the mutant HN proteins were expressed and stable within the cell for at least a 2 h chase period.

Cell surface proteins were analyzed by SDS-PAGE under reducing or non-reducing conditions (not shown) and amounts detected were quantitated by densitometry. All mutant proteins were detected at the cell surface, although at differing levels (Table 2). Disulfide-linked dimers were observed for each mutant protein at the cell surface although the apparent size of the dimers observed varied slightly from that of the wild-type protein in some experiments.

Biological activities of mutant proteins. The effect of each mutation on the three activities (attachment, neuraminidase and fusion promotion) was determined. We analyzed attachment activity by assaying red blood cell binding or hemagglutination (HA). Chicken red blood cells were bound to the surface of Cos cells expressing either wild-type or mutant proteins (Table 2). In cells expressing vector alone, virtually no binding was observed, while bound red blood cells were seen in cells expressing the wild-type HN protein (+). The mutant V88A bound red blood cells at least at wild-type levels while the other mutant HN proteins bound at levels greater than wild-type. Clearly, all mutant proteins retained attachment activity.

NA activity of each mutant protein was determined by quantitating the ability of each mutant protein to cleave the substrate neuraminlactose (Table 2). L74A and V81A had greater NA activity than the wild-type protein. The other mutant proteins had decreased NA activity ranging from 14-91%. Interestingly, two mutant proteins (I103A and L110A) had little NA activity and greater than wild-type levels of attachment activity. These data suggest that NA and HA

activities of the NDV HN protein can be genetically separated as has been previously reported (55).

Fusion promotion was determined by analyzing syncytia formation after coexpression of each mutant protein with the NDV fusion (F) protein in Cos cells (Fig. 17). Every mutation negatively affected fusion although to varying degrees. Mutations in the first heptad repeat decreased activity to 8 to 13% of the wild-type protein. Mutations in the second heptad repeat had slightly less effect as fusion activity decreased to 16 to 31% of the wild-type protein. Thus a conservative substitution of any one of these hydrophobic amino acid residues produced an HN protein with greatly decreased fusion activity.

Expression of proteins with double substitutions. After determining that alanine substitutions for "a" position amino acids negatively affected fusion, we asked if mutant proteins with double substitutions would further inhibit fusion. Similar double mutations in the F protein had been previously shown to decrease fusion to a much greater degree than single mutants (52). Thus the double mutants V81/L110A and L96/L110A were generated. These mutants were expressed at the cell surface and appeared to be folded correctly (Fig. 16, Table 2). Both mutants bound red blood cells at higher levels than the wild-type protein, as was observed for each of the corresponding single mutant proteins (Table 2). NA activities for both proteins were much lower than observed for the wild-type protein, but not as low as was observed for the single mutant L110A (Table 2). Surprisingly, the fusion activity of each mutant

was not decreased further than the activities of the single substitution mutants (Fig. 17).

Mutant proteins containing methionine substitutions. We next asked if we could further disrupt fusion promotion with the substitution of a bulky amino acid for leucine in the "a" position of each heptad repeat. Methionine residues have a longer side chain than leucine and could, therefore, more efficiently inhibit the HN protein heptad repeats from interacting with the F protein by stearic hindrance. Alternatively, the methionine residue may substitute for leucine and restore activity. The mutant proteins L74M and L96M were generated to explore these possibilities (Fig. 14).

Immunoprecipitated mutant proteins, analyzed under reducing conditions (Fig. 16A), showed that each of these mutants was as stable as the wild-type protein and, like the wild-type HN protein, was recognized by both NDV antisera and conformation-specific monoclonal antibodies (Fig. 16C). Disulfide-linked dimers were observed for each of the mutant proteins (Fig. 16B). Mutant HN proteins expressed at the cell surface were immunoprecipitated and analyzed by SDS-PAGE under reducing and non-reducing conditions (not shown). As observed for the "a" position alanine mutants, these proteins were expressed at the cell surface (Table 2) and formed disulfide-linked dimers.

Biological activities of methionine mutant proteins. The NA activity of L74M dropped from 122% (observed for L74A) to 53% (Table 2), and there was a

decrease in HA levels from above wild-type to slightly below wild-type (Table 2).

Little change in NA activity was observed for L96M (as compared to L96A),

however HA activity decreased from above wild-type to levels equal to wild-type.

Fusion promotion activity of L74M was increased slightly over that observed for

L74A (8% vs. 15%), but a decrease was observed for L96M (19% vs. 30% for

L96A) (Fig. 17). While this decrease was substantial, it was not lower than

levels observed for some of the other heptad repeat mutants.

Substitutions in other positions of the predicted helices. Coiled-coil

interactions are mediated by hydrophobic or neutral amino acids in the "a" and

"d" positions of a heptad repeat. To address whether alanine substitutions in

heptad repeat positions other than "a" influenced the fusion promotion activity

of the HN protein, the following mutants were generated. Leucine 97 ("b"

position) and isoleucine 102 ("g" position), which are positioned in the second

heptad repeat region of the stalk domain, were mutated resulting in L97A and

I102A, respectively. A residue between the two repeats, Leu 90, was also

mutated (L90A) (Fig. 14).

Biological activities of alanine mutant proteins in other positions of the

predicted helices. Alanine substitutions for hydrophobic residues in a "b"

position (L97A), in a "g" position (I102A) or between the heptad repeats (L90A)

generated proteins with wild-type epitopes, stability, and expression levels (Fig.

16). These mutations had little or no effect on the NA activities of the proteins

(Table 2). L90A and L97A had wild-type HA activities, while HA activity was

increased to a very high level for I102A (Table 2). Fusion promotion activity was decreased to 13% of wild-type for L90A, a level observed for the other substitutions in heptad repeat 1 (Fig. 17). L97A also showed a decrease in fusion (18% of wild-type) comparable to levels observed for other substitutions in heptad repeat 2. I102A had less of an effect on fusion promotion than any of the other mutants with fusion promotion at 60% of wild-type.

Discussion

Heptad repeat motifs are important for fusion activity and are found in the fusion proteins of a variety of viruses including retroviruses (envelope protein), coronaviruses (peplomer protein), paramyxoviruses (fusion protein) and influenza viruses (HA protein) (10). Heptad repeats in many of these proteins have been shown both by site-directed mutagenesis and peptide inhibition studies to be critical for fusion (7, 33, 51, 70, 71, 73). Indeed, for the NDV F protein it has been shown that mutations of heptad repeat 1 which is adjacent to the fusion peptide (58), as well as the transmembrane adjacent heptad repeat 2 (the leucine zipper) domains abrogate fusion (7, 52). Furthermore, peptides with sequences from two heptad repeats inhibit fusion (75, 77). The transmembrane-spanning region of the NDV HN protein also contains a heptad repeat of leucine residues which, when mutated, destabilized the tetrameric structure of the mature protein and altered the biological activities of the protein including fusion promotion (40).

A heptad repeat motif is that in which a hydrophobic amino acid is repeated every seven (heptad) residues, denoted as a, b, c, d, e, f, g (37). Heptad repeats which contain hydrophobic or neutral residues in the "a" and "d" positions of the repeat can form alpha-helices and are able to interact with other heptad repeats by forming coiled-coils (10, 37). Proteins which interact in

this matter are diverse and include cFos-cJun heterodimer, the catabolite gene activator protein (CAP) in *E. coli*, GCN4 in yeast and the influenza HA protein (37). Clearly, the coiled-coils are involved in protein-protein interactions important in many diverse systems.

Chimeric studies of paramyxovirus HN proteins have shown that the presumed stalk domains of various HN proteins confer F protein specificity in fusion promotion (17, 62, 67). One interpretation of these data is that the stalk domain of the HN protein is important for interactions with the F protein. Because of the importance of paramyxovirus F protein heptad repeat motifs, we wanted to investigate a region containing two heptad repeats in the presumed stalk domain of the NDV HN protein (amino acids 74-110). This region of the HN protein was analyzed for secondary structure using prediction software from the Baylor College of Medicine (Fig. 15). Two alpha-helical heptad repeat regions separated by a non-helical region of 7 amino acids were predicted. The structure of proteins with amino acid substitutions presented here were similarly analyzed (Fig. 18). Importantly, none of the substitutions resulted in a decrease of predicted alpha-helical structure. Furthermore, the 7 amino acid region between the two helices was predicted to gain helical structure in the mutants V88A, L96A, I103A and L96/L110A.

The paramyxoviruses SV41, MuV, SV5, hPIV3, avian parainfluenza 4 and SeV HN proteins were similarly analyzed for secondary structure and found to contain heptad repeat regions predicted to form alpha-helices (Fig. 15). All but

one of the viruses analyzed (SeV) were predicted to contain a non-helical space between the two helices. Disruptions of alpha-helical regions such as these are known as discontinuities and may introduce fixed bends, flexible regions or provide boundaries between coiled-coils (48). Discontinuities are quite common in coiled-coils and consist of several groups (37, 48). *Non-helical regions* do not have the structure of an alpha-helix (these are present in all but one of the paramyxoviruses analyzed), *skip residues* are the addition of extra amino acids in the heptad repeat (observed for NDV, SV41, SeV), and *stutters* occur when 3 residues are dropped from the heptad repeat (MuV and SV5 have one stutter, hPIV3 has two stutters in a row). Clearly such discontinuities potentially impart many different structures to alpha-helical regions of proteins. Conservation of heptad repeats, presumed alpha-helical regions, as well as discontinuities in the presumed stalk of paramyxovirus HN proteins suggest that these structural determinants may be important to the structure and function of the protein.

We generated four sets of mutants to begin to elucidate the mechanism by which the heptad repeat domain of the HN protein may contribute to fusion. The first set of mutants changed the hydrophobic "a" position residues of the first (more amino terminal) heptad repeat to the hydrophobic residue alanine generating L74A, V81A and V88A. Similarly, a second set of mutants L96A, I103A, and L110A were generated in the "a" position residues of the second heptad repeat. A double mutation containing an "a" position substitution in

each repeat (V81/L110A), as well as a double mutation with two changes in the second heptad repeat (L96/L110A) were generated as well. A third set of mutants introduced a methionine residue in place of leucine in heptad repeat "a" positions generating L74M and L96M. Because methionine is bulkier than leucine, these substitutions could potentially stearically prevent a possible HN and F protein interaction and thus more efficiently inhibit fusion. Alternatively, the methionine could substitute for leucine, restoring activity. A final set of mutations introduced alanines into heptad repeat positions other than the "a" position creating L97A and I102A, and into the 7 amino acid space between the two heptad repeats generating L90A.

All the mutant HN proteins appeared to fold correctly and to retain wild-type epitopes as determined by immunoprecipitation with polyclonal as well as conformationally sensitive monoclonal antisera against the HN protein. Furthermore, that the oligomeric structure was not disrupted was shown by the formation of disulfide-linked dimers. In addition, sucrose density gradients showed no shifts in sedimentation from the tetramer position to a monomer or dimer position which would indicate a loosely associated or absent tetramer (not shown).

All the mutant proteins were able to bind red blood cells. Indeed, most of these mutations resulted in mutant proteins with increased, in some cases substantially greater abilities, to bind red blood cells. These results argue that specific amino acids in the stalk domain of the HN protein are not critical for

attachment activity because the domain is highly tolerant of amino acid substitutions which appear for the most part to increase its activity.

Neuraminidase activities of the mutant proteins varied greatly with V81A having 157% of wild-type NA activity and L110A having 14% of wild-type NA activity. Approximately half of the mutant proteins had wild-type or slightly lower NA activities while the other half showed decreased NA activities. There was not an obvious pattern of preferred amino acids in specific heptad repeat positions for NA activity. The presence of mutants with less than wild-type activity, however, would argue that individual amino acids in this region appear to be important for NA activity. While these results suggest that the overall conformations of the mutant proteins may be abnormal, the presence of epitopes similar to the wild-type protein as well as wild-type levels of oligomerization suggest that any conformational alterations are subtle. Levels of NA activity do not correlate in any obvious way with the fusion activities of the mutants.

All the mutant proteins negatively affected fusion. Defects in fusion promotion were not due to defects in HA activity as all the mutant proteins were able to bind red blood cells. These results reinforce the previous conclusion that fusion promotion and attachment (as well as NA activity) can be genetically separated (56). Furthermore and most importantly, these mutant proteins (L74A, V81A, L96A, L97A, I102A, L96M and L90A) illustrate a requirement in the HN protein for specific amino acids in this region of the protein for fusion

promotion. Single, extremely conservative changes (L74A, V81A) virtually eliminated fusion promotion activity. Additionally, these mutations illustrate that the "a" positions of the helices were not more critical than other positions for fusion promotion. These results suggest that the presumed alpha helical structure of the heptad repeats is not sufficient for fusion, although one cannot rule out the possibility that a helical structure is necessary for fusion promotion as none of the mutants generated were predicted to lessen the probability of forming an alpha-helix.

As mentioned previously, paramyxovirus F proteins contain two heptad repeat regions which are conserved and have been shown to be critical for fusion. We propose that it is possible for the conserved heptad repeat region of the paramyxovirus HN protein to interact with the heptad repeats of the F protein, since the helical nature of these regions in both proteins presents the possibility of coiled-coil interactions between the proteins. The importance of specific residues for fusion promotion may indicate specific interactions between the proteins. One intriguing possibility is that the HN heptad repeats may bind to the heptad repeats HR1 and HR2 of the F protein, serving to keep these two regions apart. The discontinuity between the helices would give the HN protein the flexibility to participate in such an interaction. Upon binding of the HN protein to its receptor, a conformational change may occur in both proteins, disrupting the HN-F interaction and resulting in the release of the fusion peptide into the target membrane. Individual amino acids would create

a level of specificity which agrees with the observations that HN and F proteins from different paramyxoviruses do not complement each other to promote fusion.

TABLE 2. Biological activities of mutant proteins

HN	% of wild-type activity			
	Cell surface (mean +/- SD)	NA (mean +/- SD)	NA(CS)[a]	HA[b]
L74A	102+/-29	124+/-20	122	++
V81A	128+/-10	201+/-59	157	+++
V88A	81+/-13	21+/-6	26	+
L96A	77+/-20	70+/-13	91	++
I103A	59+/-26	10+/-3	17	++
L110A	76+/-21	11+/-7	14	++
V81/L110A	112+/-20	43+/-15	38	+++
L96/L110A	55+/-22	12+/-1	22	+++
L74M	102+/-7	54+/-0	53	+/-
L96M	72+/-21	69+/-7	96	+
L90A	67+/-8	59+/-5	88	+
L97A	79+/-14	79+/-7	100	+
I102A	111+/-19	136+/-20	123	+++

[a]NA(CS), NA value normalized to cell surface (CS) expression.

[b]+/-, slightly lower than wild-type level of binding; +, wild-type level of binding; ++, greater than wild-type level of binding; +++, much greater than wild-type level of binding.

Legend to figure 14: Linear map of mutations. Amino acid sequence for the wild-type HN protein showing residues 74-110. The heptad repeat "a" positions are shown in a larger font, heptad repeat regions A (HRA) and B (HRB) are denoted by a line below the sequence, and the seven-amino-acid (7 AA) sequence between the heptad repeats is denoted by a line below the sequence. Mutations of the wild-type protein are indicated with arrows.

SSP: NDV LGSNQDVVDRIYKQVALESPLALLNTESIIMNAITSL
++++++++++++++++ +++++++++++++++

SV41 IKSMIALLNQILYNAAIALPLKIDSTESVLLAAIKDL
++++++++++++++ +++++++++++++

MuV AAVIASAVGVMNQVIHGVTVSLPLQIEGNQNQLLSTLATICTNRNQV
+++++++++ +++++++++++++++++

SV5 ITDLLNNILSVANQIIYNSAVALPLQLDTLESTLLTAIKSL
++++++++++ ++++++++++++++++++

hPIV3 INNEFMEITEKIQMASDNTNDLIQSGVNTRLLTIQSHV
+++++++++++++++++ +++++++ +++++++++++

avian LDTRNAALLHIPLQLNTLEANL
para 4 +++++++++++ ++++++++++++

SeV LNMSSREVKESLTSLIRQEVIARAVNIQSSVQTGIPVL
++++++++++++++++++++

NNSSP: NDV LGSNQDVVDRIYKQVALESPLALLNTESIIMNAITSL
+++++++++++++++++ ++++++++++++

SV41 IKSMIALLNQILYNAAIALPLKIDSTESVLLAAIKDL
++++++++++++++++++++++++++ +++++++++++++

MuV AAVIASAVGVMNQVIHGVTVSLPLQIEGNQNQLLSTLATICTNRNQV
+++++++++ +++++++++++++++

SV5 ITDLLNNILSVANQIIYNSAVALPLQLDTLESTLLTAIKSL
+++++++++++++++++++ ++++++++++++++++++

hPIV3 INNEFMEITEKIQMASDNTNDLIQSGVNTRLLTIQSHV
++++++++++++++++++ +++++ +++++

avian LDTRNAALLHIPLQLNTLEANL
para 4 ++++++ ++++++++

SeV LNMSSREVKESLTSLIRQEVIARAVNIQSSVQTGIPVL
+++ +++++++++++++++++++++++++++++

Legend to figure 15: Secondary structure prediction of paramyxoviruses and rubulaviruses. Secondary structure prediction of NDV amino acids 74-110 using Baylor College of Medicine SSP (segment-oriented prediction) and NNSSP (nearest-neighbor prediction) programs (1). The corresponding regions of other rubulaviruses and paramyxoviruses were similarly analyzed. + denotes areas predicted to be alpha-helical; a line above the sequence denotes a space between the two heptad repeats. Para, parainfluenza virus.
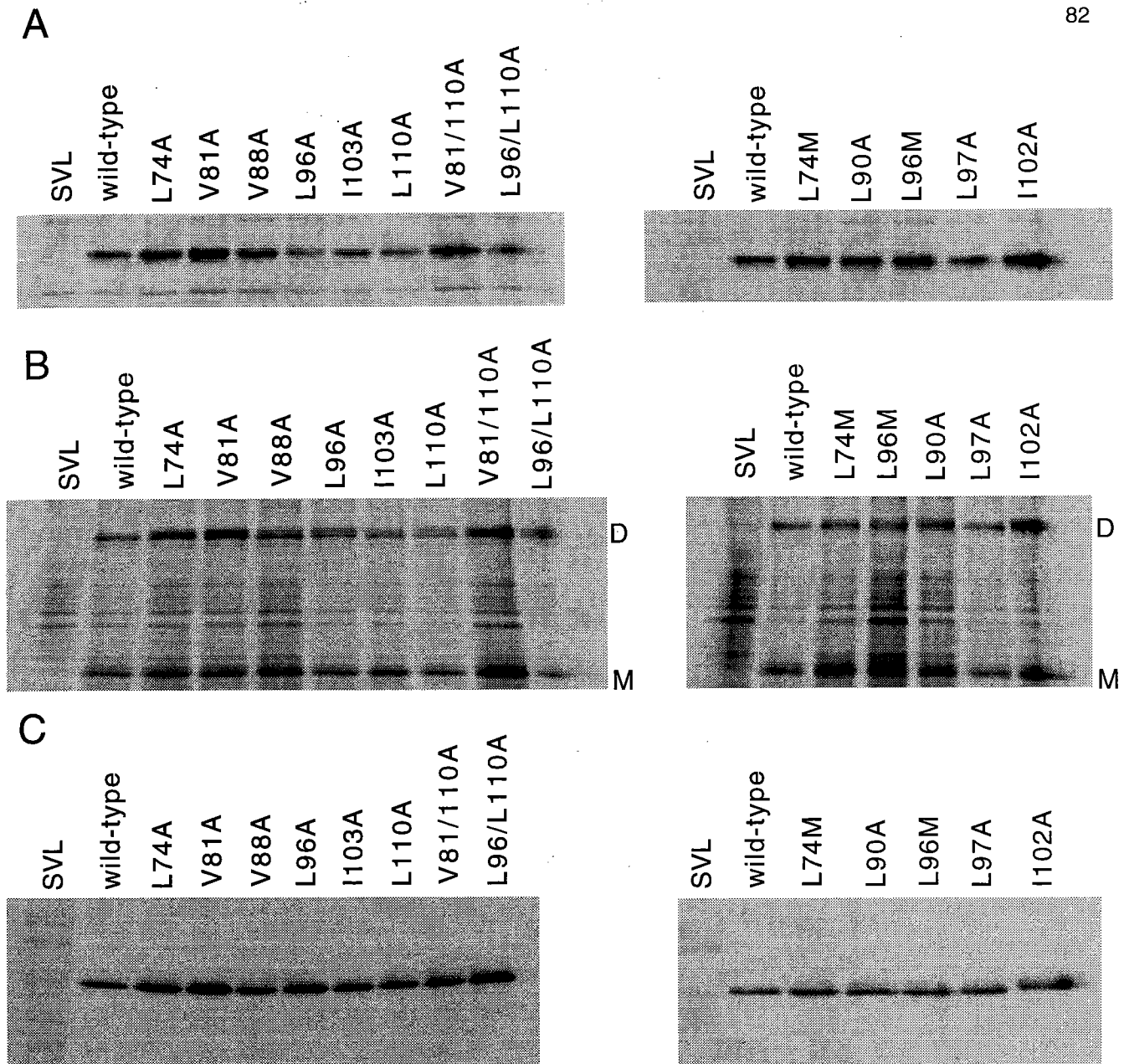
Legend to figure 16: Expression of mutant HN proteins. At 48 h posttransfection, cells transfected with wild-type or mutant cDNAs were radiolabeled for 2 h and chased for 2 h in non-radioactive media. Cells were lysed, post-nuclear supernatants were immunoprecipitated with polyclonal antisera and analyzed by SDS-PAGE on 8% gels in the presence (A) or absence (B) of reducing agent. (C) Proteins in post-nuclear supernatants were immunoprecipitated with the conformation-specific monoclonal antibody anti-2b, which is representative of results obtained with four other monoclonal antibodies, as described in Materials and Methods. SVL, vector; wild-type, wild-type HN protein; D, disulfide-linked HN protein dimer; M, monomeric HN protein. A Molecular Dynamics densitometer was utilized to image the auto-radiograph and this figure was generated from Adobe photoshop without enhance-ment. The images presented accurately represent the origional autoradiographs.
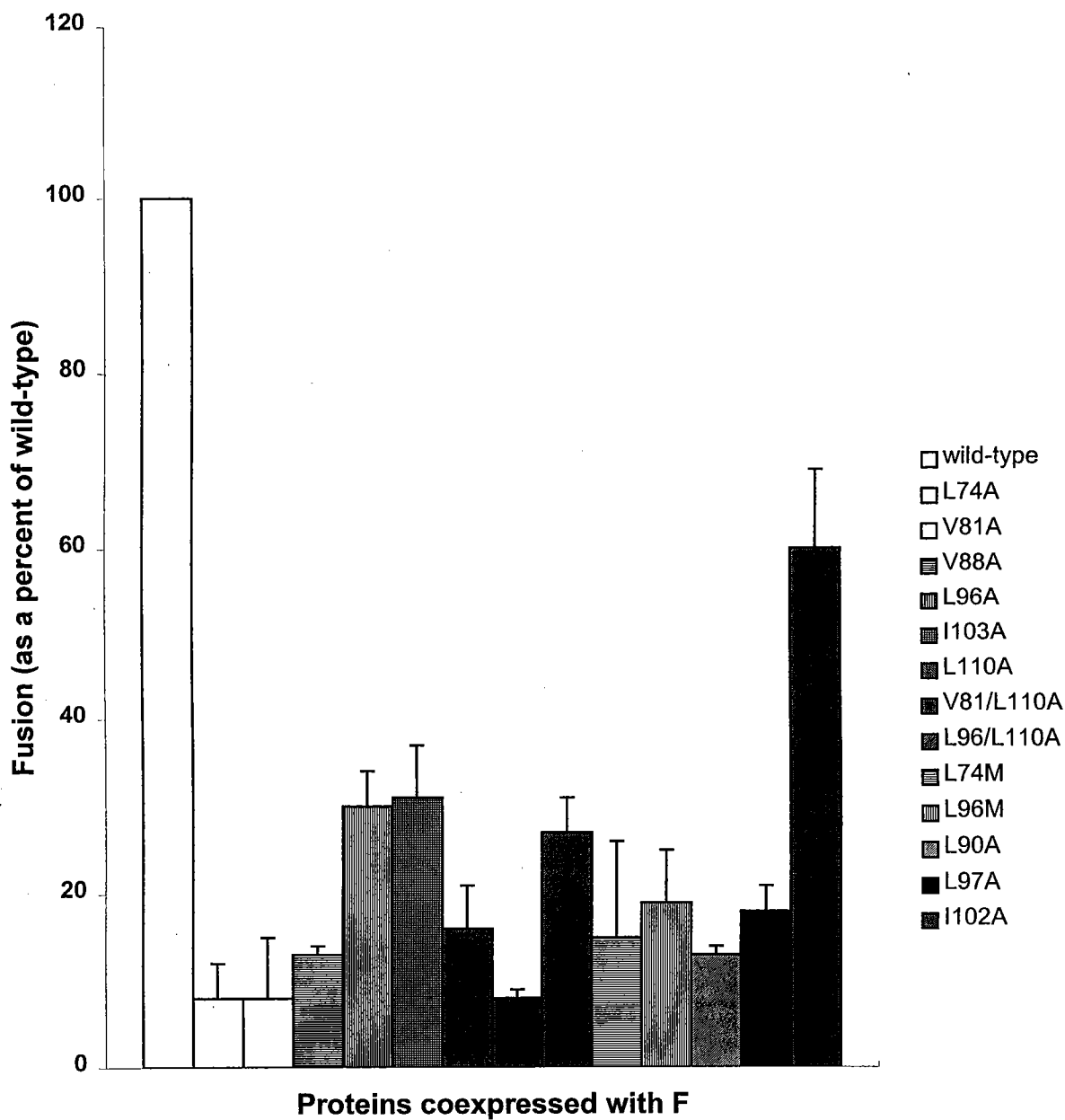
Legend to figure 17: Syncytium formation promoted by mutant HN proteins. At 48 hr post-transfection, twenty of the largest syncytia were counted. The background fusion from cells expressing vector alone was subtracted and values were normalized to cell surface expression.

NDV   LGSNQDVVDRIYKQVALESPLALLNTESIIMNAITSL
               ++++++++++++++++   +++++++++++++

L74A   AGSNQDVVDRIYKQVALESPLALLNTESIIMNAITSL
               ++++++++++++++++   +++++++++++++

V81A   LGSNQDVADRIYKQVALESPLALLNTESIIMNAITSL
               ++++++++++++++++   +++++++++++++

V88A   LGSNQDVVDRIYKQAALESPLALLNTESIIMNAITSL
                ++++++++++++++++ ++++++ +++++++++++++

L96A   LGSNQDVVDRIYKQVALESPLAALNTESIIMNAITSL
                ++++++++++++++++ ++++   +++++++++++++

I103A   LGSNQDVVDRIYKQVALESPLALLNTESIAMNAITSL
                ++++++++++++++++ ++++++++++++++++++++++

L110A   LGSNQDVVDRIYKQVALESPLALLNTESIIMNAITSA
               ++++++++++++++++   +++++++++++++

V81/L110A   LGSNQDVADRIYKQVALESPLALLNTESIIMNAITSA
               ++++++++++++++++   +++++++++++++

L96/L110A   LGSNQDVVDRIYKQVALESPLAALNTESIIMNAITSA
               ++++++++++++++++   ++++++ +++++++++++++

L74M   MGSNQDVVDRIYKQVALESPLALLNTESIIMNAITSL
               ++++++++++++++++   +++++++++++++

L96M   LGSNQDVVDRIYKQVALESPLALLNTESIIMNAITSL
               ++++++++++++++++   +++++++++++++

L90A   LGSNQDVVDRIYKQVALESPLAMLNTESIIMNAITSL
               ++++++++++++++++   +++++++++++++

L97A   LGSNQDVVDRIYKQVALESPLALANTESIIMNAITSL
               ++++++++++++++++   +++++++++++++

I102A   LGSNQDVVDRIYKQVALESPLALLNTESAIMNAITSL
               ++++++++++++++++   +++++++++++++

Legend to figure 18: Secondary structure prediction of mutant HN proteins. Secondary structure prediction (NNSSP program) of amino acids 74-110 for the wild-type HN protein and mutant HN proteins. "+" denotes areas predicted to have alpha-helical structure.

CHAPTER 4

Discussion

It has been known for many years that, for NDV, there is a strict requirement for HN and F proteins to be expressed in the same cell in order for syncytia formation to occur (30). The attachment protein requirement is observed for a majority of the members of the paramyxovirus family (30). The mechanism of cooperativity between the surface glycoproteins which leads to membrane fusion is unknown and has long been a controversial topic. When I began my thesis research, I set out to elucidate the mechanisms by which viral-mediated membrane fusion ensues. Initial experiments to detect coimmunoprecipitation of HN and F proteins were carried out in transfected cells. It was hoped that mutant proteins could be identified that were negative in fusion and concomitantly negative for coimmunoprecipitation as well. I thus hoped to map regions of both proteins that were responsible for the protein-protein interactions necessary for membrane fusion. Coimmunoprecipitation proved to be elusive in a transfected cell system, so I utilized an infected cell system. Infected cells enabled me to characterize where in the cell an HN-F protein interaction may occur as well as which populations of proteins may be involved and sizes of potential complexes that may be formed. Having

identified an interaction between HN and F proteins during an infection, and having failed at any attempts to coimmunoprecipitate the two proteins in transfected cells, I next looked for regions in the HN protein that may be critical for fusion promotion. Two heptad repeats were identified carboxy terminal to the transmembrane domain. These heptadic residues were mutated singly and doubly and were indeed found to be important for fusion promotion.

Infected cells. Whether the HN and F proteins of paramyxoviruses interact has long been a controversial subject in the field. Utilizing NDV infected Cos cells enabled us to detect a potential interaction between the HN and F glycoproteins. Not only was the HN protein found to be associated with the F_1 protein, it was determined to be associated with the F_0 protein as well. These results are different from two reports which investigated crosslinking of MV and SV5 HA/HN and F proteins. Malvoisin and Wild reported that when they crosslinked MV proteins expressed from a vaccinia virus vector driven system, they were able to coimmunoprecipitate the HA protein with the F_1 protein but not with the F_0 protein (38). When NDV infected cells that had been treated with crosslinker were lysed under the conditions that Malvoisin and Wild used, we found that only the HN and F_1 proteins could be coimmunoprecipitated. It follows that the likely reason for not detecting a MV HA-F_0 interaction was due to the insolubility of this crosslinked species in the lysis buffer used. In another report Russell at al. did not detect HN-F protein complexes in SV5 infected cells that were treated with crosslinker (53). In this study, infected cells were

removed from a monolayer and proteins were crosslinked when the cells were in suspension, while our infected cells were exposed to crosslinker as an intact monolayer. Removal of cells from a monolayer may result in rearrangement of the viral proteins and could explain why our results differ from those of Russell et al.

When crosslinked complexes obtained from NDV infected cells were immunoprecipitated and analyzed by SDS-PAGE, complexes of various sizes were observed. When immunoprecipitated with either HN or F specific antisera, three discrete bands were detected on SDS-PAGE gels between 160-300 kDa as well as larger, heterogeneous species. Given the sizes of the HN and F proteins (F_0~66 kDa, F_1~ 64 kDa, HN~74 kDa), the three distinct species observed are consistent with complexes containing 1-4 F proteins and 1-4 HN proteins. Each distinct species was shown to contain HN, F_0 and F_1 proteins when analyzed by SDS-PAGE in the second dimension whether immunoprecipitated with HN or F specific antisera. F-F protein complexes may be present as well as these have been detected in transfected cells treated with crosslinker (unpublished results). Three distinct bands migrating between 160-300 kDa may represent the minimal subunits of a larger structure that is not completely stabilized by crosslinking and is partially disrupted upon lysis. Such a structure may be too large to be resolved by SDS-PAGE as there was immunoprecipitated material that remained in the loading wells and did not enter the gel in the presence of crosslinker. Gradient studies would be

useful in determining the sizes of very large potential HN-F protein complexes. The formation of large complexes has been observed when synthetic peptides of the NDV F protein HR1 and HR2 regions were mixed together. The sizes of such complexes were determined to be 30mers in solution (unpublished data), which supports the notion that very large structures may be present in the fusion pore.

Alternatively, the association observed between the HN and F proteins in infected cells could be due to associations present during the budding process and not due to viral-mediated membrane fusion. This possibility appears unlikely for several reasons. The M protein, which is thought to be a driving force in budding, has been shown to interact with the HN and F proteins (31). No association of the M protein in the HN-F protein complexes was ever detected. One explanation for the lack of a detectable association among the three proteins is that the time point examined was early in infection; budding is a process which occurs late in infection and thus should not be observed. Indeed, the HN-F protein interaction was observed as early as 4.25 h post-infection arguing that the interaction observed was not involved in the budding process.

Another explanation for the HN-F protein association could be an artifactual association observed due to the high levels of viral protein expression during an infection. This possibility is unlikely, however, because

associations were observed early in infection when the levels of viral proteins were low.

The interaction between the HN and F proteins occurred independent of F_0 cleavage. This observation supports the notion that the HN and F proteins may interact intracellularly and may interact prior to transport to the trans Golgi where F_0 is processed into F_1 and F_2. Furthermore, the interaction was detected as early as a 5 min pulse. This result is consistent with a protein-protein association occurring in the rough endoplasmic reticulum. An intracellular interaction between HN and F proteins is supported by the results of Tanaka et al. who reported that an ER retention signal on hPIV3 F protein resulted in the retention of the hPIV3 protein in cells coexpressing both proteins, implying an intracellular interaction (63). Tong and Compans also reported that an hPIV2 F protein with an ER retention signal could retain the hPIV2 HN protein within the cell, also supporting that notion that an interaction between the HN and F proteins occurs in the ER (65). Paterson et al., however, found that when SV5 or hPIV3 glycoproteins with ER retention signals were expressed in cells, the ER tagged attachment protein did not cause retention of the F protein and ER tagged F protein did not promote the retention of the attachment protein (49). This study concluded that any potential HN-F protein interactions do not occur intracellularly and they may occur instead at the cell surface. One explanation for the conflicting results of Paterson et al. is the construction of the F proteins. In the Tong and Compans and the Tanaka et al.

reports, the F protein with the ER retention signal lacks a cytoplasmic tail as well as a transmembrane domain while the HN and F proteins described in the Peterson et al. report contained an ER retention signal on the cytoplasmic tails. The cytoplasmic tails and/or transmembrane domains may play a role in HN-F interactions. Modifications of these domains may change the conformation of the proteins and therefore possibly alter any potential HN-F protein interactions. Indeed, mutations made in the transmembrane region of the NDV HN protein and the cytoplasmic tail of NDV and SV5 F proteins have a negative effect on fusion promotion (2, 40, 57).

The inability to detect a homotypic protein-protein interaction in cells expressing SV5 or hPIV3 HN and F proteins (49) differs from the observation of HN-F protein interactions in NDV infected cells (reported in Chapter 2). Lack of an observable retention of HN and F proteins by F and HN proteins containing an ER retention signal does not rule out the interaction of a small population of HN and F proteins in transfected cells which serve to promote fusion. Alternatively, the interaction may be weak and pulled apart when one of the proteins is forced to remain in the ER.

The HN-F protein interaction observed in NDV infected cells was observed to the same extent in the presence or absence of HN binding to its receptor (Chapter 2). This data conflicts with a recent report by Deng et al. where an HN mutant protein which was both attachment and fusion deficient was unable to coprecipitate the homologous F protein (16). Based on the

properties of this mutant protein the authors concluded that the first step of fusion is mediated by the HN protein binding to its receptor which then triggers an interaction between the HN and F proteins leading to fusion. It was reported that the mutant HN protein was recognized by a panel of conformation-sensitive monoclonal antisera although the data was not shown. Aberrant migration of the protein was observed on SDS-PAGE gels, however, suggesting altered glycosylation or an aberrant conformation. A slightly misfolded HN protein may not interact with the F protein due to an altered conformation and not necessarily due an inability to bind the receptor.

Transfected cells. After detecting an HN-F protein interaction in infected cells, I sought evidence of a similar interaction in transfected cells, a system in which mutant proteins could be characterized. Proteins deficient in fusion could be assayed to determine which regions of both proteins functionally interact to promote fusion. Proteins deficient in attachment (HN) or cleavage (F) could determine if such activities are necessary for an interaction to occur in transfected cells. Further, proteins deficient in transport to the plasma membrane as well as proteins containing retention signals could be utilized to explore how early in intracellular transport the proteins interact.

Preliminary investigations of potential interactions between the two proteins in transfected cells focused upon the wild type HN and F proteins. No interactions were detected by coimmunoprecipitation or after chemical crosslinking utilizing various crosslinking compounds. The inability to detect

an interaction may have been due to differences in infected cells relative to transfected cells. One major difference between transfected and infected cells is the different levels of expression of viral proteins. In transfected cells the level of protein expression is low and, if only a small population of proteins is involved in the fusion event, perhaps detecting complexes is beyond our level of sensitivity. In order to increase the number of HN and F proteins involved in the fusion event at the cell surface, attempts were made to synchronize fusion. This was accomplished by various methods of blocking fusion and then removing the block so that fusion could occur in a synchronous fashion among all fusion-competent proteins. One method of blocking fusion was to incubate cells in neuraminidase to cleave receptors followed by the addition of an overlay of cells containing receptors to enable many proteins to bind and potentially fuse at the same time. Another approach was to express an F protein which was not cleaved intracellularly and cleave it at the cell surface in order to allow all the F proteins to become fusogenic and engage in fusion at the same time. Evidence of an interaction was not obtained using either of these methods. It is possible that the HN-F protein interaction occurs intracellularly as was observed in infected cells, so that synchronization at the cell surface did not increase the numbers of proteins fusing to levels of detection by our system. Alternatively, a potential interaction at the plasma membrane of transfected cells may be transient and thus difficult to detect.

In order to address the question of a transient interaction, cotransfection experiments were performed using various mutant HN and F proteins. It was hoped that the altered fusion phenotypes of some mutant proteins would be due to a lack of dissociation of the complex at some step during the fusion process, thus enabling detection of a normally transient interaction. A non-fusion promoting HN protein as well as an HN protein which fused at levels much higher than wild type were investigated. Fusion negative F proteins, which contained mutations in HR1, HR2, the cytoplasmic tail, and the fusion peptide were also utilized. Attempts to detect an interaction between mutant and wild type as well as mutant and mutant proteins proved to be unsuccessful. Perhaps none of the mutant proteins remained associated with its heterologous partner.

Infected cells contain other viral proteins and thus differ from transfected cells. In order to investigate whether the presence of other viral proteins would lead to the detection of an HN-F interaction, we cotransfected F, HN, NP and M cDNAs, but were unable to detect an interaction between the glycoproteins under these conditions. Perhaps the P and L proteins or the presence of a nucleocapsid structure have an effect on the HN-F protein interaction. The generation of an infectious clone would be interesting in this regard.

Another difference between transfected and infected cells is the inhibition of host cell protein synthesis during an infection. We attempted to imitate this effect by adding cycloheximide to cells to inhibit protein synthesis

after the expressed HN and F proteins were pulse-labeled. Once again an interaction was not detected.

It is quite possible that there are other differences between infected and transfected cells that are unknown and have, therefore, not been taken into account. In this regard it is interesting to note that the only reports of HN-F protein interactions in transfected cells were in the context of a vaccinia virus driven system (described in Chapter 1).

Heptad repeat mutants. The heptad repeat region of the HN protein was found, like HR1 and HR2 of the F protein, to be critical for fusion promotion. Single point mutations were generated which had wild type or greater levels of HA and NA activities, but were diminished in the ability to promote fusion. Heptad repeats have been shown to bind to one another, forming coiled-coils and to mediate protein-protein interactions. One intriguing model based upon the phenotypes of these mutant proteins is that the HN HRA and HRB regions interact via coiled-coils with the HR1 and HR2 regions of the F protein. Support for a potential interaction between these regions of the NDV HN protein and the F protein comes from recent work by Deng et al (16). This group coprecipitated NDV HN chimeras containing regions from hPIV3 (the same region of the stalk where the point mutations in Chapter 3 were generated) with the NDV F protein. A correlation between the inability to fuse and the inability to coimmunoprecipitate both proteins was observed for three of the mutant proteins investigated. One mutant protein was coimmunoprecipitated at low

levels although the mutant was capable of promoting fusion at lower levels than observed for the wild-type protein. One interpretation of this data is that the HN-F protein interaction was dissociated during the immunoprecipitation due to a weaker interaction than was observed for the wild-type protein. Chemical crosslinking of the mutant HN and F proteins could address whether this is the case.

Viral fusion in other systems. The best characterized viral fusion system is influenza virus (31). X-ray crystallography studies of both the pre-fusogenic as well as the fusogenic HA protein have been analyzed (8, 9, 72). It was discovered that during activation the HA protein undergoes major conformational changes mediated by the formation of a large, coiled-coil which relocates the fusion peptide 70 A to promote its interaction with the target membrane. Recent structural work has shown that the N- and C-terminal residues of the flu HA protein are in very close proximity which would enable a close approach between the target and attack membranes (12).

Structural studies have shown that like influenza, HIV-1 also undergoes major conformational changes during membrane fusion which involve the formation of coiled-coils (11, 29). Common features observed in the HIV and influenza viral fusion systems suggest that there are many similarities in how viruses mediate membrane fusion. These viruses may therefore serve as model systems to aid in elucidating the mechanisms of NDV-mediated membrane fusion.

The role of the F protein in viral fusion. As stated earlier, paramyxovirus F proteins contain two heptad repeat regions (HR1 and HR2) which are critical for viral fusion. Mutations of either region diminish the protein's ability to fuse (7, 52, 58). Furthermore, a synthetic peptide corresponding to the HR1 region of NDV was able to inhibit fusion in cells coexpressing the HN and F proteins (75). This inhibition of fusion was found to be virus specific as the peptide did not inhibit fusion in cells coexpressing SeV HN and F proteins. Peptides corresponding to the NDV HR2 region were also found to inhibit fusion (33, 76, 77). Inhibition by synthetic peptides with sequences from heptad repeat domains has been observed for many other viruses (including paramyxoviruses) as well (31, 51, 70, 71, 73). Peptides corresponding to HR1 and HR2 have also been shown to form complexes with one another in solution, and, when the two peptides were mixed prior to addition to HN and F expressing cells, there was minimal inhibition of fusion, suggesting a physiologically relevant interaction (26, 75). Studies with SV5 have further shown that the complexes between HR1 and HR2 form a structure with helices arranged in an anti-parallel orientation which is very stable as determined by protease digestion and thermostability assays (3, 26). FPLC studies have also shown the formation of NDV HR1-HR2 peptide complexes, although unlike the SV5 report where a structure consistent with a trimer of heterodimers was detected, the formation of very large structures was observed (in preparation). Similar results were obtained with SeV synthetic peptides (18). The stability of

the SV5 HR1-HR2 complex implies that this interaction would only be observed during or after the fusion event, as a large amount of energy would be required to release the peptide into the target membrane if this stable interaction occurred prior to fusion. Indeed, this agrees with results from other viral systems such as influenza and HIV where fusion is thought to cause an irreversible conformational change in the fusion protein. Recent studies with NDV HR1 and HR2 peptides have determined that these peptides can self-associate forming homotrimers (in preparation). If one assumes that the thermostable heterooligomers observed in the SV5 system are an end-product of fusion, then perhaps the homotrimers represent the structure of the F protein prior to fusion. Influenza studies support this hypothesis in that the more stable alpha-helical form of HA2 is thought to be the post-fusion form while the less stable form where two alpha-helices are disrupted by a bend is thought to be the pre-fusion form of the protein.

A fusion model for NDV. Drawing from what is known about fusion from the influenza virus and HIV1 systems as well as paramyxovirus studies, certain assumptions about NDV may be made. First, the F protein most likely exists in two forms: a metastable pre-fusion form, and a stable, irreversible post-fusion form. The peptide studies from paramyxoviruses mentioned above certainly support the idea of the interaction of regions of the F protein in two separate forms: F proteins containing interacting HR1-HR1 and HR2-HR2 regions, as well as F proteins containing interacting HR1-HR2 regions. Second, a trigger

for the release of the fusion protein into the target membrane is necessary. During the pH-independent fusion process observed in HIV infections, the attachment protein is thought to provide this trigger. For NDV, the HN protein likely serves this function. Another assumption that may be made is that heptad repeat regions which form coiled-coils are critical for fusion promotion. Indeed, these regions have been shown to be critical for viral fusion in many viral systems including paramyxoviruses.

The following model is one possible mechanism by which the HN and F proteins of NDV may cooperate in order to promote membrane fusion. As stated earlier, evidence exists for the association of HR1 and HR2 peptides forming both homologous and heterologous oligomers. If indeed the stable heterologous form represents the post-fusion conformation of the protein, then these regions of the protein should be kept apart prior to the fusion event. The HN protein may serve this role by interacting with the F protein either during or shortly after synthesis. Evidence for an early interaction in NDV-infected cells was reported in Chapter 2. A protein-protein interaction may be mediated via the formation of coiled-coils between the membrane proximal HRA of the HN protein and HR2 of the F protein and between the membrane distal HRB and HR1 regions (Fig. 19). The potential non-helical structure predicted to exist between HRA and HRB would impart flexibility in the HN protein and possibly enable it to bend, forming a structure by which the interacting HRB and HR1 regions point back down toward the membrane. Upon binding of the HN

protein to its receptor, a conformational change may occur causing a rearrangement of the HN protein at the bend between the helices and thus a rearrangement of the associated F protein. This could potentially enable the F protein to relocate the HR1 region toward the target membrane in an event somewhat analogous to that observed for influenza virus and HIV. This rearrangement would result in the insertion of the fusion peptide into the attack membrane and potentially lead to a close approach of the attack and target membranes (Fig. 20A). Studies with synthetic peptides from NDV have shown that the HR1 peptide can self associate into very large complexes while the HR2 peptide self associates as trimers (unpublished data, Matthew Abramowitz and Trudy Morrison, Morrison laboratory). After insertion of the fusion peptides into the target membrane, the adjacent HR1 region may self associate into a large structure while the HR2 region associates as a trimer, placing a strain on the membranes. Such a strain may further the dimpling that was initiated by the insertion of the fusion peptide into the target cell, and lead to hemifusion. The final stages of fusion may involve the association of HR1 and HR2 in the same membrane (Fig. 20B). Whether the HN protein is involved in these latter steps of the fusion process is unknown, although infected cell studies have shown that the HN and F proteins could be coimmunoprecipitated immediately after fusion was stimulated (Chapter 2). It does appear likely, however, that the HN protein would dissociate from HR1 and HR2 in order for the association of these regions to occur. The HN protein

may still associate with the F protein, however, via other regions in either or both proteins.

Many steps of this model could be examined experimentally and would serve to further define the roles of each protein in the fusion event. The hypothesis that the HN and F proteins interact in order to prevent an HR1-HR2 association could be investigated by peptide studies. Studies where HR1 and HRB or HR2 and HRA peptides are mixed and then assayed for binding to HR2 and HR1, respectively, would show whether the HN peptides do indeed prevent HR1-HR2 associations. Analyzing peptide associations in solution would enable one to evaluate if there is the potential for the formation of HR1-HRB and HR2-HRA heterooligomers and further, whether large complexes, which could be the protein components of potential fusion pores, are formed. Finally, such studies could be utilized to analyze peptides containing point mutations, particularly the HRA and HRB mutant proteins characterized in Chapter 3. This approach would enable the elucidation of which residues are critical for HN-F protein associations as well as allow the investigation of both proteins for other regions which potentially interact with the heterologous protein.

A mutational analysis of the putative non-helical region between HRA and HRB whereby mutant proteins are generated that would cause a predicted increase in alpha-helical character of the region could be analyzed for the ability to promote fusion. If a bend between the helices is important for fusion promotion, one would expect such mutant proteins to be deficient in fusion

promotion. The importance of a highly conserved proline residue which is located in this region could also be assayed for the ability to promote fusion as it may be responsible for ensuring that the potential bend in the HN protein is present at a specific angle necessary in the pre-fusion conformation of the proteins.

Conformational changes in both proteins either before and after the initiation of fusion would answer questions about the mechanism of fusion. Cells expressing both proteins could be incubated in the presence of neuraminidase in order to prevent attachment and subsequent fusion. The neuraminidase could then be removed and the addition of an overlay of cells containing receptor would then be used to promote maximal attachment and fusion. Conformational changes could be monitored in a time course assay by analysis with conformationally sensitive monoclonal antisera and by Triton X114 binding.
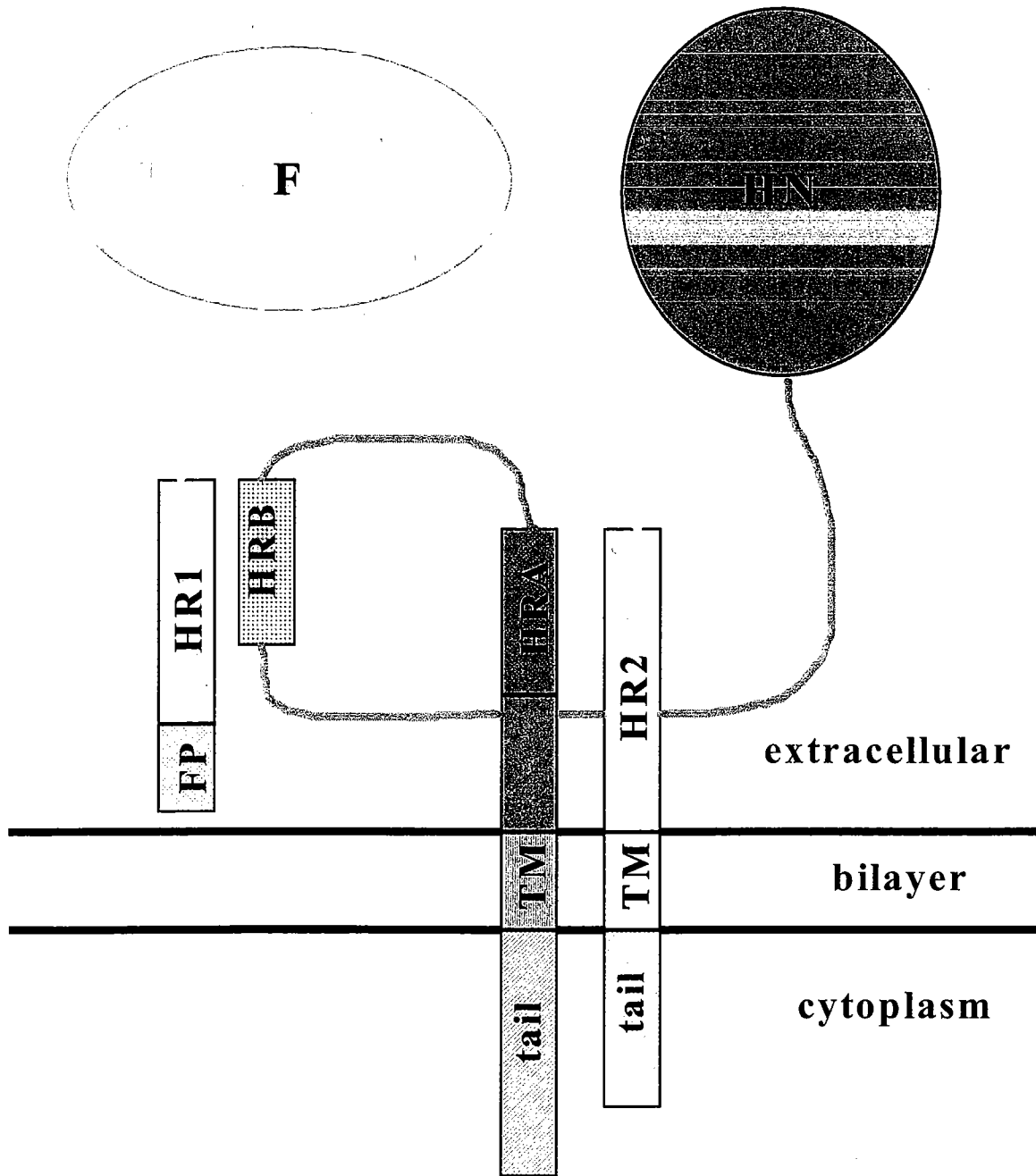
Legend to Figure 19. Potential interactions between the HR1 and HRB regions and the HR2 and HRA regions of the NDV F and HN proteins, respectively. FP, fusion peptide; TM, transmembrane domain; tail, cytoplasmic tail domain; HR, heptad repeat domain.

A

target cell

FP

HR1

HR2

HRB

HRA

attack cell

B

HR 2

HR1   HRA

FP

fused cell
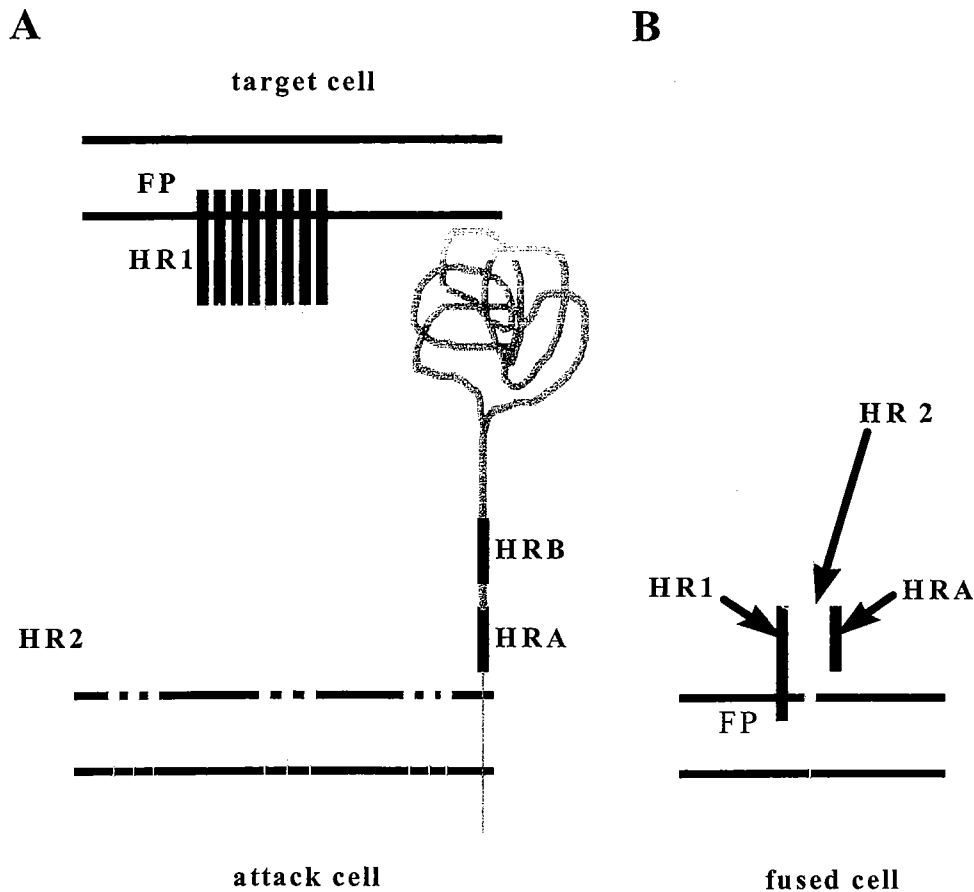
Legend to Figure 20. A fusion model. This is one possible model for NDV-mediated membrane fusion. A conformational change in the HN protein triggered by receptor binding in turn triggers a conformational change in the F protein whereby HRB and HR1 dissociate, leading to the release of the fusion peptide into the target membrane (A). HRA may or may not remain associated with HR2. The HR1 peptides associate in large structures while the HR2 peptides associate as trimers. Because the HR2 peptides don't associate as closely as the HR1 peptides, there is a strain on the membrane of the attack cell causing it to dimple and allowing close approach of the target and attack membranes. (B) Once lipid mixing occurs, the HR1 and HR2 regions of the F protein may associate as a trimer of heterodimers. HRA from the HN protein may or may not be a part of this complex. FP, fusion peptide; HR, heptad repeat domain.

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