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Requirements for Assembly and Release of Newcastle Disease Virus-Like Particles: A Dissertation

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REQUIREMENTS FOR ASSEMBLY AND RELEASE OF NEWCASTLE DISEASE VIRUS-LIKE PARTICLES

A Dissertation Presented

By

Homer Dadios Pantua

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

October 27, 2006

Program in Immunology and Virology
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Parts of this dissertation have appeared in separate publications:


REQUIREMENTS FOR ASSEMBLY AND RELEASE OF NEWCASTLE DISEASE VIRUS-LIKE PARTICLES

A Dissertation Presented

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October 27, 2006
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There were a lot of people who have been instrumental in making this work successful. I would like to thank my mentor, Dr. Trudy Morrison for the guidance, and invaluable support and encouragement during my entire thesis work. I also thank her for allowing me to freely drive my research project. There can be no mentor as excellent as her. I am also thankful to my thesis research committee members, Dr. Timothy Kowalik, Dr. Raymond Welsh, Dr. Elizabeth Luna for their excellent direction and guidance through the conduct of this thesis work. I would also like to thank Dr. Mark Peeples and Dr. Paul Clapham for their helpful suggestions during my defense.

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I thank the ALMIGHTY for blessing me with all these wonderful people in my life, for the strength to overcome the trials and for all the abilities that He blessed me.
ABSTRACT

The final step of paramyxovirus infection requires the assembly of viral structural components at the plasma membrane of infected cells followed by budding of virions. While the matrix (M) protein of some paramyxoviruses has been suggested to play a central role in the assembly and release of virus particles, the specific viral and host protein requirements are still unclear. Using Newcastle disease virus (NDV) as a prototype paramyxovirus, we explored the role of each of the NDV structural proteins in virion assembly and release. For these studies, we established a virus-like particle (VLP) system for NDV. The key viral proteins required for particle formation and the specific viral protein-protein interactions required for assembly and release of particles were explored in chapter 2. First we found that co-expression of all four proteins resulted in the release of VLPs with densities and efficiencies of release (1.18 to 1.16 g/cm$^3$ and 83.8%±1.1, respectively) similar to that of authentic virions. Expression of M protein alone, but not NP, F-K115Q or HN proteins individually, resulted in efficient VLP release. No combination of proteins in the absence of M protein resulted in particle release. Expression of any combination of proteins that included M protein yielded VLPs, although with different densities and efficiencies of release.

To address the roles of NP, F and HN proteins in VLP assembly, the interactions of proteins in VLPs formed with different combinations of viral proteins were characterized by co-immunoprecipitation. The co-localization of M protein with cell surface F and HN proteins in cells expressing all combinations of viral proteins was characterized. Taken together, the results show that M protein is necessary and
sufficient for NDV budding. Furthermore, they suggest that M protein – HN protein and M protein - NP interactions are responsible for incorporation of HN protein and NP proteins into VLPs and that F protein is incorporated indirectly due to interactions with NP and HN protein.

Since the vacuolar protein sorting (VPS) system is involved in the release of several enveloped RNA viruses, chapter 3 describes studies which explored the role of the VPS system on NDV particle release. First, we characterized the effects of three dominant negative mutant proteins of the VPS pathway on particle release. Expression of dominant negative mutants of CHMP3, Vps4 and AIP1 proteins inhibited M protein particle release as well as release of complete VLPs. Mutation of a YANL sequence in the NDV M protein to AANA inhibited particle release while replacement of this sequence with either of the classical late domain motifs, PTAP or YPDL, completely restored particle release. The host protein AIP1, which binds YXXL late domain sequences, is incorporated into M protein particles. These results suggest that an intact VPS pathway is necessary for NDV VLP release and that the YANL sequence is an NDV M protein L domain.

The sequence and structure of the Newcastle disease virus (NDV) fusion (F) protein are consistent with its classification as a type 1 glycoprotein. We have previously reported, however, that F protein can be detected in at least two topological forms with respect to membranes in both a cell-free protein synthesizing system containing membranes as well as infected COS-7 cells (J. Virol. 2004 77:1951). One form is the classical type 1 glycoprotein while the other is a polytopic form in which approximately
200 amino acids of the amino terminal end as well as the cytoplasmic domain (CT) are translocated across membranes. Furthermore, we detected CT sequences on surfaces of F protein expressing cells and antibodies specific for these sequences inhibited red blood cell fusion to HN and F protein expressing cells suggesting a role for surface expressed CT sequences in cell-cell fusion. In chapter 4, we extended these findings and found that the alternate form of the F protein can also be detected in infected and transfected avian cells, the natural host cells of NDV. Furthermore, the alternate form of F protein was also found in virions released from both infected COS-7 cells and avian cells by Western analysis. Mass spectrometry confirmed its presence in virions released from avian cells. Two different polyclonal antibodies raised against sequences of the CT domain of the F protein slowed plaque formation in both avian and COS-7 cells. Antibody specific for the CT domain also inhibited single cycle infections as detected by immunofluorescence of viral proteins in infected cells. The potential roles of this alternate form of the NDV F protein in infection are discussed.

Virus-like particles (VLPs) generated from different viruses have been shown to have potential as good vaccines. Chapter 5 explored the potential of NDV VLPs as a vaccine for NDV or as a vaccine vector for human pathogens. Significant quantities of NDV VLPs can be produced from tissue culture cells. These VLPs are as pure as virions prepared in eggs. In addition, some rules for incorporation of viral proteins into VLPs were also explored. We found that the cytoplasmic domain of the fusion (F) protein is necessary for its incorporation into VLPs. We found that an HN protein with an HA tag at its carboxyl terminus was incorporated into VLPs. We also found that the HN and F
proteins of NDV, strain B1, can be incorporated into VLPs with M and NP of strain AV. The demonstration of specific domains required for protein incorporation into particles is important in using NDV VLPs as a vaccine vector for important human pathogens.

In conclusion, this dissertation presents results that show that the M protein plays a central role in NDV assembly and release, a finding that is consistent with findings with other paramyxoviruses. More importantly, this work extends the current knowledge of paramyxovirus assembly and release by providing the first direct evidence of interactions between paramyxovirus proteins. These interactions between viral proteins provide a rational basis for incorporation of viral proteins into particles. This work also provides a clearer understanding of the role of the host vacuolar protein sorting machinery in NDV budding. A clear understanding of virus assembly and budding process contributes to the design of strategies for therapeutic intervention and in the development of safer, more economical and effective vaccines.
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<tr>
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<td>AP</td>
</tr>
<tr>
<td>Australia-Victoria strain</td>
<td>AV</td>
</tr>
<tr>
<td>Blue tongue virus</td>
<td>BTV</td>
</tr>
<tr>
<td>B-mercaptoethanol</td>
<td>BME</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>BSA</td>
</tr>
<tr>
<td>Cytoplasmic domain</td>
<td>CT</td>
</tr>
<tr>
<td>Diamidino-2-phenylindole</td>
<td>DAPI</td>
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<tr>
<td>Dominant negative</td>
<td>dn</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>DOC</td>
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<tr>
<td>Dulbecco's Modified Eagle Medium</td>
<td>DMEM</td>
</tr>
<tr>
<td>East Lansing Strain</td>
<td>ELL-0</td>
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<tr>
<td>Endosomal sorting complexes required for transport</td>
<td>ESCRT</td>
</tr>
<tr>
<td>Equine infectious anemia virus</td>
<td>EIAV</td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>FBS</td>
</tr>
<tr>
<td>Fluorescence-Activated Cell Sorting</td>
<td>FACS</td>
</tr>
<tr>
<td>Foot and mouth disease virus</td>
<td>FMDV</td>
</tr>
<tr>
<td>Fusion protein</td>
<td>F</td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>GST</td>
</tr>
<tr>
<td>Hemagglutinin-neuraminidase protein</td>
<td>HN</td>
</tr>
<tr>
<td>Hepatitis B virus</td>
<td>HBV</td>
</tr>
<tr>
<td>Hepatitis B core antigen</td>
<td>HBcAg</td>
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</table>
Hepatitis B surface antigen (HBsAg)
Hepatitis C virus (HCV)
Human immunodeficiency virus type 1 (HIV-1)
Human papilloma virus (HPV)
Human parainfluenza virus type 1 (hPIV1)
Immune complex (IC)
Immunofluorescence (IF)
Immunoglobulin G (IgG)
Immunoprecipitation (IP)
Kilodalton (kDa)
Large protein (L)
Late domain (L)
Mason-Pfizer monkey virus (M-PMV)
Mass spectrometry (MS)
Matrix protein (M)
Measles virus (MV)
Mock infected (UI)
Moloney leukemia virus (MLV)
Multivesicular bodies (MVBs)
N-ethylmaleimide (NEM)
Neural precursor cell expressed, developmentally down regulated gene (Nedd)
Neuraminidase
Neurotrophic velogenic Newcastle disease virus
Newcastle disease virus
Nucleocapsid protein
Phosphate-buffered saline
Phosphoprotein
Red blood cells
Red fluorescent protein
Ribonucleoprotein
Rous sarcoma virus
Sendai virus
Simian virus 5
Sodium deoxycholate
Sodium dodecyl sulfate
Subacute sclerosing panencephalitis
Temperature-sensitive
Transmembrane domain
Tris-HCL, NaCl, EDTA
Tumor susceptibility gene
Untransfected
Untranslated region
Vacuolar protein sorting
Vesicular stomatitis virus  VSV
Virus-like particles  VLP
Viscerotrophic velogenic Newcastle disease virus  VVNDV
CHAPTER I
INTRODUCTION

A. General statement of topic

Enveloped RNA viruses must cross the plasma membrane of the host cell twice during the virus life cycle. The first is during virus entry and second is during particle egress. The latter process involves several steps including assembly of viral components at the plasma membrane and the subsequent release of particles by budding. The aim of this work is to understand the mechanisms of assembly and release of Newcastle disease virus (NDV) by using a virus-like particle (VLP) system. Using this system, this thesis has defined the viral determinants and host cellular machinery that participate in particle assembly and budding. The results of this work are also important in designing and developing VLPs as a vaccine for Newcastle disease or as a vaccine vector for important human diseases.

B. Newcastle disease virus

Newcastle disease virus (NDV) causes a highly contagious disease of many species of domestic and wild birds. The disease was first reported in Java in 1926 and was brought to international attention in Newcastle on Tyne, England, in 1926 (reviewed in (Shope 1964; Alexander 2001)). The disease is characterized by respiratory, digestive and neurological signs. The severity of clinical signs ranges from inapparent infection to a rapidly fatal condition. Different strains of virus are categorized based on their ability to cause nervous, respiratory, and visceral lesions, or death (Alexander 2001). Virulence differentiations among strains are determined by standard pathotyping assays, which
utilize inoculation of embryonated eggs and live chickens. NDV strains are classified as velogenic (highly virulent), mesogenic (moderately virulent) and lentogenic (avirulent), depending on the severity of the disease they cause (Beard and Hanson 1984; Alexander 2000). The velogenic strain is further divided into two pathotypes, the viscerotrophic velogenic (VVNDV) and the neurotrophic velogenic (NVNDV), which cause acute lethal disease with visceral hemorrhage or neurological and respiratory signs, respectively.

NDV has been noted in a great majority of the countries in the world, possibly due to the wide range of the reservoir host. NDV is an economically important pathogen, since periodic outbreaks affect the poultry industry. Recently, a VVNDV epidemic in the United States resulted in the destruction of about 4 million birds. Because of its potential as an agent of agrobioterrorism, the United States government has classified virulent strains as select agents under the Patriot Act.

Vaccination against NDV is part of standard poultry vaccination protocols. The widely used NDV vaccines are the lentogenic, live attenuated B1 or La Sota strains. Although these strains do not have pathogenic effects on the birds, vaccination using these strains still produces mild respiratory and digestive disorders, which affect the general productivity of birds in the poultry industry.

Classification and general properties of Newcastle disease virus

Newcastle disease virus (NDV) is classified as the only member of Avulavirus genus within the subfamily Paramyxovirinae in the family Paramyxoviridae within the order Mononegavirales (Table 1.1). This family of viruses also includes other important
<table>
<thead>
<tr>
<th>Order Mononegavirales</th>
<th>Examples of Specific virus</th>
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<tr>
<td>Family Paramyxoviridae</td>
<td></td>
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<tr>
<td>Subfamily Paramyxovirinae</td>
<td></td>
</tr>
<tr>
<td>Genus Respirovirus</td>
<td><em>Bovine parainfluenza virus</em> &lt;br&gt;<em>Human parainfluenza virus type 1</em> &lt;br&gt;<em>Sendai virus</em></td>
</tr>
<tr>
<td>Genus Morbillivirus</td>
<td><em>Measles virus</em> &lt;br&gt;<em>Canine distemper virus</em> &lt;br&gt;<em>Phocine distemper virus</em></td>
</tr>
<tr>
<td>Genus Rubulavirus</td>
<td><em>Simian virus 5</em> &lt;br&gt;<em>Mumps virus</em></td>
</tr>
<tr>
<td>Genus Henipavirus</td>
<td><em>Hendra virus</em> &lt;br&gt;<em>Nipah virus</em></td>
</tr>
<tr>
<td>Genus Avulavirus</td>
<td><em>Newcastle disease virus</em></td>
</tr>
<tr>
<td>Subfamily Pneumovirinae</td>
<td></td>
</tr>
<tr>
<td>Genus Pneumovirus</td>
<td><em>Bovine respiratory syncytial virus</em> &lt;br&gt;<em>Human respiratory syncytial virus</em></td>
</tr>
<tr>
<td>Genus Metapneumovirus</td>
<td><em>Avian metapneumovirus</em> &lt;br&gt;<em>Human metapneumovirus</em></td>
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human and animal pathogens that cause measles, mumps, respiratory diseases (e.g. respiratory syncytial virus and parainfluenza viruses), canine distemper, and encephalitis (e.g. Hendra and Nipah viruses) (Table 1.1).

Virions range in size range from 150 to 300 nm in diameter. Purified virion buoyant density is 1.18-1.20 g/cm$^3$. Particles are pleiomorphic but usually spherical in shape (Figure 1.2A). They consist of a host derived lipid-containing envelope and a helical nucleocapsid (Lamb and Kolakofsky 2001).

The negative stranded NDV genome is about 15,186 nucleotides and encodes 6 genes, positioned 3’-NP-P-M-F-HN-L-5’, which are separated in the positive sense by short untranslated regions (UTRs) (Figure 1.1). The six NDV genes encode structural proteins that are categorized into membrane and core components. The membrane components consist of two transmembrane glycoproteins, the F and HN proteins, and the matrix (M) protein (Figure 1.2B). The virus envelope encases the ribonucleoprotein (RNP) core, which is formed by the RNA genome associated with the nucleocapsid protein (NP) and the polymerase complex composed of phosphoprotein (P) and large (L) proteins (Figure 1.2B). The inner surface of the virion is lined by the M protein which is thought to mediate interaction between the RNP complex and lipid-bilayer, as well as the cytoplasmic tails of the spike glycoproteins (Figure 1.2B) (Cathomen, Mrkic et al. 1998).

**Paramyxovirus replication**

All steps of NDV replication take place in the cytoplasm of the infected host cell (Figure 1.3). NDV infection of the host cell is initiated by first binding to the sialic-acid
Figure 1.1. Linear genome diagram of NDV. Schematic shows NDV genes from 3’ to 5’. NP, nucleocapsid protein; P, phosphoprotein; V and W, accessory proteins; M, matrix; F, fusion; HN, hemagglutinin-neuraminidase; L, large. Vertical bars show intervening regions (some sequences are transcribed but not translated).
containing receptors in the plasma membrane. Attachment of the HN protein to the receptor activates and leads to conformational changes in the F protein, which directs fusion of the viral membrane with the host cell plasma membrane at neutral pH (White 1990). For most paramyxoviruses, co-expression of the F and HN proteins from the same virus is necessary for efficient membrane fusion (reviewed in White 1990; Lamb 1993; Morrison 2003). After fusion of the viral membrane and the host cell membrane, the viral nucleocapsid dissociates from the M protein by an unknown mechanism and is delivered into the cytoplasm of the cell. The viral polymerase complex transcribes each gene in the RNA genome. Following translation of the primary transcripts and accumulation of the viral proteins, antigenome synthesis commences. The products of this synthesis are then used by the same polymerase complex to produce exact, complementary copies of full length, negative-sense genomes that will be packaged into virions (Lamb and Kolakofsky 2001).

The viral nucleocapsids are assembled in two steps; first, the newly synthesized NP associates with nascent genomic RNA to form the helical nucleocapsid structure, and second, the P-L polymerase complex associates with the nucleocapsid. The well conserved N-terminal region of NP contains the RNA binding domain and the determinants for the NP-NP interaction that forms the helical structure (Curran, Homann et al. 1993; Bankamp, Horikami et al. 1996; Myers and Moyer 1997; Myers, Pieters et al. 1997). The C-terminal region of NP contains the domains that bind with the P protein (Buchholz, Retzler et al. 1994). The C-terminal region is also responsible for its
Figure 1.2. Morphology of Paramyxovirus particle. A, Electron micrograph of a typical paramyxovirus (Lamb, R.A. and D Kolakofsky (2001). Paramyxoviridae: The Viruses and Their Replication. Philadelphia, Lippincott Williams & Wilkins.) B, Schematic of a typical paramyxovirus showing the membrane components (F, HN, M proteins) and core components (RNA with NP, P, and L).
specific interaction with the M protein and is hypothesized to be necessary for the incorporation of nucleocapsid into virions (Coronel, Takimoto et al. 2001).

The two newly synthesized HN and F glycoproteins are transported to the surface of infected cells. Viral glycoproteins are synthesized in the endoplasmic reticulum and undergo a series of conformational maturation steps prior to transport through the secretory pathway (Lamb and Kolakofsky 2001). NDV has two forms of F protein, the classical type I transmembrane glycoprotein and the partially translocated alternate form of F protein in which both the amino terminus and CT domain of the protein are translocated across the membrane (McGinnes, Reitter et al. 2003).

Paramyxovirus assembly takes place at the plasma membrane of infected cells (Lamb and Kolakofsky 2001). All components of the virus, the nucleocapsid, M protein, and the envelope glycoproteins are transported to the plasma membrane and virions are assembled and particles are released by the process of budding. NDV virion components are enriched in lipid rafts within the plasma membrane, and it has been suggested that these specific domains serve as sites of virus assembly and budding (Laliberte, McGinnes et al. 2006).

C. Mechanisms of enveloped RNA virus assembly and budding

Many enveloped RNA virus particles are formed by a process that includes assembly of viral components at the plasma membrane of infected host cells and the subsequent release of particles by budding. Buds emerge from sites on the plasma membrane where viral components have assembled and then pinch off, resulting in the
Figure 1.3. Schematic of paramyxovirus replication. 1, Attachment of HN to receptor; 2, Fusion of viral membrane with plasma membrane; 3, Delivery of RNA genome into the cytoplasm; 4, Transcription; 5, Translation; 6, Anti-genome synthesis and genome synthesis; 7, Transport of membrane components to the cell surface; 8, Assembly and budding of progeny virions.
release of particles. A number of steps appear to be involved in the formation of the infectious virus particles, including the transport of viral glycoproteins to the cell surface, the transport of assembled RNPs and matrix proteins to the plasma membrane of the cell, interactions between viral proteins in the budding site, and interactions between viral components and host cell machinery that allow budding and release of particles. However, details of these processes in paramyxovirus infection are unclear. Important questions that will be addressed in this thesis include the following:

(i) Which viral structural components actively contribute to the budding process?
(ii) What is the minimum viral component required for budding to occur?
(iii) Do glycoproteins play an important role in virus budding?
(iv) Does assembly require specific viral protein-protein interactions?
(v) Do paramyxoviruses recruit host machinery to assist in the budding process?
(vi) Are these interactions with host proteins mediated by late domains?

D. Contributions of viral factors in enveloped RNA virus budding

For some enveloped RNA viruses, the viral components that participate in virus assembly and budding have been well characterized. The key role played by the viral matrix-like proteins in assembly of particles, as well as roles played by additional viral components such as the viral glycoproteins, are some of the important topics of discussion in this part of this dissertation. In addition, interactions between viral components that are necessary for assembly of particles are also discussed.
Role of matrix protein

In most enveloped RNA virus systems, it has been shown that the matrix-like proteins play an important role in assembly and budding. Matrix-like proteins are situated in virions beneath the lipid envelope so that they have the potential to contact both the RNP core and envelope glycoprotein cytoplasmic tails. Given this position, the matrix proteins are likely to play a central role in the organization of virus assembly perhaps by inducing ordered concentration of viral components at defined budding sites in the plasma membrane of infected cells.

The importance of matrix-like proteins in assembly and budding of enveloped RNA viruses has been shown in experiments using temperature-sensitive viruses that have defective M genes, by direct manipulation of M genes in recombinant viruses by reverse genetics, and by reconstitution of budding directed by matrix proteins in transfected cells. Evidence for the essential role of matrix-like proteins in enveloped RNA virus assembly is summarized below.

A role of matrix-like proteins in virus budding was first demonstrated by use of temperature-sensitive (ts) mutants. A variety of mutant vesicular stomatitis viruses (VSV) with ts mutations in their M genes have been characterized and found to be defective in assembly and budding of particles at restrictive temperature (Knipe, Baltimore et al. 1977). In addition to inefficient release of virions, most of the particles released did not contain an RNA genome and the morphology of the particles was altered, being spherical or pleiomorphic instead of the normal bullet-shaped morphology (Lyles, McKenzie et al. 1996).
Another group found that ts mutations in the M gene of Sendai virus (SV) are defective in assembly and budding of particles at restrictive temperature (Yoshida, Nagai et al. 1979; Kondo, Yoshida et al. 1993). Interestingly, massive over-expression of ts mutant M protein of SV restored normal release of particles suggesting that the budding defect is due to insufficient accumulation of M protein and not to a defect in function of the mutant M protein (Kondo, Yoshida et al. 1993). This finding also suggests that SV budding may require a threshold level of M protein accumulation.

Further studies which confirmed the key role played by matrix-like proteins in assembly and budding have utilized reverse genetics techniques, which allow the generation of recombinant enveloped negative-strand RNA viruses from plasmid DNA. Rabies virus (Cathomen, Mrkic et al. 1998; Mebatsion, Weiland et al. 1999), measles virus (MV) (Mebatsion, Weiland et al. 1999) and SV (Inoue, Tokusumi et al. 2003) generated to lack the entire M gene demonstrated impaired budding. Moreover, MV containing mutant M protein derived from subacute sclerosing panencephalitis (SSPE) virus was also defective in release of particles (Patterson, Cornu et al. 2001). These reports together with the ts mutant M protein results strongly support the idea that the M protein plays a central role in virus assembly and budding.

The importance of matrix-like proteins for enveloped RNA virus budding has also been shown by reconstituting budding in cells that express matrix-like protein alone. The usefulness of this approach was first demonstrated by generating virus-like particles from insect cells transduced with a recombinant baculovirus vector containing a VSV M gene (Li, Luo et al. 1993). Other important contributions in the field of enveloped RNA virus
assembly are studies on the retrovirus system. Self-budding activity of Gag protein has been demonstrated in several different retroviruses (reviewed in (Morita and Sundquist 2004)).

The first direct evidence of budding mediated by paramyxovirus M protein was demonstrated by characterizing the M protein expressed from cDNA. Similar to results obtained with the matrix-like proteins of retroviruses (Morita and Sundquist 2004), filoviruses (Jasenosky and Kawaoka 2004), rhabdoviruses (Jayakar, Jeetendra et al. 2004), and influenza viruses (Barman, Adhikary et al. 2004), expression of human parainfluenza virus type 1 (hPIV1) M protein alone or the SV M protein alone resulted in the release of M protein containing particles (Coronel, Murti et al. 1999; Takimoto, Murti et al. 2001). These results indicated that these M proteins contain all the functional elements necessary for formation and release of particles. The VLPs released into the culture medium varied in size and were smaller than the regular virus particles. The density of particles was less than that of virus particles released from infected cells, probably due to the lack of other structural proteins and the RNA genome.

However, the role of the M protein in the budding of virus or VLPs seems to vary among paramyxoviruses. In some cases, co-expression of M proteins along with additional viral components can substantially increase the efficiency of release of particles. Unlike the expression of the M proteins of SV and hPIV1, expression of the simian virus 5 (SV5) M protein was not sufficient for particle release. Although the M protein was necessary, expression of two other proteins (NP and either glycoprotein) along with M protein was required for release of SV5 virus like particles (VLPs).
Particle budding that is normally observed with SV M protein alone is also enhanced by co-expression of SV F protein (Takimoto, Murti et al. 2001). Thus, although M proteins of most paramyxoviruses are capable of directing budding of particles in the absence other viral components, in many cases, budding is enhanced by co-expression of multiple viral components. The only data that implicates the NDV M protein in virus budding was a report that mutations in the M protein resulted in decreased F glycoprotein incorporation into virions (Peeples and Bratt 1984). However, there is no direct evidence that shows that the NDV M protein plays an important role in budding of particles.

**Role of glycoproteins in promoting efficient budding of enveloped RNA viruses**

Glycoproteins from different RNA viruses have been observed to play a role in the assembly and budding of particles. The assembly and release of retroviral particles are mediated by Gag protein, though in some retroviral genera, the Env glycoproteins play a major role in directing Gag to the cell surface (Perez, Davis et al. 1987; Owens, Dubay et al. 1991; Yu, Yuan et al. 1993; Poumbourios, Wilson et al. 1997). The glycoproteins of rhabdoviruses and filoviruses have also been demonstrated to have self-budding activity as shown by budding of vesicles containing the rhabdovirus G protein from cells expressing the VSV or rabies virus G glycoproteins (Rolls, Webster et al. 1994; Mebatsion, Konig et al. 1996). Ebola virus GP expressed alone also induced release of particles with variable morphology (Volchkov, Volchkova et al. 1998; Noda, Sagara et al. 2002). However, when GP was co-expressed with Ebola virus matrix protein, VP40, there was an enhancement in the release of particles with uniform
filamentous morphology (Noda, Sagara et al. 2002). These findings in the rhabdovirus and filovirus systems demonstrated that glycoproteins play an important role in assembly and budding of these viruses particles. While the orthomyxovirus influenza A matrix protein, M1, induces production of VLPs in the absence of other viral components, the efficiency of release is strongly increased by the neuraminidase (NA) protein. Co-expression of influenza A structural proteins in the absence of NA protein resulted in the formation of VLPs that aggregated at the surfaces of cells (Gomez-Puertas, Mena et al. 1999; Gomez-Puertas, Albo et al. 2000). The enzymatic activity of NA prevents newly formed particles from aggregating at the surfaces of infected cells.

Paramyxoviruses encode two glycoproteins, the fusion protein and attachment protein. Early studies have shown that the HN protein of SV is not necessary for the budding of virus particles (Portner, Marx et al. 1974; Stricker and Roux 1991). Using the VLP system, the conclusion that the HN protein is not necessary for budding was further confirmed. Co-expression of SV proteins in the absence of HN protein resulted in efficient release of particles, suggesting that SV HN protein has no effect on VLP budding (Leyrer, Bitzer et al. 1998). In contrast, budding of M protein containing particles was enhanced by co-expression of F protein, while co-expression of HN protein with M protein had no effect on budding efficiency (Takimoto, Murti et al. 2001). These results suggested that the SV F protein but not the HN protein is important for efficient budding of particles. Consistent with this view, the F protein was found to have self-budding activity while HN protein was not observed to have this activity (Takimoto, Murti et al. 2001; Sugahara, Uchiyama et al. 2004).
The cytoplasmic domain of the F protein of paramyxoviruses has been shown to likely contain the domain required for efficient budding. The cytoplasmic tails (CT) of SV and hPIV1 F protein share five consecutive amino acids (TYTLE). Mutational analysis showed that these residues are important for particle formation (Takimoto, Murti et al. 2001). Similar studies have been done with SV5, to define further the role of glycoproteins in paramyxovirus budding. Budding of recombinant SV5 with truncated F or HN protein cytoplasmic tails were found to be inefficient (Schmitt, He et al. 1999; Schmitt, Leser et al. 2002). It is still unclear which of the NDV glycoproteins are important for assembly and budding of virions.

**Interactions between components of enveloped RNA viruses during assembly**

For efficient assembly and budding of enveloped RNA viruses, two steps are essential. First, all viral structural components must be transported to the site of assembly and budding. Second, these components must interact with each other in an orderly manner to initiate the budding process that leads to the production of infectious progeny virus. This process appears to be accomplished through a series of viral protein-protein interactions, many of which involve the matrix protein. The M protein potentially interacts with membranes, with glycoproteins likely via cytoplasmic tails and with RNP in the cytoplasm of infected cells.

Matrix-like proteins of VSV (Chong and Rose 1993; Chong and Rose 1994), Ebola virus (Jasenosky, Neumann et al. 2001), Marburg virus (Kolesnikova, Bugany et al. 2002), influenza virus (Kretzschmar, Bui et al. 1996), SV (Stricker, Mottet et al. 1994) and MV (Riedl, Moll et al. 2002) were shown to have intrinsic membrane-binding
properties. Membrane association of NDV M protein was demonstrated by the reconstitution of matrix proteins and synthetic liposomes into vesicles (Faaberg and Peeples 1988).

While paramyxovirus M proteins are clearly pivotal in the release of assembled virus, the interactions between M protein and other viral proteins required for the assembly of complete particles are less well defined. Indeed, available information, based on properties of VLPs formed after co-expression of different viral proteins with M protein or on co-localization or co-fractionation of M protein with other viral proteins have often led to contradictory conclusions. For example, some reports suggest that M protein binds to F protein while others suggest a specific interaction with the attachment protein (SV HN or RSV G) or both attachment and F proteins (Sanderson, Wu et al. 1994; Ali and Nayak 2000; Ghildyal, Li et al. 2005). It is also proposed by Schmitt et al that the cytoplasmic domain of F and HN proteins have redundant functions in mediating viral protein-protein interactions (Schmitt, He et al. 1999; Schmitt, Leser et al. 2002). M protein is also proposed to interact with NP protein in some studies (Stricker, Mottet et al. 1994).

E. Contributions of host factors in enveloped RNA virus budding

Budding through the host cell plasma membrane is a crucial step in the life cycle of many viruses. Recent studies of retroviruses and a number of other enveloped RNA viruses have now demonstrated that cellular proteins that are intimately involved in intracellular membrane trafficking and receptor relocalization play key roles in
facilitating the virus budding process (reviewed in (Bieniasz 2006)). Below is a brief review of the important role played by cellular proteins in virus budding.

**Role of the vacuolar protein sorting pathway in enveloped RNA virus budding**

The role of host cellular machinery in enveloped virus budding has been excellently demonstrated in the retrovirus system. It has been shown that retroviruses co-opt the cellular endosomal sorting machinery, vacuolar protein sorting (VPS) system, for use in virus egress. The VPS machinery, first defined in yeast as class E Vps proteins, is normally used by cells to maintain surface transmembrane protein concentration and to deliver proteins targeted for degradation in the lysosomes (Babst 2005). These functions are accomplished by the formation of multivesicular bodies (MVBs). MVBs are structures formed from the invagination of the endosomal membrane into the endosomal lumen thus forming vesicles inside a vesicle (Figure 1.4) (Babst 2005; Bieniasz 2006). The formation of MVBs are coordinated mainly by three endosomal sorting complexes required for transport (ESCRT I, II and III) first characterized in yeast (reviewed in (Babst 2005)) (Figure 1.5). ESCRT I and ESCRT II both contain a subunit that binds to ubiquitin. ESCRT I recognizes the ubiquitinated protein cargos and recruits ESCRT II and ESCRT III that participate in protein sorting and vesicle formation (Figure 1.5A) (reviewed in (Pornillos, Garrus et al. 2002; Babst 2005)). ESCRT II functions to recruit ESCRT III to the membrane. The pathway also requires an ATPase, Vps4, which is responsible for the dissociation of the full ESCRT complex and subsequent recycling of its components (Babst, Sato et al. 1997; Babst, Wendland et al. 1998).
**Figure 1.4. Protein transport pathway in eukaryotic cells.** Endocytosed surface proteins from the secretory pathway are delivered into the endosomal system. Cargo proteins destined for degradation in the lysosome are sorted into luminal vesicles of multivesicular bodies (MVBs). Mature MVBs fuse with the lysosome and deliver the MVB vesicles to the lysosomal lumen where the vesicles and cargo are degraded. (Adapted from published reports and Babst, M.B. et al. 2005. Traffic 6:2-9)
A key advance in understanding the budding process of enveloped RNA viruses came with the demonstration that retrovirus Gag proteins interact with components of the VPS machinery, presumably hijacking the system to direct virus release (Pornillos, Garrus et al. 2002; Martindale 2003; Morita and Sundquist 2004). HIV-1 budding from infected human cells is modeled in analogy to vesicle formation in the yeast MVB (Pornillos, Garrus et al. 2002) (Figure 1.5B). It is thought that the topology of vesicle formation is similar to virus particle budding in the sense that they are both directed away from the cytoplasm (Martindale 2003). It has been shown that that HIV-1 Gag interacts with the tumor susceptibility gene (TSG) 101 protein (Pornillos, Garrus et al. 2002), a subunit of ESCRT I, which suggests that ESCRT I participates in the virus budding process. HIV-1 budding has also been shown to be blocked by dominant negative mutations that disrupt the activity of the CHMP3, a subunit of ESCRT III, as well as the ATPase, Vps4 (Garrus, von Schwedler et al. 2001; Strack, Calistri et al. 2003). Interestingly, other enveloped RNA viruses, most notably the filoviruses and rhabdoviruses, have evidently evolved a similar exit strategy by interaction of their matrix proteins with the endosomal sorting machinery (reviewed in (Jasenosky and Kawaoka 2004; Jayakar, Jeetendra et al. 2004; Schmitt and Lamb 2004; Hartlieb and Weissenhorn 2006)). However, the role of the host VPS machinery in paramyxovirus budding is not well understood.
Figure 1.5. Comparison of the model of vacuolar protein sorting pathway and HIV-1 budding. A, Multivesicular bodies formation showing ESCRT I, II and III and the ATPase, Vps4. B, Interplay between VPS molecules and Retrovirus Gag in the budding process.
Viral Late Domains

The essential role of the matrix-like proteins in virus release is due in part to short motifs in the protein sequence, collectively termed as late (L) domains to reflect their function late in the budding process (reviewed in (Martin-Serrano, Zang et al. 2001; Strack, Calistri et al. 2002; Jasenosky and Kawaoka 2004; Morita and Sundquist 2004; Schmitt and Lamb 2004; Takimoto and Portner 2004; Patton, Morris et al. 2005; Bieniasz 2006)). L domains are highly conserved short peptide motifs that promote virus release by mediating interactions with a member of the class E proteins, components critical for the formation of vesicles that inwardly bud into MVBs. (Pornillos, Garrus et al. 2002; Freed 2003; Bieniasz 2006). It is thought that matrix-like proteins with L domain motifs co-opt the host VPS pathway for use in virus budding (Freed 2002; Martindale 2003; Strack, Calistri et al. 2003; Morita and Sundquist 2004). The first compelling evidence that a viral L domain functions in budding was shown with an HIV-1 mutant with a deletion in the p6 Gag. In this study, mutant HIV-1 failed to detach from the cell plasma membrane (Gottlinger, Dorfman et al. 1991). A highly conserved PTAP motif located near the N terminus was shown to play a crucial role in the budding activity of p6 (Gottlinger, Dorfman et al. 1991). This result was recapitulated in the Rous sarcoma virus. Furthermore, it was shown that a highly conserved PPPY motif, which mapped in the p2b, plays an important role in virus particle production. Three classes of motifs have since been identified to have L domain activity; PTAP, PPXY and YXXL. In each case, the integrity of these motifs is essential for L domain function, suggesting that L domain serves to mediate interaction with host factors (Bieniasz 2006).
Figure 1.6. **Viral late domains in matrix-like proteins of different viruses.** L domain sequences in the matrix-like protein of different enveloped RNA viruses. HIV-1, Human immunodeficiency virus type 1; MLV, Moloney murine leukemia virus; RSV, Rous sarcoma virus; M-PMV, Mason-Pfizer monkey virus; EIAV, Equine infectious anemia virus; VSV, Vesicular stomatitis virus (Freed EO 2002 J. Virol. 76:4679-4687).
Subsequently, several L domain sequences have been identified in many different viruses (reviewed in (Freed 2004)). L domains in the VP40, the M protein of filoviruses, and rhabdoviruses M protein have been identified (Figure 1.6). An L domain sequence, FPIV, in the SV5 M protein has also been defined (Schmitt, Leser et al. 2005). However, this sequence cannot be sufficient for SV5 release since the M protein of this virus, when expressed alone, does not direct particle release. Therefore, no classical L domain sequence has been identified in the M proteins of the paramyxoviruses.

F. Contents of dissertation

The viral and host protein requirements for NDV assembly and budding remain to be determined. Results in this thesis demonstrate the following:

(i) NDV VLPs are efficiently released from cells expressing the NP, M, F and HN proteins;
(ii) NDV M protein is required and sufficient for particle assembly and budding;
(iii) Co-expression of NDV M protein with at least two viral proteins is necessary for efficient incorporation of other proteins into particles;
(iv) Specific viral protein-protein interactions are required for particle assembly;
(v) Vacuolar protein sorting machinery is essential for particle budding;
(vi) YANL sequence in the NDV M protein has late domain function;
(vii) The alternate form of F protein also exists in virus, suggesting a role in virus assembly and budding;
(viii) NDV VLPs have significant potential as a vaccine for Newcastle disease;

They may also provide framework for vaccines for other viruses.
Evidence supporting these conclusions are parts of a series of papers, two published, one submitted and one in preparation. The Abstracts of each paper are shown below.

**Chapter 2: Requirements for assembly and release of Newcastle disease virus-like particles** (Published in Journal of Virology November 2006 v80)

Paramyxoviruses such as Newcastle disease virus (NDV) assemble in and bud from plasma membranes of infected cells. To explore the role of each of the NDV structural proteins in virion assembly and release, virus-like particles (VLPs) released from avian cells expressing all possible combinations of the nucleoprotein (NP), membrane or matrix protein (M), an uncleaved fusion protein (F-K115Q), and hemagglutinin-neuraminidase (HN) protein were characterized for densities, protein content, and efficiencies of release. Co-expression of all four proteins resulted in the release of VLPs with densities and efficiencies of release (1.18 to 1.16 g/cm$^3$ and 83.8%±1.1, respectively) similar to that of authentic virions. Expression of M protein alone, but not NP, F-K115Q or HN proteins individually, resulted in efficient VLP release. No combination of proteins in the absence of M protein resulted in particle release. Expression of any combination of proteins that included M protein yielded VLPs, although with different densities and efficiencies of release. To address the roles of NP, F and HN proteins in VLP assembly, the interactions of proteins in VLPs formed with different combinations of viral proteins were characterized by co-immunoprecipitation. The co-localization of M protein with cell surface F and HN proteins in cells expressing all combinations of viral proteins was characterized. Taken together, the results show that M protein is necessary and sufficient for NDV budding.
Furthermore, they suggest that M-HN and M-NP interactions are responsible for incorporation of HN and NP proteins into VLPs and that F protein is incorporated indirectly due to interactions with NP and HN protein.

Chapter 3: Release of Newcastle disease virus-like particles requires the host VPS system (Submitted, Journal of Virology)

Newcastle disease virus-like particles (VLPs) are released from avian cells expressing the viral NP, F, HN, and M proteins and M protein is both necessary and sufficient for particle release (Pantua et al, J. of Virol 80: Nov). Here we show that VLPs were also released from HEK 293T cells expressing either M protein alone or in combination with NP, F and HN proteins. Since the vacuolar protein sorting (VPS) system is involved in release of several enveloped RNA viruses, we explored the role of the VPS system on NDV particle release using three dominant negative mutant proteins of the VPS pathway. Expression of dominant negative mutants of CHMP3, Vps4 and AIP1 proteins inhibited M protein particle release as well as release of complete VLPs. Mutation of a YANL sequence in the NDV M protein to AANA inhibited particle release while replacement of this sequence with either of the classical late domain motifs, PTAP or YPDL, completely restored particle release. The host protein AIP1, which binds YXXL late domain sequences, is incorporated into M protein particles. These results suggest that an intact VPS pathway is necessary for NDV VLP release, that the YANL sequence is an NDV M protein L domain, and that NDV may access the VPS system using the PTAP late domain.
Chapter 4: Characterization of an alternate form of Newcastle disease virus fusion protein (Published in Journal of Virology, 2005 v79 pp.11660-11670)

The sequence and structure of the Newcastle disease virus (NDV) fusion (F) protein are consistent with its classification as a type 1 glycoprotein. We have previously reported, however, that F protein can be detected in at least two topological forms with respect to membranes in both a cell-free protein synthesizing system containing membranes as well as infected COS-7 cells (J. Virol. 2004 77:1951). One form is the classical type 1 glycoprotein while the other is a polytopic form in which approximately 200 amino acids of the amino terminal end as well as the cytoplasmic domain (CT) are translocated across membranes. Furthermore, we detected CT sequences on surfaces of F protein expressing cells and antibodies specific for these sequences inhibited red blood cell fusion to HN and F protein expressing cells suggesting a role for surface expressed CT sequences in cell-cell fusion. Extending these findings we have found that the alternate form of the F protein can also be detected in infected and transfected avian cells, the natural host cells of NDV. Furthermore, the alternate form of F protein was also found in virions released from both infected COS-7 cells and avian cells by Western analysis. Mass spectrometry confirmed its presence in virions released from avian cells. Two different polyclonal antibodies raised against sequences of the CT domain of the F protein slowed plaque formation in both avian and COS-7 cells. Antibody specific for the CT domain also inhibited single cycle infections as detected by immunofluorescence of viral proteins in infected cells. The potential roles of this alternate form of the NDV F protein in infection are discussed.
Chapter 5: NDV virus-like particles as vaccine for Newcastle disease (In preparation)

Virus-like particles (VLPs) generated from different viruses have been shown to have potential as good vaccines. Significant quantities of NDV VLPs can be produced from tissue culture cells. These VLPs are as pure as virions prepared in eggs. We also found that the cytoplasmic domain of the fusion (F) protein is necessary for its incorporation into VLPs. We found that an HN protein with an HA tag at its carboxyl terminus was incorporated into VLPs. We also found that the HN and F proteins of NDV strain B1 can be incorporated into VLPs with M and NP of strain AV. The demonstration of specific domains required for incorporation into particles is important in using NDV VLPs as a vaccine vector for other important human pathogens.
CHAPTER II

REQUIREMENTS FOR THE ASSEMBLY AND RELEASE

OF NEWCASTLE DISEASE VIRUS-LIKE PARTICLES

A. Introduction

Paramyxoviruses, such as Newcastle disease virus (NDV), assemble progeny virions at infected cell plasma membranes and release these particles by budding from cell surfaces (Lamb and Kolakofsky 2001). Paramyxovirus assembly requires the packaging of genomic RNA with the nucleoprotein (NP) as well as phosphoprotein (P) and large (L) polymerase (Lamb and Kolakofsky 2001), components of the polymerase complex. This ribonucleoprotein core is encased in a host-derived membrane modified by two transmembrane glycoproteins, the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins, as well as the matrix or membrane (M) protein, which is associated with the inner surface of the viral membrane (Peeples 1991; Lamb and Kolakofsky 2001). The paramyxovirus protein-protein interactions required for particle assembly and the viral and cellular proteins necessary for particle release are not well defined.

The matrix-like proteins of many enveloped RNA viruses play a pivotal role in virus assembly and release (Peeples 1991; Pornillos, Garrus et al. 2002; Freed 2003; Jasenosky and Kawaoka 2004; Jayakar, Jeetendra et al. 2004; Schmitt and Lamb 2004; Takimoto and Portner 2004). These proteins are often sufficient for release of particles. For example, expression of the retroviral gag precursor protein, in the absence of other viral components, results in the assembly and release of gag virus-like particles (VLPs) (Delchambre, Gheysen et al. 1989; Gheysen, Jacobs et al. 1989; Demirov and Freed
Matrix proteins from Ebola virus (Jasenosky, Neumann et al. 2001; Timmins, Scianimanico et al. 2001; Jasenosky and Kawaoka 2004), vesicular stomatitis virus (Li, Luo et al. 1993; Sakaguchi, Uchiyama et al. 1999; Jayakar, Jeetendra et al. 2004) and influenza virus (Gomez-Puertas, Albo et al. 2000), when expressed alone, are released as VLPs. The human parainfluenza virus type 1 (hPIV1) and the Sendai virus (SV) M proteins expressed alone induced release of VLPs (Coronel, Murti et al. 1999; Sakaguchi, Uchiyama et al. 1999; Takimoto, Murti et al. 2001; Sugahara, Uchiyama et al. 2004). Expression of M protein was also required for simian virus 5 (SV5) VLP formation (Schmitt, Leser et al. 2002). However, in contrast to PIV1 and SV, the SV5 M protein was not sufficient for VLP release.

Matrix proteins are also often necessary for particle release. For example, M protein deficient rabies virus generated through reverse genetics was severely impaired in virion formation (Mebatsion, Weiland et al. 1999). Measles virus (MV) (Cathomen, Mrkic et al. 1998) and Sendai virus (SV) (Inoue, Tokusumi et al. 2003) modified by reverse genetics to lack the M protein genes were impaired in budding and release. Moreover, MV containing mutant M protein derived from subacute sclerosing panencephalitis (SSPE) virus was also defective in release of particles (Patterson, Cornu et al. 2001).

The essential role of these matrix proteins in release is due in part to short motifs, called late domains, in these proteins that interact with components of the host vacuolar protein sorting (VPS) system (Martin-Serrano, Zang et al. 2001; Strack, Calistri et al. 2002; Jasenosky and Kawaoka 2004; Morita and Sundquist 2004; Schmitt and Lamb 2004; Morita and Sundquist 2004).
2004; Takimoto and Portner 2004; Patton, Morris et al. 2005; Bieniasz 2006). It is thought that these proteins hijack the host VPS pathway for use in virus budding (Freed 2002; Martindale 2003; Strack, Calistri et al. 2003; Morita and Sundquist 2004). A late domain sequence in the SV5 M protein has been defined (Schmitt, Leser et al. 2005). However, this sequence cannot be sufficient for SV5 release since the M protein of this virus, when expressed alone, does not direct particle release.

While paramyxovirus M proteins are clearly pivotal in the release of assembled virus, the interactions between M protein and other viral proteins required for the assembly of complete particles are less well defined. Indeed, available information, based on properties of VLPs formed after co expression of different viral proteins with M protein or on co-localization or co-fractionation of M protein with other viral proteins have often led to contradictory conclusions. For example, some reports suggest that M protein binds to F protein while others suggest a specific interaction with HN protein or both HN and F proteins (Sanderson, Wu et al. 1994; Ali and Nayak 2000; Ghildyal, Li et al. 2005). M protein is also proposed to interact with NP protein in some studies (Stricker, Mottet et al. 1994).

Thus general rules for assembly and release of paramyxoviruses are not yet clear. Important questions include (i) the role of each viral protein in virus assembly, (ii) the full definition of paramyxovirus late domains in viral structural proteins, and (iii) the cellular factors involved in the budding process.

Using NDV as a prototype paramyxovirus, we sought to clarify the role of each paramyxovirus protein in assembly and release. We combined a definition of the viral
protein requirements for assembly and release of VLPs with a characterization of the protein-protein interactions in VLPs formed with different combinations of viral proteins. We also characterized the co-localization of M protein with the viral glycoproteins in plasma membranes. Our results show that particle assembly involves a network of specific protein-protein interactions and likely correct targeting of proteins to specific cellular domains.
B. Materials and Methods

Cells and viruses. A spontaneously transformed fibroblast cell line derived from the East Lansing strain (ELL-0) of chicken embryos (UMNSAH/DF-1) was obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS). NDV, strains Australia-Victoria (AV) and B1 were propagated in embryonated chicken eggs by standard protocols (McGinnes, Pantua et al. 2006). Strain AV was grown in BCL-3 containment.

Plasmids. NDV cDNA sequences encoding NP, M, HN, and uncleaved F (F-K115Q) proteins were subcloned into the expression vector pCAGGS (Miyazaki, Takaki et al. 1989; Niwa, Yamamura et al. 1991) to generate pCAGGS-NP, pCAGGS-M, pCAGGS-HN and pCAGGS-F-K115Q, respectively. The F protein cDNA contained a point mutation in the cleavage site sequence at residue 115 (K115Q) which eliminated the furin recognition site (Li, Sergel et al. 1998).

Transfection, infection and metabolic labeling. Transfections of subconfluent ELL-0 cells were accomplished using Lipofectamine (Invitrogen) as recommended by the manufacturer. The following amounts of plasmid DNA were used per 35mm dish: 1.0 μg pCAGGS-NP, 1.0 μg pCAGGS-M, 0.75 μg pCAGGS-F-K115Q, and 1.0 μg pCAGGS-HN. These amounts were previously determined to yield levels of expression similar to cells infected with NDV at a multiplicity of infection of 5. A total of 3.75 μg of plasmid DNA per 35mm plate was used in all transfection experiments. When only one, two, or three cDNAs were used, the total amount of transfected DNA was kept constant by
adding vector pCAGGS DNA. For each transfection, a mixture of DNA and 5 μl of Lipofectamine in OptiMEM media (Gibco/Invitrogen) was incubated at room temperature for 45 minutes and then added to cells previously washed with OptiMEM. The cells were incubated for 5 hours, the Lipofectamine-DNA complexes were removed, and 2 ml of supplemented DMEM was added. After 36 hours, the medium was replaced with 0.7 ml DMEM without cysteine and methionine and supplemented with 100 μCi of [³⁵S] methionine and [³⁵S] cysteine mixture (NEG-772 EASYTAG™ Express Protein Labeling Mix, [³⁵S], Perkin Elmer Life Sciences Inc.). After 4 hours of pulse label, one set of transfected plates was lysed, while in another set the medium was replaced with 1.0 ml of supplemented DMEM with 0.1 mM cold methionine (Nutritional Biochemicals Corporation). After 8 hours of chase, the medium was collected and the cells were lysed in 0.5 ml lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH7.4) containing Triton-DOC (1% Triton X-100, 1% sodium deoxycholate) and 1.25 mg N-ethylmaleimide (NEM). Cells were harvested with a cell scraper and homogenized by passing through a 26 gauge needle 10 to 15 times.

ELL-0 cells were infected at an MOI of 5 pfu for 5 hours and labeled with [³⁵S] methionine and [³⁵S] cysteine mixture for 30 min, and chased in nonradioactive medium for 8 hours as described above. Cell supernatant was harvested and virions purified as described below. Cells were lysed and homogenized as described above.

**Virus and VLP purification.** VLPs as well as virions were purified from cell supernatants in protocols previously developed for virus purification (Levinson and Rubin 1966). The cell supernatants were clarified by centrifugation at 5000 rpm for 5 min
at 4°C, overlaid on top of a step gradient consisting of 3.5 ml 20% and 0.5 ml 65% sucrose solutions (g/ml) in TNE buffer (25mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA), and centrifuged at 40,000 rpm for 12 hours at 4°C using a SW50.1 rotor (Beckman). The interface (containing concentrated particles) was collected in 0.5 ml, mixed with 2.0 ml of 80% sucrose, and overlaid on top of 1.0 ml 80% sucrose cushion. Additional layers of sucrose (1.0 ml of 50 % and 0.5 ml of 10% sucrose) were layered on top of the sample. The gradient was centrifuged at 38,000 rpm for 20 h at 4°C. The gradient was collected from the bottom into one 1ml fraction and eight 0.5 ml fractions using a polystaltic pump. Densities of each fraction were determined using a refractometer. Particles derived from expression of all combinations of proteins were prepared in a single experiment, thus enabling direct comparison of results. The experiment was repeated three times.

**Antibodies.** Antiserum used to precipitate NP (anti-NDV) was rabbit polyclonal antibody raised against UV inactivated NDV by standard protocols. Anti-NDV also contained antibodies specific for HN, F and M proteins. Antisera used to precipitate F protein were raised against glutathione S-transferase (GST) fusion proteins that contained amino acid sequences 130 to 173 (anti-HR1) (McGinnes, Gravel et al. 2002), 470 to 500 (anti-HR2) (Dolganiuc, McGinnes et al. 2003), or 96 to 117 (anti-F2-96). Antiserum used to precipitate HN protein was raised against HN protein sequences from amino acid 96 to 117 (anti-A) (McGinnes and Morrison 1998). Antiserum used to precipitate M protein was a mouse monoclonal antibody raised against purified M protein (Faeberg and Peeples 1988).
**Immunoprecipitation and polyacrylamide gel electrophoresis.** Immunoprecipitation was accomplished by combining one volume of cell lysate or sucrose gradient fraction with two volumes of TNE buffer with 1% Triton X-100. Samples were incubated with specific antibodies for 16 hours at 4°C. Immune complexes (ICs) were adsorbed to Protein A (Pansorbin Cells, CALBIOCHEM) for 2 hours at 4°C, pelleted, and then washed three times in immunoprecipitation (IP) wash buffer (phosphate buffer saline (PBS) containing 0.5% Tween-20 and 0.4% sodium dodecyl sulfate (SDS)). ICs were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer (125 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.4% Bromphenol blue) with 1 M β-mercaptoethanol (BME) and boiled. Proteins were separated on 8% polyacrylamide-SDS gel and subjected to autoradiography. Quantification of resulting autoradiographs was accomplished using a Fluor-S™ MultiImager (BioRad).

**Co-immunoprecipitation.** Purified VLPs were incubated in ice cold TNE buffer (25 mM Tris HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100, 2.5 mg/ml N-ethylmaleimide for 15 min. Excess primary antibody was added and VLPs were incubated at 4°C overnight. Pansorbin cells, blocked overnight in TNE buffer containing 1% Triton X-100 and 5 mg bovine serum albumin (BSA) and then prewashed in TNE containing 1% Triton X-100 and 1 mg/ml BSA, were added in excess as determined in preliminary experiments, and incubation was continued at 4°C with constant mixing for at least 2 h. Immune complexes were collected by centrifugation (10,000 rpm for 30 s in a microcentrifuge) and washed three times in ice-cold TNE containing 0.5% Triton X-100. The pelleted complexes were resuspended in gel sample buffer.
**Protease Protection Assay.** Protease digestion of M protein from avian cell extracts and VLPs was accomplished by adding 0.25, 0.5, 1, 5, 10, and 20 μg of proteinase K per ml of sample and incubating for 30 min on ice. In parallel, VLPs were also made 0.5 % with respect to Triton X-100 prior to incubation with proteinase K. After digestion, phenylmethylsulfonyl fluoride (PMSF) (0.1 M) was added. For subsequent immunoprecipitation, the reaction mixtures were made 1% with respect to Triton X-100 and 0.5% with respect to sodium deoxycholate.

**Immunofluorescence Microscopy.** Avian cells, grown in 35 mm dish containing glass cover slips, were transfected with different combinations of NDV cDNAs as described above. After 40 h, nuclei were stained with 5 μg/ml 4’,6-Diamidino-2-phenylindole (DAPI) for 30 min at 37°C. Cells were washed twice with ice-cold immunofluorescence (IF) buffer (PBS containing 1% bovine serum albumin, 0.02 % sodium azide, and 5 mM CaCl2), fixed with 2% paraformaldehyde, blocked with IF buffer for 2 h, and incubated for 1 h at 4°C in IF buffer containing polyclonal antibodies against HN and F proteins. Cells were washed twice with ice-cold IF buffer, permeabilized with 0.05% Triton X-100, blocked with IF buffer for at least 2 h and incubated for 1 h at 4°C in IF buffer containing purified ascites fluids containing anti-M protein monoclonal antibody. Cells were then washed twice with ice-cold buffer followed by incubation for 1 h at 4°C in IF buffer containing fluorescein conjugated goat anti-rabbit IgG (Alexa 488) and rhodamine conjugated goat anti-mouse IgG (Alexa 568) (Molecular Probes) secondary antibodies. Cells were washed with ice-cold IF buffer, mounted onto slides using Vectashield mounting medium (Vector Labs, Inc) for immunofluorescence microscopy. Fluorescence
images were acquired using a Nikon fluorescence microscope and Openlab software and processed using Adobe Photoshop.

**Electron Microscopy.** Particles were purified as described above except that, after flotation they were concentrated by centrifugation into a pellet (40000 rpm for 4h at 4\(^\circ\)) and resuspended in TNE buffer containing 2% glutaraldehyde. Virus and particles were adsorbed to Formvar carbon coated nickel grids, negatively stained with 4 % uranyl acetate, and examined by transmission electron microscopy.
C. Results

Cells expressing NDV NP, M, F-K115Q, and HN proteins released VLPs. To determine if VLPs could be released from cells co-expressing the major structural proteins of NDV, radioactively labeled particles released from a chicken fibroblast cell line co-expressing NP, M, F and HN proteins were purified by sucrose density ultracentrifugation. We used an uncleaved version of F protein to eliminate any potential effects of cell-to-cell fusion on particle release. Cells were co-transfected with plasmids at concentrations of DNA we previously determined to result in expression levels and ratios of proteins comparable to infected cells (data not shown). Cells were pulse-labeled with $[^{35}\text{S}]$ methionine and $[^{35}\text{S}]$ cysteine and then chased for 8 hours, a time which we determined in preliminary experiments to result in maximal particle release (data not shown).

Co-expression of NP, M, F and HN proteins resulted in the release of VLPs with a density of 1.18 to 1.16 g/cc (Figure 1, panel A). Virus particles purified in parallel from NDV, strain AV, infected cells had a density of 1.21 to 1.19 g/cc (Figure 2.1, panel B). The slightly lighter density of VLPs compared to authentic virus was likely due to the absence of the virion RNA within the VLPs. The efficiencies of VLP and virus release were calculated as the percentage of M protein remaining in the cell extracts after the chase relative to the amount of protein in the pulse. By this criterion, the efficiency of VLP release was 83.8%±1.1, while the efficiency of NDV release was 91%±1.4 (Figure 2.1, panel C). It is assumed that M protein lost in the chase was incorporated into VLPs. However, it is possible that the decrease during the chase may also be due to some
Figure 2.1. Co-expression of NP, F, HN and M proteins resulted in VLP formation and release. Panel A: avian cells, co-transfected with pCAGGS-NP, -M, -F-K155Q, and –HN, were radioactively labeled with $^{[35\text{S}]\text{methionine}}$ and $^{[35\text{S}]\text{cysteine}}$ for 4 hours (P) and then chased in non-radioactive medium for 8 hours (C). Panel B: avian cells, infected with NDV, strain AV, at an MOI of 5 for 5 hours, were pulse-labeled for 30 minutes and chased in non-radioactive medium for 8 hours. Radioactively labeled proteins in the extracts were immunoprecipitated with a cocktail of antibodies specific for all viral proteins and the precipitated labeled proteins are shown on the left side of each panel. Particles in cell supernatants were purified as described in Materials and Methods. After flotation into sucrose gradients (right side of each panel), each gradient fraction was mixed with TNE containing 1% Triton X-100 and immunoprecipitated with the antibody cocktail. The density of each fraction (g/cc) is shown at the bottom. Panel C shows the quantitation of efficiency of virion and VLP release as determined by the amount of M.
protein in the pulse and chase extracts and is the result of averaging 3 separate experiments. The standard deviation is shown. Panel D shows non-immunoprecipitated radiolabeled particles released from NDV, strain B1, virus infected avian cells and cells expressing NP, M, F-K115Q and HN proteins. Particles were isolated by concentrating onto the 20% and 65% sucrose interface and then floated into a step gradient. Particles in the gradient fractions were then pelleted and resuspended in TNE buffer. HN, hemagglutinin-neuraminidase protein; F₀, uncleaved fusion protein; NP, nucleocapsid protein; F₁, cleaved fusion protein; M, membrane or matrix protein.
degradation although we have not seen evidence for degradation of a mutant M protein that is retained within cells (Manuscript in preparation). These results demonstrate that NDV VLPs are efficiently assembled and released from avian cells expressing the four major structural proteins.

To determine the purity of released particles, we compared, without immunoprecipitation, the protein content of radiolabeled particles released from cells expressing NP, M, F-K115Q and HN proteins with that of radiolabeled tissue culture grown virus. NDV B1 strain was used in this experiment for two reasons. First, this strain packages only a uncleaved F protein like the VLPs. Second, B1 infection results in less inhibition of host protein synthesis than infection with the more virulent NDV strain AV (unpublished observation). Thus, any host proteins that are incorporated into virus are more likely detected in B1 virus. Figure 2.1, panel D, shows that the profile of proteins present in purified VLPs is similar to that in virions (compare virus and VLP lanes). This result showed that particles released from cells expressing the four major structural proteins are as pure as virions released from infected cells. The identity of the major band below NP in both VLPs and virions is unknown.

**M protein was sufficient for release of membranous particles.** To determine the minimum protein requirements for particle release, we asked if any of the NDV proteins was individually capable of directing particle release. Cells expressing each of the viral proteins individually were radioactively labeled in a pulse-chase protocol and particles were isolated as described above. Figure 2.2, panel B, shows that particles were released only from cells expressing the M protein. Furthermore almost no M protein could be
Figure 2.2. M protein is sufficient for particle release. Avian cells were transfected with pCAGGS-NP, -M, -F-K115Q, and -HN individually. Panel A shows radioactively labeled proteins in the extracts at time of pulse (left) and chase (right). Particles in the supernatants of avian cells, expressing NP, M, F, and HN individually, were concentrated and floated into sucrose gradients as described in legend to Figure 1. Panel B shows the distribution in the gradients of radioactively labeled proteins derived from each supernatant. Densities of gradient fractions are shown at bottom (g/cc). Quantification of the amounts of proteins in particles present in all fractions is shown in panel C. The results of three separate experiments were averaged and the standard deviation is shown.
detected in cell extracts after the 8 hour chase (Figure 2.2A, right panel) indicating that much of the pulse-labeled protein was released from cells. By comparing the levels of M protein in the pulse labeled extract and the chase extract, the efficiency of release was calculated to be 90%±3.0. In contrast, most of the pulse labeled NP, F and HN proteins remained in extracts after the chase (Figure 2.2, panel A). This result correlated with the lack of significant amounts of particles detected in the corresponding cell supernatant (Figure 2.2, panel B) although there was a trace of very light density material released from HN protein expressing cells.

Figure 2.2, panel C, shows the quantification of VLPs produced from cells expressing each protein individually. Interestingly, the amount of M protein-containing particles from cells expressing M protein alone was greater than when all four structural proteins were expressed. However, the M protein-only particles had a very heterogeneous density, with values ranging from 1.24 to 1.12 g/cc (Figure 2.2, panel B). These results show that M protein is sufficient for the release of particles.

The observation that M protein, released from cells, floated into a sucrose gradient indicates that the protein was associated with membrane. To confirm that the M protein-containing particles were membrane-bound particles, these particles were incubated with protease. In this assay, VLPs and cell extracts were either left untreated (Figure 2.3, panel A, lane 1) or treated with different concentrations of Proteinase K (lanes 2 to 7). As expected, the M protein in cell extracts was sensitive to low concentrations of protease (Figure 2.3, panel A, extract panel). The lower band below the M protein is a protease digestion product indicating that M protein has a protease
**Figure 2.3. M proteins are encased in membranous particles.** Panel A: Avian cells were transfected with pCAGGS-M and radioactively labeled VLPs were isolated and purified as described in Materials and Methods. Extract (upper panel) and VLPs, which were concentrated onto 20%-65% sucrose interface (middle panel), were treated with different concentrations (0.25, 0.5, 1, 5, 10, and 20 μg/ml; lanes 2 to 7 respectively) of Proteinase K for 30 min on ice. In parallel, particles were incubated in 1% Triton X-100 prior to Proteinase K treatment (bottom panel). After incubation with protease, reactions were stopped by adding 0.1 mM PMSF. M proteins were immunoprecipitated as described in Materials and Methods. Panel B: Electron microscopy of particles released from NDV infected cells (B1), M protein expressing cells (M), or cells expressing NP, M, F-K115Q and HN proteins (VLPs). Particles were purified as described in Materials and Methods. Middle and left panels show two representative particles from each preparation. Right panels show enlargements of the edges of typical particles.
resistant core. However, M proteins in particles were largely protected from protease digestion (Figure 2.3, panel A, VLP panel). In contrast, disruption of the particle membrane with detergent resulted in digestion of the M protein (Figure 2.3, panel A, VLP with Triton X-100 panel). Taken together, these results demonstrated that the M protein particles are membrane-bound particles.

**Particles visualized by electron microscopy.** Visualization of particles released from cells expressing M protein only (Figure 2.3, panel B, M panel) as well as from cells expressing all four proteins (Panel B, VLP panel), and purified tissue culture virions (Panel B, B1 panel) was accomplished using electron microscopy. Particles formed with all four proteins looked very similar to authentic virus. Both B1 virions and VLPs released (B1 and VLP panels) from cells expressing all four proteins have membrane structures that were suggestive of spike glycoproteins in the envelope (see arrows). M protein particles were more variable in size and had no evidence of glycoprotein spike structures.

**M protein was required for VLP release.** Since the M protein is sufficient for particle release, we next asked whether the M protein is required for the release. To answer this question, cells were transfected with all possible combinations of NP, F, and HN cDNAs in the absence of the M gene. Cells expressing any combination of proteins without M protein did not release particles (Figure 2.4). Furthermore, in the absence of M protein, NP, F and HN proteins expressed in pair wise combinations were retained in cell extracts after the 8 hour chase (Figure 2.5, panel A, lanes 2, 4 and 5). These results strongly suggest that M protein is required for particle release.
Figure 2.4. **M protein is required for particles release.** Avian cells were transfected with all possible combinations of cDNAs in pCAGGS vector encoding NP, F, and HN proteins in the absence of M cDNA (F-K115Q+HN, F-K115Q+NP, HN+NP, NP+F-K115Q+HN). Particles in cell supernatants were purified as described in legend to Figure 1. Panels show proteins present in each gradient fraction. Radioactively labeled infected cell extract was used as marker. Densities of fractions are shown at the bottom (g/cc). The control for this experiment is shown in Figure 1, panel A.
Minimal amounts of glycoprotein or NP were incorporated into VLPs after pairwise expression with M protein. To determine the contribution of NP, F or HN proteins to M protein-driven particle release, particles released from cells expressing all possible combinations of two proteins were isolated and characterized as described above. Pairwise expression of NP, F or HN proteins with M protein resulted in the release of particles containing both proteins (Figure 2.5, panel B). Intriguingly, however, particles contained only trace amounts of NP, F or HN proteins, while M protein was the predominant protein (Figure 2.5, panel B). The distribution of NP, F or HN proteins in the gradients was identical to that of M protein (Figure 2.5, panel B). In addition, the particle densities were very heterogeneous and were much like that of M protein-only particles. Surprisingly, the amounts of M protein containing particles were decreased upon co-expression of M protein with particularly NP but also with F or HN proteins (Figure 2.5, panel C).

Efficient incorporation of other viral proteins into VLPs required the expression of M protein and at least two of the other proteins. To examine the effects of co-expression of three viral proteins on particle release, cells were transfected with all possible combinations of three cDNAs (Figure 2.6, panel A). In contrast to the expression of a single glycoprotein with the M protein, co-expression of both F and HN glycoproteins with M protein resulted in significantly increased incorporation of both glycoproteins into particles (Figure 2.6, panels B and C). The F and HN proteins were detected in the same gradient fractions as M protein. Furthermore, the densities of the particles were more homogenous compared to those generated from cells expressing M
Figure 2.5. Effect of expressing NP, F or HN proteins with M protein on particle release. Avian cells, transfected with cDNAs encoding structural protein genes F-K115Q+M, HN+M or NP+M, as well as all four cDNAs were labeled in a pulse-chase protocol as described in legend to Figure 1. Particles present in the supernatants were concentrated and then floated into sucrose gradients as described in Materials and Methods. Panel A shows labeled proteins in cell extracts at end of pulse (top) and chase (bottom). Panel B shows the proteins present in each gradient fraction after immunoprecipitation of each fraction with an antibody cocktail. Densities (g/cc) of the fractions are shown at the bottom. Panel C show the quantification of each protein in particles in all fractions. Autoradiographs of gradient fractions from F-K115Q+NP, F-K115Q+HN and HN+NP expressing cells did not contain any protein and are shown with gels in Figure 4. Results are the average of three experiments and the standard deviation is shown.
protein alone (compare Figure 2.6, panel B and Figure 2.2, panel B) or M protein with a single glycoprotein. These results indicate that expression of both F and HN proteins with M protein is necessary for efficient incorporation of either glycoprotein into particles.

Expression of M protein with NP and either F or HN protein resulted in increased incorporation of NP as well as the glycoprotein into particles (Figure 2.6, panels B and C). The distribution of NP protein-containing particles in the gradient was similar to that of particles released from cells expressing all four structural proteins (Figure 2.6, panel B). Importantly, the densities of these particles were more homogeneous compared to particles released from cells expressing M protein alone, and were analogous to the density of the authentic virus or complete VLPs (compare Figure 2.6, panel B and Figure 1, panel B). Overall, these results indicate that M protein is necessary and sufficient for particle release and that expression of M protein with at least two other proteins is required for efficient incorporation of other proteins into particles.

**M protein co-localized with viral glycoproteins.** To explore further the role played by each protein in VLP assembly, we characterized, by immunofluorescence, the plasma membrane localization of M, F and HN proteins after their expression individually or after expression of combinations of NP, M, F and HN proteins. Transfected cells were incubated with anti-F protein or anti-HN protein antibodies prior to cell permeabilization to limit binding of antibodies to cell surface F or HN proteins. Cells were then permeabilized using 0.05% Triton X-100 and then incubated with M protein specific antibody. Figure 2.7, panel A, shows vector transfected control cells and as well as cells expressing individually M, F-K115Q or HN proteins. The F-K115Q and HN proteins
Figure 2.6. Effect of expressing all combinations of three viral proteins on particle release. Avian cells, transfected with all possible combinations of three NDV structural protein genes including M cDNA, were labeled in a pulse-chase protocol and particles in the supernatant were concentrated and floated into a sucrose gradient as in Figure 1. The proteins in the cell extracts were immunoprecipitated with the antibody cocktail. Panel A show labeled proteins in cell extracts at end of pulse (top) and chase (bottom). Panel B shows the proteins present in each gradient fraction after immunoprecipitation of each fraction with an antibody cocktail. Densities (g/cc) of the fractions are shown at the bottom. Panel C shows quantification of the amounts of each protein in particles in all fractions. Autoradiograph of particles released from NP, F, and HN protein expressing cells is shown in Figure 4.
were diffusely distributed on the surface of the cells (F-K115Q and HN panels). M protein exhibited diffuse cytoplasmic staining as well as punctate structures of various sizes (Figure 2.7, panel A, anti-M and merged image). Co-expression of either F or HN proteins with M protein (panel B) had little effect on the distribution of M protein, F protein, or HN protein (Figure 2.7, panel B, anti-M, anti-F and anti-HN images) and little to no co-localization of F or HN glycoproteins with M protein was observed (Figure 2.7, panel B, merged image). This finding correlates with the very low incorporation of F or HN proteins into M protein containing particles after pair-wise co-expression.

Co-expression of M protein with at least two other proteins slightly changed the distribution of M protein (Figure 2.7, panel C, anti-M images) and F and HN proteins (Figure 2.7, panel C, anti-F and anti-HN images) and increased the co-localization of M protein with either F or HN proteins (Figure 2.7, panel C, merged images). This result is consistent with increased incorporation of HN, F, or NP proteins when two proteins are co-expressed with M protein.

When all four proteins were co-expressed, the distribution of M protein was changed to more punctuate structures distributed mostly along the edges of the cells (Figure 2.7, panel D, anti-M images). Importantly, most of the F or HN protein signal co-localized with the M protein (Figure 2.7, panel D, merged images). This result is consistent with the more ordered assembly of VLPs when all four proteins are co-expressed.

Altogether, these results suggest that co-localization of viral proteins is detected with expression of three proteins and is most dramatic when NP, M, F and HN proteins...
**Figure 2.7. Co-localization of M protein with F and HN proteins.** The cell surface localization of NDV F and HN proteins and the cellular localization of M proteins were analyzed by immunofluorescence microscopy. Avian cells were either transfected individually (A) or with F-K115Q+M or HN+M (B), with NP+M+F-K115Q, NP+M+HN or M+F-K115Q+HN (C) and all 4 cDNAs (D). Nuclei were stained with DAPI (blue) 40 h post-transfection. Intact transfected cells were stained with rabbit anti-F protein antibodies or anti-HN protein antibodies as indicated in the panels. Cells were permeabilized with 0.05% Triton X-100 prior to incubation with anti-M protein antibody. Secondary antibodies were anti-rabbit Alexa 488 conjugate (green) and anti-mouse Alexa 568 conjugate (red). Images were taken at 60x magnification and were merged using Adobe Photoshop. Results shown are representative of all cells in the slide.
are co-expressed. These results also suggest that there are specific protein-protein interactions involved in assembling particles.

**There are specific viral protein interactions in VLPs.** To identify the specific protein interactions involved in VLP assembly, radioactively labeled particles formed with different combinations of proteins were solubilized in 1% Triton X-100 and proteins present were precipitated, separately, with cocktails of monospecific antibodies for M, HN or F proteins. Proteins were also precipitated with a mix of antibodies with specificities for all proteins in order to precipitate total proteins. First, each antibody cocktail precipitated all proteins from VLPs formed with M, HN, F and NP, although the efficiency of precipitation for each protein varied with the antibody specificity (Figure 2.8, panel A). These results are consistent with a network of interactions between all four proteins such that precipitation of one resulted in the precipitation of the other three proteins. The results also suggested that proteins indirectly linked to the precipitated protein were less efficiently precipitated than a protein directly linked to a precipitated protein. For example, anti-F protein antibody precipitated NP very efficiently (lane 3) but M protein very inefficiently (lane 3). This result suggests that there may be a direct link between F protein and NP but not F protein and M protein.

The interactions in VLPs were more clearly defined by precipitation of proteins from particles formed with all combinations of three proteins. When particles released from cells expressing M, F-K115Q and HN proteins were used in a similar co-immunoprecipitation procedure (Figure 2.8, panel B), anti-F protein precipitated only F protein and traces of HN protein (Figure 2.8, panel B, lane 3). This result indicates that
Figure 2.8. Co-immunoprecipitation of viral proteins in particles. Radioactively labeled particles released from cells expressing NP+M+F-K115Q+HN (A), M+F-K115Q+HN (B), NP+M+F-K115Q (C) and NP+M+HN (D) were purified into 20%-65% sucrose interface. Particles were solubilized in TNE buffer containing 1% Triton X-100 at 4°C for 15 min. Solubilized particles were then incubated with excess amounts of cocktail of anti-F protein antibodies (anti-HR1, anti-HR2, anti-Ftail, anti-F2-96 and monoclonal anti-F (G5)), anti-HN protein antibodies (mix of monoclonal antibodies), anti-M protein monoclonal antibody or cocktail of NDV-specific antibodies for overnight at 4°C. No antibody as well as pre-immune sera were used as negative controls. Immune complexes were precipitated with prewashed Pansorbin A for at least 2 h at 4°C with constant mixing. Samples were washed three times in cold TNE with 0.5% Triton X-100. All steps of co-immunoprecipitation were accomplished at 4°C. Proteins were resolved by SDS-PAGE as described in Materials and Methods. Results show one of three independent experiments, all with identical results.
the F protein does not directly complex with the M protein. Immunoprecipitation with anti-HN protein antibody precipitated M protein with HN protein (Figure 2.8, panel B, lane 4) and immunoprecipitation with anti-M protein antibody brought down HN protein with M protein (Figure 2.8, panel B, lane 5). These results strongly suggest that the M protein interacts with HN protein but not with the F protein.

When particles containing NP, M and F-K115Q were used in a similar immunoprecipitation protocol, complexes formed with anti-F protein antibody contained NP, as well as F protein, but not M protein (Figure 2.8, panel C, lane 3). Complexes formed with anti-M protein antibody contained NP as well as M protein but no F protein (Figure 2.8, panel C, lane 4). These observations indicate that M protein directly interacts with NP and that the F protein interacts with NP and confirm the lack of F and M protein interactions. Anti-M protein antibody does not indirectly precipitate detectible amounts of F protein. This result may be due to inefficient precipitation of NP protein, decreasing the amounts of F protein precipitated to very low levels. Alternatively, NP-NP interactions required to precipitate F protein with anti-M protein antibody may be disrupted by particle lysis.

When particles containing NP, M and HN were used, complexes formed with anti-HN protein antibody contained NP and M proteins as well as HN protein (Figure 2.8, panel D, lane 3). In addition, anti-M protein antibody precipitated NP and HN proteins (Figure 2.8, panel D, lane 4). These observations are consistent with the conclusion that the M protein interacts with both NP and HN proteins. These results cannot rule out an interaction between HN protein and NP.
Overall, results of co-immunoprecipitation of proteins in particles as well as results of cellular co-localization studies provide a rational basis for the incorporation of viral proteins into VLPs and suggest that specific protein interactions are involved in the assembly of an NDV virus-like particle.
D. Discussion

Paramyxoviruses spread through cell monolayers or through an organ by two mechanisms, successive rounds of virus infection and cell-cell fusion. Thus infected cell surfaces produce virus particles as well as fuse with adjacent cells. In order to begin to understand the relationships between these two processes in NDV infected cells, the protein requirements for assembly and release of virus-like particles were characterized. Avian cells, expressing the viral NP, M, HN and F proteins, released VLPs nearly as efficiently as virus. These particles released had protein ratios similar to infectious virus and their densities were homogeneous and only slightly less than that of authentic virus. The efficiencies of release of VLPs produced in three other paramyxovirus systems were 10% (SV), 34% (SV5), and 70% (SV) (Takimoto, Murti et al. 2001; Schmitt, Leser et al. 2002; Sugahara, Uchiyama et al. 2004). All previously described paramyxovirus systems have utilized human 293T cells for expression of viral proteins. We have found that NDV VLP release from avian cells, the natural host cell for NDV, had an efficiency of nearly 84%, but the efficiency of NDV VLP release from 293T or COS-7 cells was approximately 50% (unpublished observations). Therefore, differences between the efficiencies of VLP formation in the NDV system reported here and the efficiencies reported in other systems may be due to a cell type dependent effect. Thus NDV VLP assembly in avian cells represents an ideal system to explore protein requirements for assembly and release of virus particles.

Using avian cells, we found that the NDV M protein, and only M protein, was sufficient for particle release. Expression of M protein alone resulted in release of M
protein containing particles with an efficiency comparable to that observed when all four proteins were expressed, suggesting that no other protein is required for efficient release. Particles released from cells expressing NDV M protein alone were, however, very heterogeneous with respect to density. While the reasons for this finding are unclear, it is possible that budding of M protein particles occurred indiscriminately from different cell membranes with differing densities. Alternatively, it is possible that particles contained different lipid to protein ratios due to variable oligomerization of the M protein. M proteins of other negative stranded RNA viruses are reported to form oligomeric structures (Hewitt and Nermut 1977; Garoff, Hewson et al. 1998; Panchal, Ruthel et al. 2003). Particles formed from monomer M protein may have a higher lipid to protein ratio than particles formed from M protein in an oligomeric state. While M proteins of SV and hPIV1 were also shown to be sufficient for particle release (Coronel, Murti et al. 1999; Takimoto, Murti et al. 2001; Sugahara, Uchiyama et al. 2004), the SV5 M protein was not sufficient (Schmitt, Leser et al. 2002).

The NDV M protein was also necessary for particle release. In the absence of M protein expression, no other viral protein or combination of proteins resulted in significant particle release. By contrast, previous studies by two different groups reported that SV F protein exhibited an autonomous exocytosis activity demonstrated by the release of vesicles containing only the F protein (Takimoto, Murti et al. 2001; Sugahara, Uchiyama et al. 2004), although the level of release was very low. We found that cells expressing the NDV F protein alone did not release F protein containing material, results similar to those reported by Schmitt et al. in the SV-5 system (Schmitt,
Leser et al. 2002). We did observe a trace amount of very light density material that contained HN protein when this protein was expressed alone but no HN protein was released when it was co-expressed with combinations of NP and F-K115Q.

Although all studies agreed upon the central role played by the M protein in virus release, specific interactions of other viral proteins with M protein required for the assembly of complete VLPs are still poorly understood. To define these interactions required for NDV assembly, we used three approaches. First, we determined the requirements for efficient incorporation of NP, F, and HN proteins into particles by expressing all combinations of these proteins with M protein. Second, the protein interactions in particles formed with all combinations of three and four proteins were defined by co-immunoprecipitation. Lastly, the co-localization of cell surface HN and F proteins with M protein when expressed in different combinations with M and NP proteins were characterized.

Pairwise expression of NP, HN or F proteins with M protein resulted in only trace amounts of NP, HN or F proteins incorporated into M containing particles. In addition, expression of NP, F or HN proteins with M protein did not change the heterogeneous density of M protein containing particles. In contrast, co-expression of M protein with two other proteins significantly increased the incorporation of NP, HN, or F proteins into particles. The released particles had more homogenous density similar to that of particles containing all four proteins, a result that suggested necessary and specific interactions between the three proteins resulted in both efficient incorporation of NP or glycoproteins as well as more ordered particles. Furthermore, co-expression of two proteins with M
protein also significantly increased the co-localization of M protein with either HN or F proteins in the plasma membrane indicating increased interactions with M protein.

To define these protein-protein interactions, particles formed with different combinations of three and four proteins were solubilized with nonionic detergent and proteins precipitated with cocktails of monospecific antibodies for M, HN, or F proteins. Each antibody cocktail precipitated all proteins from VLPs formed with M, HN, F and NP, although the efficiency of precipitation for each protein varied with the antibody specificity. These results are consistent with a network of interactions between all four proteins such that precipitation of one resulted in the precipitation of the other three proteins but with efficiencies that varied determined by how directly a protein was linked to the precipitated protein.

The protein-protein interactions were more clearly defined by immunoprecipitation of proteins from particles formed with all combinations of three proteins. These results show a specific interaction between HN and M proteins, between NP and M protein, and between F protein and NP (diagramed in Figure 9). There is no evidence for a direct interaction between F protein and M protein. There is likely a weak interaction between F and HN proteins, since anti-F protein antibodies precipitated HN protein from particles containing M, HN, and F proteins. In addition, since there is no interaction between F and M proteins, incorporation of F protein into these particles must be accomplished by interactions with HN protein. Our results cannot rule out an interaction between HN protein and NP.
Figure 2.9. Protein-protein interactions in particles. Inset shows viral protein-protein interactions detected by co-immunoprecipitation of proteins in particles. Also shown are interactions proposed to result in assembly of particles formed by co-expression of all combinations of NP, F, and HN proteins with M protein.
Thus, when all four proteins are co-expressed, NP and HN protein are incorporated into VLPs by a direct interaction with M protein (Figure 2.9). F protein is likely incorporated indirectly due to interactions with NP and HN protein. An ordered complex of the four proteins is supported by the dramatic co-localization of M protein with F protein and M protein with HN protein in the plasma membrane when all four proteins are co-expressed.

However, when only F is expressed with M protein, F protein was likely not significantly incorporated into particles because there was no direct interaction between the two proteins (Figure 2.9). Supporting this conclusion is the observation that there was no co-localization of F and M proteins in the plasma membrane in these cells.

In spite of direct associations of M with NP, there was little NP protein incorporation into particles when NP and M proteins were co-expressed in the pair-wise combination. Previous reports show that the M protein of Sendai virus is recruited in the cytoplasm by the viral nucleocapsid (Stricker, Mottet et al. 1994). Perhaps NP causes the retargeting of M protein to this compartment. Indeed, co-expression of M protein with NP resulted in a 2.5 fold suppression of M protein containing particle release, a result also consistent with retention of M protein in cells by NP protein.

Although precipitations of particles proteins formed with M, HN, and F protein indicated a direct interaction of HN protein with M protein, there were only low levels of incorporation of HN protein into particles when HN and M proteins were co-expressed in a pairwise combination. Furthermore, there was little co-localization of the two proteins in the plasma membrane. Perhaps in the absence of other proteins, HN and M proteins
are never localized in the same regions of the cell, preventing their association. It is also possible that the conformation of the HN protein transmembrane or cytoplasmic tail may be different in the absence of expression of F protein or NP protein inhibiting association of HN protein with M protein. The reason for the 50% reduction of M protein particles upon co-expression of HN protein with M protein is unclear but has been previously reported in Sendai virus system (Sugahara, Uchiyama et al. 2004).

Particles formed with NP, M and F proteins are likely due to interactions between M and NP and interactions between F and NP (Figure 2.9). F protein may relocate NP to the plasma membrane drawing M to specific domains containing F protein. Indeed, addition of NP increases the co-localization of M protein with F protein in the plasma membrane. Particles formed with NP, M and HN proteins likely form due to interactions of both HN protein and NP with M protein (Figure 2.9). Expression of NP with HN and M proteins certainly increases the co-localization of M and HN proteins in the plasma membrane. Perhaps NP-M protein interactions alter the conformation of M facilitating its interaction with HN protein. Indeed, surface HN protein in the presence of NP appears more punctuate along the cell edges.

The network of interactions proposed here could account for the conclusions of Schmidt, et al that the cytoplasmic domains (CT) of the HN and F proteins have redundant functions (Schmitt, Leser et al. 2002). The CT domain of the F protein may target NP-M complexes to the plasma membrane by interactions with NP protein while the HN protein CT domain targets these complexes by virtue of direct interactions with M protein.
The proposed interaction of M protein and NP is supported by studies of Stricker, et al, of Sendai virus (Stricker, Mottet et al. 1994). Interaction of HN protein with M protein is consistent with numerous studies suggesting an interaction of M protein with viral glycoproteins in paramyxovirus-infected cells or in cells transfected with paramyxovirus cDNAs (Ali and Nayak 2000). Indeed, it has been reported that the respiratory syncytial virus G protein specifically interacts with M protein (Ghildyal, Li et al. 2005). However, there are no previous reports of a direct interaction between F protein and NP. It is possible that interactions between viral proteins vary within paramyxoviruses and the requirements for formation of VLPs may depend upon the distribution of late domains on the viral proteins.

The results presented here are consistent with our proposal that the NDV M protein buds indiscriminately from different cellular membranes in the absence of other viral proteins. When both glycoproteins are present in the plasma membrane, the M protein association with the plasma membrane may stabilize. NP association with F and M protein may also further stabilize and organize the network of interactions within the assembling particle.

In summary, we have established a VLP production system for NDV. We also showed that the M protein is sufficient and required for NDV particle budding. Moreover, there are specific protein-protein interactions in VLPs involved in the ordered assembly of particles. Interactions identified between M and HN or F and NP may play role in targeting M and NP into assembly sites in the plasma membrane.
CHAPTER III
RELEASE OF NEWCASTLE DISEASE VIRUS-LIKE PARTICLES REQUIRES
THE HOST VACUOLAR PROTEIN SORTING SYSTEM

A. Introduction

Paramyxoviruses, such as Newcastle disease virus (NDV), assemble in and bud from plasma membranes of infected cells. Assembly involves packaging of the approximately 16 KB negative-stranded RNA genome with the nucleoprotein (NP), the phosphoprotein (P), and the polymerase (L), to form the ribonucleoprotein (RNP) core. The core associates with cellular membranes modified with the hemagglutinin-neuraminidase (HN), and fusion (F) glycoproteins, and the matrix (M) protein, which lines the inner surfaces of the membrane (reviewed in (Lamb and Kolakofsky 2001; Takimoto and Portner 2004).

Assembly of paramyxovirus components involves specific viral protein-protein interactions, which are poorly defined. However, the M protein is thought to play a central role in these interactions, complexing with both the viral glycoproteins and the RNP core in the viral assembly site (Peeples 1991). This hypothesis is supported by the observation that the co-localization of viral glycoproteins with nucleocapsid was diminished in cells infected with measles virus that lacked the M gene (Cathomen, Mrkic et al. 1998). In addition, numerous studies have reported specific interactions between the M protein and NP (Stricker, Mottet et al. 1994; Schmitt and Lamb 2004; Takimoto and Portner 2004), attachment proteins (Sanderson, McQueen et al. 1993; Sanderson, Wu
et al. 1994; Schmitt, He et al. 1999; Ghildyal, Li et al. 2005), or F protein (Sanderson, Wu et al. 1994; Henderson, Murray et al. 2002).

Several reports have suggested that the M protein also plays a direct role in paramyxovirus budding and release. Sendai virus (SV) and measles virus (MV) with mutations in the M gene (Yoshida, Nagai et al. 1979; Yoshida, Hamaguchi et al. 1982) or which lack the M gene (Cathomen, Mrkic et al. 1998; Mebatsion, Weiland et al. 1999) are impaired in virion release, indicating that the M protein is required for release of particles. There is also direct evidence that some paramyxovirus M proteins are sufficient for budding (reviewed in (Schmitt and Lamb 2004; Takimoto and Portner 2004)). Similar to results obtained with the matrix-like proteins of retroviruses (Morita and Sundquist 2004), filoviruses (Jasenosky and Kawaoka 2004), rhabdoviruses (Jayakar, Jeetendra et al. 2004), and influenza viruses (Barman, Adhikary et al. 2004), expression of SV (Takimoto, Murti et al. 2001) and human parainfluenza virus type 1 (hPIV1) (Coronel, Murti et al. 1999) M proteins alone resulted in the release of M protein containing particles. The M protein of NDV is also both necessary and sufficient for release of membranous particles (Pantua, McGinnes et al. 2006). It has also been reported that the Nipah virus M protein is sufficient for release of particles (Ciancanelli and Basler 2006). These results indicated that these M proteins contain all the functional elements necessary for release of particles. In contrast, expression of the simian virus 5 (SV5) M protein, while necessary, was not sufficient for particle release (Schmitt, Leser et al. 2002).
It has been shown that the matrix-like proteins of many different groups of RNA viruses contain short sequences required for virus budding and are thus called viral late (L) domains (reviewed in (Freed 2002)). A key advance in understanding the budding process of enveloped RNA viruses came with the demonstration that the L domains in matrix-like proteins interact with components of the host vacuolar protein sorting system (VPS), presumably hijacking this pathway to direct virus release.

The VPS pathway has been implicated in release of simian virus 5 (SV5), since a dominant-negative mutant of Vps4 (Vps4-E228Q) inhibited release of SV5 virions as well as VLPs (Schmitt, Leser et al. 2005). In addition, a FPIV sequence in the SV5 M protein was identified as the functional L domain (Schmitt, Leser et al. 2005). However, since the SV5 M protein is not sufficient for VLP release (Schmitt, Leser et al. 2002), the FPIV sequence cannot be sufficient for SV5 VLP release. It has been shown that AIP1, a component of the host VPS system, (Strack, Calistri et al. 2003), binds to the SV accessory protein C. Furthermore, increased expression of AIP1 enhanced virus budding (Sakaguchi, Kato et al. 2005). However, no functional L domain sequence has been identified in the SV C protein. It has been reported that a mutation of the sequence YMYL in the Nipah virus M protein inhibits release (Ciancanelli and Basler 2006).

Here, we investigated the role of the host cell VPS pathway in NDV VLP release. We found that three different dominant negative mutant protein components of the VPS pathway inhibited particle release. Furthermore, a YXXL sequence (YANL) in the NDV M protein has properties of an L domain. Mutation of this sequence abolished particle release while substitution of this sequence with the classical late domains YPDL
or PTAP fully restored particle release. We also found that AIP1, which binds YXXL late domains (Strack, Calistri et al. 2003) is incorporated into VLPs.
B. Materials and Methods

Cells. Human renal epithelial cells expressing the SV40 T antigen (293T) were propagated in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, vitamins, non-essential amino acids, and glutamine.

Plasmids. The expression vector pCAGGS (Miyazaki, Takaki et al. 1989; Niwa, Yamamura et al. 1991) containing NDV cDNA sequences encoding NP, M, HN, or uncleaved F (F-K115Q) was used to transfect 293T. The F protein cDNA contained a point mutation in the cleavage site sequence at residue 115 (K115Q), which eliminated the furin recognition site (Li, Sergel et al. 1998). The pBJ5 expression vector containing the gene encoding a Flag-tagged Vps4A with the E228Q mutation (Strack, Calistri et al. 2003), N- and C-terminally HA-tagged AIP1, wild type Vps4A or wild type CHMP 3 were previously described (Strack, Calistri et al. 2003). The pDsRed2-N1 vector (Clontech) containing the gene encoding the CHMP3- and AIP1-Red fluorescent protein (RFP) fusion protein were previously described (Strack, Calistri et al. 2003).

Site-specific mutagenesis. Mutations in the M protein PKSP and YANL sequences at amino acids 216 and 219 and amino acids 232 and 235 were introduced by PCR to yield M-A_{216}A_{219} and M-A_{232}A_{235}, respectively. Specific sited-directed mutagenic primers were designed to substitute the proline residues at positions 216 and 219 and tyrosine and leucine residues at positions 232 and 235, respectively, with alanine. Additional mutant M genes were constructed by substituting PTAP or YPDL sequences for YANL at amino acid positions 232 to 235 using PCR and mutagenic primers designed to substitute these
sequences. The entire genes of each M protein mutant DNA were sequenced to verify that no additional mutation was introduced by the mutagenesis protocol. Mutations generated are illustrated in Figure 5.

**VLP production.** To produce VLPs, 293T cells were transfected with cDNAs using Lipofectamine (Invitrogen) and metabolically labeled with $[^{35}\text{S}]$ methionine and cysteine as described previously (Pantua, McGinnes et al. 2006). Briefly, sub-confluent 293T cells growing in 35-mm dishes were transfected with either pCAGGS M or mixture of pCAGGS-M, pCAGGS-NP, pCAGGS-F-K115Q and pCAGGS–HN. After 36 hours, media were replaced with DMEM without methionine and cysteine containing 50 μCi of $[^{35}\text{S}]$ methionine and $[^{35}\text{S}]$ cysteine mixture (NEG-772 EASYTAG™ Express Protein Labeling Mix, $[^{35}\text{S}]$, Perkin Elmer Life Sciences Inc.). After 4 hours of pulse label, one set of transfected plates was lysed, while in another set the medium was replaced with 1.0 ml of supplemented DMEM with 0.1 mM cold methionine (Nutritional Biochemicals Corporation). After 8 hours of nonradioactive chase, the medium was collected and the cells were lysed in 0.5 ml lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH7.4) containing Triton-DOC (1% Triton X-100, 0.05% sodium deoxycholate) and 1.25 mg N-ethylmaleimide (NEM). Cells were harvested and homogenized.

To determine if the VPS pathway is involved in VLP budding, sub confluent 293T cells were simultaneously transfected with either pCAGGS-M or all four major structural NDV protein genes (HN, F, M, and NP) and cDNAs encoding for dominant-negative mutants of AIP1, CHMP3 and Vps4A (Strack, Calisti et al. 2003).
Corresponding empty vectors or wild type genes were used as controls. Cells were incubated for 36 hours and cells were radioactively labeled as described above.

**VLP purification.** VLPs were purified from cell supernatants in protocols previously developed for virus purification (Levinson and Rubin 1966; McGinnes, Pantua et al. 2006). Briefly, cell supernatants were clarified by centrifugation at 5000 rpm for 5 min at 4°C, overlaid on top of a step gradient consisting of 3.5 ml 20% and 0.5 ml 65% sucrose solutions in TNE buffer (25mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA), and centrifuged at 24,000 rpm for 12 hours at 4°C using a SW50.1 rotor (Beckman). The 20%-65% sucrose interface (containing concentrated particles) was collected in 0.5 ml, mixed with 2.0 ml of 80% sucrose, and overlaid on top of 1.0 ml 80% sucrose (weight/volume) cushion. Additional layers of sucrose (1.0 ml of 50 % and 0.5 ml of 10% sucrose) were layered on top of the sample. The gradient was centrifuged at 38,000 rpm for 20 h at 4°C. The gradient was collected from the bottom into one 1ml fraction and eight 0.5 ml fractions using a polystaltic pump. Densities of each fraction were determined using a refractometer. For most experiments, VLPs used were from the interface of 20-65% sucrose of the first gradient.

**Antibodies.** Antisera used to precipitate viral proteins were a cocktail of anti-NDV antibodies. Antiserum used to precipitate NP was rabbit polyclonal antibody raised against UV inactivated NDV by standard protocols. Antiserum used to precipitate F protein were raised against glutathione S-transferase (GST) fusion proteins that contained amino acid sequences 130 to 173 (anti-HR1) (McGinnes, Gravel et al. 2002), 470 to 500 (anti-HR2) (Dolganiuc, McGinnes et al. 2003), or 96 to 117 (anti-F2-96). Antiserum used
to precipitate HN protein was raised against HN protein sequences from amino acid 96 to 117 (anti-A) (McGinnes and Morrison 1998). Antiserum used to precipitate M protein was a mouse monoclonal antibody raised against purified M protein (Faeberg and Peeples 1988). Antibody used to precipitate HA-tagged proteins was a mouse monoclonal HA antibody conjugated to agarose beads (Sigma). Secondary antibody used for immunoblotting was a peroxidase conjugated mouse monoclonal anti-HA antibody (Sigma).

**Immunoprecipitation, polyacrylamide gel electrophoresis and immunoblotting.**

Immunoprecipitation was accomplished using protocols described previously (Pantua, McGinnes et al. 2006). Briefly, one volume of cell lysate or sucrose gradient fraction were combined with two volumes of TNE buffer containing 1% Triton X-100. Samples were incubated with specific antibodies for 16 hours at 4°C with constant mixing. Immune complexes (ICs) were adsorbed to Protein A (Pansorbin Cells, CALBIOCHEM) for 2 hours at 4°C, pelleted, and then washed three times in immunoprecipitation (IP) wash buffer (phosphate buffer saline (PBS) containing 0.5% Tween-20 and 0.4% sodium dodecyl sulfate (SDS)). Anti-HA agarose beads (Sigma) were used to precipitate HA-tagged AIP-1. ICs were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer (125 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.4 % Bromphenol blue) with 1 M β mercaptoethanol (BME) and boiled. Proteins were separated on 8% polyacrylamide-SDS gels, dried and subjected to autoradiography. Immunoblotting for HA-tagged AIP1 was accomplished by using anti-HA antibody conjugated to horseradish
peroxidase (Sigma). Quantification of resulting autoradiographs and chemiluminescence gels were accomplished using a Fluor-S\textsuperscript{TM} MultiImager (BioRad).

**Immunofluorescence Microscopy.** Subconfluent 293T cells grown in 35 mm dish containing glass coverslips, were transfected with pCAGGS-Mwt, pCAGGS- M-A\textsubscript{232}A\textsubscript{235} or empty vector control alone. After 24 h, nuclei were stained with 5 μg/ml 4’,6-Diamidino-2-phenylindole (DAPI) for 30 min at 37°C. Cells were washed twice with ice-cold immunofluorescence (IF) buffer (PBS containing 1% bovine serum albumin, 0.02 % sodium azide, and 5 mM CaCl2), fixed with 2% paraformaldehyde, blocked with IF buffer for 2 h, and incubated for 1 h at 4°C in IF buffer containing purified ascites fluids containing anti-M protein monoclonal antibody. Cells were washed twice with ice-cold IF buffer, permeabilized with 0.05% Triton X-100, blocked with IF buffer for at least 2 h and incubated for 1 h at 4°C in IF buffer containing rhodamine conjugated goat anti-mouse IgG (Alexa 568) (Molecular Probes) secondary antibody. Cells were washed with ice-cold IF buffer, mounted onto slides using Vectashield mounting medium (Vector Labs, Inc) for immunofluorescence microscopy. Fluorescence images were acquired using a Nikon fluorescence microscope and Openlab software and processed using Adobe Photoshop.
C. Results

**VLPs were released from 293T cells.** We have previously shown that expression of M protein alone or co-expression of NP, M, F and HN proteins resulted in the release of VLPs from avian cells (Pantua, McGinnes et al. 2006). Because we wished to evaluate effects on particle release of available dominant negative mutant human VPS proteins, we asked if human renal epithelial cells (293T) could support the release of NDV VLPs. Figure 1, panel A, shows particles released from 293T cells expressing M protein alone or 293T cells co-expressing NP, M, F-K115Q and HN proteins (Figure 3.1, panel B). Particles released from 293T cells expressing M protein alone were very heterogeneous with respect to density (Figure 3.1, panel A), very similar to particles released from avian cells expressing M protein alone (Pantua, McGinnes et al. 2006). In contrast, VLPs released from 293T cells expressing all 4 major structural proteins were more homogenous in density. These particles were slightly less dense (1.18 g/cc) than the authentic virus (1.2 g/cc (Lamb and Kolakofsky 2001)) due to absence of genomic RNA. These results are also similar to those obtained using avian cells (Pantua, McGinnes et al. 2006). These combined results show that M protein VLPs and complete VLPs were released from 293T cells. However, the efficiency of release of particles from 293T cells, as measured by the percentage of pulse labeled M protein remaining in cells after a long nonradioactive chase, was lower than the efficiency of release from avian cells (50% vs. 84% (Pantua, McGinnes et al. 2006), respectively).
Figure 3.1. VLPs released from 293T cells. 293T cells transfected with pCAGGS M (panel A) or with mixture of pCAGGS-NP, -M, -F-K155Q, and –HN (panel B), were radioactively labeled with $[^{35}\text{S}]$ methionine and $[^{35}\text{S}]$ cysteine for 4 hours (P) and then chased in non-radioactive medium for 8 hours (C). Proteins present in cell lysates were immunoprecipitated with a cocktail of antibodies specific for all viral proteins and the precipitated labeled proteins are shown on the left side of each panel. Particles in cell supernatants were purified as described in Materials and Methods. After flotation into sucrose gradients (right side of each panel), each gradient fraction was immunoprecipitated with the antibody cocktail. The density of each fraction (g/cc) is shown at the bottom. Panel C: Extract (upper panel) and M containing particles that were purified onto a 20%-65% sucrose interface (middle panel) were treated with different concentrations (0.25, 0.5, 1, 5, 10, and 20 $\mu$g/ml; lanes 2 to 7 respectively) of Proteinase K for 30 min on ice. In parallel, purified particles were incubated in 1% Triton X-100 prior to Proteinase K treatment (bottom panel). After incubation with protease, reactions were stopped by adding 0.1 mM PMSF. Proteins were immunoprecipitated using NDV protein-specific antibodies and resolved by SDS-PAGE. HN, hemagglutinin-neuraminidase protein; $F_0$, uncleaved fusion protein; NP, nucleoprotein; M, matrix protein.
To determine if the M protein released from these cells was enclosed in membranes, the sensitivity of M protein to protease was measured. M protein particles were either left untreated or treated with different concentrations of proteinase K. As a control, cell extracts from M protein-expressing cells were also included. As expected, M protein in cell extracts was digested by the protease (Figure 3.1, panel C, top). In contrast, M protein in particles was significantly protected from protease digestion (Figure 3.1, panel C, middle). Disruption of particle membranes by incubation with 1% Triton X-100 prior to treatment with proteinase K resulted in the digestion of M protein (Figure 3.1, panel C, bottom). These results demonstrate that particles released from 293T cells were membrane-bound particles.

**Dominant negative forms of VPS molecules inhibited VLP release.** Since previous studies have implicated the VPS pathway in budding of other enveloped RNA viruses (Pornillos, Garrus et al. 2002; Demirov and Freed 2004; Morita and Sundquist 2004), we asked whether this pathway is involved in M protein-driven particle release. To address this question, we used a dominant-negative (dn) mutant CHMP3 protein. CHMP3 is a subunit of the ESCRT III complex (von Schwedler, Stuchell et al. 2003). Fusion of CHMP3 with red fluorescent protein (RFP) transforms this protein into dn protein that has been shown to inhibit HIV-1 gag particle release (Strack, Calistri et al. 2003). 293T cells were transfected with pCAGGS-M with different concentrations of either empty vector or cDNAs encoding the dn mutant protein. Figure 3.2, panel A, shows that co-expression of M protein with different concentrations of dn-CHMP3 did not inhibit M protein expression. However, dn-CHMP3 inhibited the release of M protein VLPs, even
Figure 3.2. Titration of dominant-negative CHMP3 and Vps4-E228Q. Panel A, left, shows pulse labeled extracts of 293T cells that were simultaneously transfected with pCAGGS-M (1.0 μg) and either pDsRed2-N1 vector (0.1, 0.5 and 1.0 μg) or pDsRed2-N1-CHMP3-RFP (0.1, 0.5 and 1.0 μg). Panel A, right, shows the particles (M-VLPs) released from these cells after an 8-hour nonradioactive chase. Panel C, left, shows extracts of pulse labeled cells that were simultaneously transfected with pCAGGS-M and either pBJ5 vector or pBJ5-Vps4A-E228Q. Panel C, right, shows the particles (M-VLPs) released from these cells after an 8-hour chase. Transfected 293T cells in both A and C were labeled in a pulse-chase protocol as described in Materials and Methods. Particles in cell supernatants were purified and concentrated onto a 20% and 65% sucrose interface as described in Materials and Methods. Proteins were immunoprecipitated using NDV protein-specific antibodies and resolved by SDS-PAGE. Panels B and D show percent of particles released from cells transfected with pCAGGS-M and pDsRed2-N1-CHMP3 or pBJ5-Vps4A-E228Q, respectively, relative to those released from cells transfected with pCAGGS-M and vector only. Panels B and D show the average of two separate experiments.
at low concentrations of dn-CHMP3 DNA (Figure 3.2, panels A and B). Vps4A is dominant negative form of Vps4, which is an ATPase that catalyzes the disassembly of the ESCRT components (Babst, Sato et al. 1997; Babst, Wendland et al. 1998). As shown in Figure 3.2, panels C and D, Vps4A-E228Q inhibited release of M protein particles but not the expression of M protein.

To determine if inhibition of particle release was due only to over expression of these proteins, 293T cells were transfected with vector control, or wild type CHMP3 or wild type Vps4A as well as their dominant negative counterparts. We also included the wild type and dn mutant forms of the protein, AIP1 (Strack, Calistri et al. 2003). In all cases, the wild type form of the VPS protein had little effect on particle release. However, release of M protein particles was inhibited by dn-CHMP3 to about 90% (Figure 3.3, panels A and B). Vps4A-E228Q inhibited M protein VLP release by about 90% (Figure 3.3, panels C and D), and AIP-1-RFP inhibited particle release by 90% (Figure 3.3, panels E and F).

Figure 3.4 shows that the dominant negative forms of CHMP3, Vps4A, and AIP1 but not the wild type proteins also inhibited the release of VLPs containing all four viral proteins.

These combined results show that the inhibition of VLP release observed was not due to over expression of the VPS protein, but rather due to specific effects of the dn mutant proteins. These results support the conclusion that an intact VPS pathway is essential for M protein particle release.
Figure 3.3. Effect of wild type and dominant-negative VPS mutant proteins on M protein-VLP release. Panel A shows pulse labeled cell extracts (top), extracts from cells subjected to an 8 hour chase (middle), and corresponding released particles (bottom) from cells co-transfected with pCAGGS-M and either pDsRed2-N1 vector (lane 1), pBJ5-WT-CHMP3 (lane 2) or pDsRed2-N1-CHMP3-RFP (lane 3). Panel C shows pulse-labeled and pulse-chase cell extracts of 293T cells (top, middle, respectively) and corresponding released particles (bottom) from cells co-transfected with pCAGGS-M and either pBJ5 vector (lane 1), pBJ5-WT-Vps4A (lane 2) or pBJ5-Vps4A-E228Q (lane 3). Panel E shows pulse labeled and pulse-chase extracts of 293T cells (top, middle, respectively) and corresponding M-VLPs (bottom) from cells co-transfected with pCAGGS-M and either pDsRed2-N1 vector (lane 1), pBJ5-AIP1-HA (lane 2) or pDsRed2-N1-AIP1-HA-CHMP3-RFP (lane 3). M-VLPs are released from pulse labeled
cells during an 8-hour nonradioactive chase. Particles were purified as described in legend to Figure 3.2. Proteins were immunoprecipitated using NDV protein-specific antibodies and resolved by SDS-PAGE. Panels B, D and F show quantification of particles released relative to those released from vector controls. Standard deviations are shown.
Figure 3.4. Effect of dominant-negative mutants CHMP3, Vps4-E228Q and AIP1 on the release of complete VLPs. Panels A shows pulse labeled extracts of 293T cells (lanes 1 to 3), extracts after an 8 hour chase (lanes 4-6) and corresponding released VLPs (lanes 7-9) from cells co-transfected with of NDV cDNAs, encoding NP, M, HN and F proteins, and either pDsRed2-N1 vector (lanes 1, 4, and 7), pBJ5-WT-CHMP3 (lanes 2, 5, and 8) or pDsRed2-N1-CHMP3-RFP (lanes 3, 6, and 9). Panel C shows pulse labeled extracts of 293T cells (lanes 1 to 3), extracts of cells after an 8 hour chase (lanes 4-6) and corresponding released VLPs (lanes 7-9) from cells co-transfected with the mixture of four NDV cDNAs and either pBJ5 vector (lanes 1, 4, and 7), pBJ5-WT-Vps4A (lanes 2,
5, and 8) or pBJ5-Vps4A-E228Q (lanes 3, 6, and 9). Panel E shows pulse labeled extracts of 293T cells (lanes 1 to 3), extracts of cells subjected to an 8 hour chase (lanes 4-6) and corresponding VLPs (lanes 7-9) from cells co-transfected with the mixture of NDV cDNAs and either pDsRed2-N1 vector (lanes 1, 4 and 7), pBJ5-AIP1-HA (lanes 2, 5, and 7) or pDsRed2-N1-AIP1-HA-CHMP3-RFP (lanes 3, 6, and 9). VLPs, released from pulse labeled cells during an 8-hour nonradioactive chase, were purified as described in legend to Figure 3.2. Proteins were immunoprecipitated using NDV protein-specific antibodies and resolved by SDS-PAGE. Panels B, D and F show quantification of VLPs released relative to wild type VPS protein controls. Standard deviations are shown.
**Mutation of the YANL sequence inhibited VLP release.** Results in Figure 1 as well as previous results (Pantua, McGinnes et al. 2006) suggest that NDV M protein contains a fully functional and necessary L domain. NDV M protein has two possible L domain sequences, PKSP and YANL, which are similar to the classical L domains PTAP and YPXL (reviewed in (Freed 2002)), respectively. To determine whether these sequence motifs play a role in M protein-driven particle budding, we substituted the proline residues in the PKSP sequence with alanine (M-A\textsubscript{216}A\textsubscript{219}) and tyrosine and leucine in the YANL sequence with alanine (M-A\textsubscript{232}A\textsubscript{235}) (Figure 3.5, panel A). These mutant M proteins were expressed either individually (Figure 3.5, panel B) or in combination with NP, F-K115Q and HN proteins (Figure 3.5, panel D). Figure 3.5, panels B-E, shows that similar amounts of particles were released from cells expressing the M-A\textsubscript{216}A\textsubscript{219} mutant and cells expressing wild type M protein. In striking contrast, there was a significant reduction of particles released from cells expressing the M-A\textsubscript{232}A\textsubscript{235} mutant (Figure 5, panels B and C). Similarly, co-expression M-A\textsubscript{232}A\textsubscript{235} mutant protein with NP, F-K115Q and HN proteins resulted in 80% reduction in particles released (Figure 3.5, panels D and E).

To determine if the inhibition of particle release by mutation of the YANL sequence was due to elimination of L domain activity or defects in conformation of the M protein, we substituted the YANL sequence separately with two known classical L domain sequences, YPDL and PTAP (Strack, Calistri et al. 2003; Morita and Sundquist 2004). Figure 3.5, panels B and C, shows that the amounts of M-particles released from cells expressing the NDV M protein containing the substituted YPDL or PTAP motifs
Figure 3.5. L domain in NDV M protein. Panel A shows wild type M protein sequences and mutant M proteins with alanine substitutions at amino acid positions 216 and 219 (M-A216A219), 232 and 235 (M-A232A235), and YPDL and PTAP substitutions at
positions 216 to 219 and 232 to 235, respectively. Panel B shows pulse lysate (top) and chase (middle) lysate, and M-VLPs released (bottom) from 293T cells expressing wild type or mutant M proteins. Panel D shows pulse lysate (top) and chase (middle) lysate, and VLPs released (bottom) from 293T cells expressing NP, F and HN proteins with either wild type or mutant M proteins. Particles were purified as described in Legend to Figure 3.2. Proteins were immunoprecipitated using NDV protein-specific antibodies and resolved by SDS-PAGE. Panels C and E shows quantification of VLPs released relative to wild type M protein. Standard deviations are shown.
Figure 3.6. Cell association of wild type M protein and M-A_{232}A_{235} mutant protein. Wild type and mutant M protein in cells were visualized by immunofluorescence microscopy using anti-M protein monoclonal antibody (left panels). 293T cells were transfected with pCAGGS-M wt, pCAGGS-M-A_{232}A_{235}, or empty vector. Cell nuclei were stained with DAPI (blue) 24 h post-transfection (middle panels). Right panels show merged images. Cells were fixed in 2% paraformaldehyde and permeabilized with 0.05% Triton X-100 prior to incubation with anti-M protein antibody. Secondary antibodies were anti-mouse Alexa 568 conjugate (red). Images were taken with a 60x objective. Results shown are representative of all positive cells in the slide.
were comparable to wild type levels. Cells expressing these substituted M proteins along with NP, F-K115Q and HN proteins released wild type levels of VLPs (panels D and E). These results strongly indicate that the YANL sequence at position 232 to 235 in the NDV M protein functions as an L domain.

**Mutation in the YANL sequence resulted in retention of M protein in cells.** Since expression of M-A$_{232}$A$_{235}$ protein did not support the release of particles, we explored the cellular localization of the mutant M proteins, by immunofluorescence microscopy. Figure 3.6 shows that both the wild type and mutant M proteins were predominantly present at the edges of the cells and no signal in the empty vector control. Interestingly, significantly greater amounts of M-A$_{232}$A$_{235}$ proteins were retained in the cells (compare top and middle panels) with a significant concentration of the signal at the cell plasma membrane, as would be expected for a mutation that inhibits particle release. There was also some mutant protein in large vesicles. This material did not appear to be in the same focal plane as the nucleus (not shown), in contrast to results reported for mutant Nipah virus M proteins (Ciancanelli and Basler 2006). The large vesicles may represent the back up of excess M protein in intracellular membranes. Altogether, these results further support our conclusion that the YANL sequence is a functional late domain in the NDV M protein.

**AIP-1 incorporated into VLPs.** Retrovirus particles, which have a gag protein with an YPXL L domain, contain AIP1 (Strack, Calistri et al. 2003). We consistently observed a polypeptide with an approximate size of 100kD, the size of AIP1, in the SDS-PAGE gels containing NDV VLP proteins (data not shown). To test the possibility that AIP1 is
Figure 3.7. Incorporation of AIP1 in VLPs. 293T cells were transfected with pCAGGS M and either empty vector, or vector with HA-tagged AIP1. Panel A shows radioactively labeled M protein precipitated (IP) from cell extracts and M-VLPs using M protein-specific monoclonal antibody. HA-AIP1 (N-terminally tagged) and AIP1-HA (C-terminally tagged) were detected in extracts and M-VLPs by immunoblotting (IB) using HA antibody conjugated with peroxidase. Panel B shows precipitated radiolabeled M protein (left) and AIP1-HA (right) from cell extracts (top) and VLPs (bottom).
incorporated into VLPs, we co-expressed the M protein with either N- (HA-AIP1) or C-terminally (AIP1-HA) HA-tagged AIP1 or with vector alone. Figure 3.7, panel A, shows that M protein particles released from both cells expressing M protein and AIP1-HA contained AIP1-HA. HA-AIP1 was not incorporated. Figure 3.7, panel B, shows that AIP1-HA can also be precipitated from purified disrupted VLPs. These results demonstrated that AIP1 is incorporated into VLPs and suggest that AIP1 may interact directly or indirectly with the M protein in particles.
D. Discussion

In order to explore the mechanisms involved in paramyxovirus assembly and release, we have characterized VLPs released from cells expressing NDV proteins. We have previously shown that VLPs with properties similar to authentic virus were very efficiently released from avian cells expressing NP, M, F and HN proteins (Pantua, McGinnes et al. 2006) and that expression of M protein is both necessary and sufficient for particle release from these cells. Here we showed that NDV VLPs were also released from the human cell line, 293T, expressing the NP, F, HN, and M proteins although the efficiency of release from these cells was less than from avian cells. M protein was also sufficient for particle release from these cells. These results allowed us to explore the role of dominant-negative mutants of human VPS proteins in NDV particle release.

The matrix-like proteins of many enveloped RNA viruses play a pivotal role in virus assembly and release due in part to the presence of short motifs, or late domains. Late domains are sequences that interact with components of host vacuolar protein sorting (VPS) system. The cellular VPS system is associated with multivesicular bodies (MVBs), structures created by invagination of endosomal membranes into the endosomal lumen, thereby resulting in vesicles inside a vesicle (reviewed in (Martindale 2003; Babst 2005)). Since the topology of MVB formation is similar to that of virus budding from plasma membranes, it has been proposed that viral proteins usurp and redirect this host cell machinery to mediate virus budding (reviewed in (Martindale 2003; Demirov and Freed 2004; Morita and Sundquist 2004)).
The formation of MVBs requires three protein complexes, first characterized in yeast, which are collectively known as the endosomal sorting complexes required for transport (ESCRT I, II, and III) (Katzmann, Babst et al. 2001; Jiang, Erickson et al. 2002; Katzmann, Stefan et al. 2003; Raiborg, Rusten et al. 2003; Babst 2005). In addition, the Vps4 protein, which is an ATPase, is required for the dissociation of the full ESCRT complex important for the recycling of the complex (Raiborg, Rusten et al. 2003). Studies on retrovirus budding have clearly shown that the VPS pathway plays an essential role in virus budding. Inhibition of the VPS pathway by either dominant-negative mutants of specific VPS proteins (Garrus, von Schwedler et al. 2001; Strack, Calistri et al. 2003) or by small inhibitory RNA (Garrus, von Schwedler et al. 2001; Martin-Serrano, Zang et al. 2003; Tanzi, Piefer et al. 2003) specific for selected VPS proteins inhibited virus or VLP release.

A dominant negative Vps4 protein blocked release of SV5 virions or VLPs composed of NP, HN, F, and M proteins, implicating the VPS system in SV5 release (Schmitt, Leser et al. 2005). Extending these results, we found that a dominant negative version of Vps4, Vps4 A-E228Q (Martin-Serrano, Yarovoy et al. 2003; Strack, Calistri et al. 2003; von Schwedler, Stuchell et al. 2003), blocked NDV VLP release. Furthermore, our results demonstrated that this dominant negative protein blocked release of particles containing only M protein. We also found that a dominant negative version of CHMP3 (Strack, Calistri et al. 2003), a subunit of the ESCRT III complex (Babst 2005), and a dominant negative mutant of AIP1, a protein that binds both ESCT I and III proteins (Strack, Calistri et al. 2003), inhibited NDV VLP release as well as release of particles
containing only M protein. This inhibition was not due to over expression of the protein since transfection of the wild type versions of these proteins had little effect on M particle release. These results show that an intact VPS pathway is essential for both complete VLP and M particle release.

Many studies have demonstrated that L domains in the matrix proteins of viruses mediate their interaction with specific molecules of the VPS pathway (reviewed in (Freed 2002; Freed 2004; Morita and Sundquist 2004; Bieniasz 2006)). Three L domain motifs, PTAP, YPXL, and PPXY (reviewed in (Freed 2002; Pornillos, Garrus et al. 2002)), have been identified in retroviruses (Puffer, Parent et al. 1997; Strack, Calistri et al. 2003), rhabdoviruses (Jayakar, Murti et al. 2000; Irie, Licata et al. 2004), and filoviruses (Irie and Harty 2005; Irie, Licata et al. 2005). An YRKL sequence has been identified as a late domain in orthomyxoviruses (Hui, Barman et al. 2006). The PTAP sequence binds TSG101 (tumor susceptibility gene 101) protein, a component of ESCRT I (Huang, Orenstein et al. 1995). The YPXL sequence has been shown to interact with AP2 (adaptor protein 2) (Chen, Vincent et al. 2005) and AIP1 (Strack, Calistri et al. 2003). The YRKL sequence in the influenza virus M1 protein binds to VSP28, an ESCRT 1 protein that binds tsg101, as well as Cdc42, a member of the Rho family of GTP-binding proteins. The PPXY motif binds to Nedd4-like (neural precursor cell expressed, developmentally down regulated gene 4) ubiquitin ligases (Xiang, Cameron et al. 1996; Vana, Tang et al. 2004).

Paramyxovirus M proteins do not have a PTAP, an YPXL, an YRKL, or a PPXY motif. Lamb and colleagues have recently reported that the sequence FPIV in the SV5 M
protein was a late domain (Schmitt, Leser et al. 2005). Mutation of this sequence inhibited release of particles and addition of this sequence in a retrovirus gag construct stimulated the release of particles. However, since the SV5 M protein is not sufficient for SV5 particle release (Schmitt, Leser et al. 2002), this sequence cannot function independently as a late domain in the context of this paramyxovirus M protein. Thus, it is not clear how SV5 uses the VPS pathway or how the FPIV sequence might function as a late domain. Sequence analysis of the NDV M protein shows the presence of this FPIV motif. In addition, NDV M protein contains a PKSP and a YANL sequence, not found in the SV5 M protein. We have shown here that the YANL motif, but not the PKSP sequence, has properties of an L domain. Mutation of this sequence eliminated particle release and resulted in significant accumulation of the mutant protein in the cell plasma membrane. There was no obvious re-localization of the protein into the nucleus as reported for mutant Nipah virus M proteins (Ciancanelli and Basler 2006). Most importantly, substitution of this sequence with known late domains, either PTAP or YPDL, fully restored particle release. Thus inhibition of particle release by mutation of the YANL sequence is not likely due to effects on protein folding since very different sequences restore activity. These results also show that the NDV M protein may access the VPS pathway using either type of late domain, YPDL or PTAP domain. These results also indicate that the FPIV sequence, in the context of the NDV M protein, cannot function as a late domain independent of the YANL sequence since the YANL mutant protein M-A232-A235 has a wild type FPIV sequence.
YPDL late domains have been shown to interact with the VPS protein AIP1 (Strack, Calistri et al. 2003). Indeed, we found that AIP1 protein can be found in released particles containing M protein further implicating the VPS pathway in NDV release. The M protein of Sendai virus has also been shown to be sufficient for release of particles (Takimoto, Murti et al. 2001; Sugahara, Uchiyama et al. 2004). This protein has a YLDL sequence, which might serve as a late domain for SV M protein. While the SV5 M protein does not have a YXXL motif, the SV5 NP protein has a number of YXXL motifs including a YPLL sequence. Perhaps the required SV5 late domain is present on the SV5 NP rather than the M protein. Indeed, Schmitt, et al (Schmitt, Leser et al. 2002) have reported that SV5 VLP release is significantly enhanced by the expression of the SV5 NP protein with M protein as well as a glycoprotein. Differences in requirements for the release of particles in different paramyxovirus systems may be due in part to different distributions of the late domains on structural proteins. However, mutation of the NDV M protein YANL sequence eliminated particle release from cells expressing not only M protein but also NP, HN and F proteins. Thus there are no late domains in the NDV NP that can function independently as a late domain in the context of the viral glycoproteins and M protein.

In summary, we have shown that the host cell VPS pathway is necessary for M protein budding. We have also shown that the YANL motif in the NDV M protein has the properties of a late domain.
CHAPTER IV
CHARACTERIZATION OF AN ALTERNATE FORM OF NEWCASTLE DISEASE VIRUS FUSION PROTEIN

A. Introduction

Newcastle disease virus (NDV) is a major agricultural pathogen that causes a fatal respiratory and neurological disease in poultry (Nagai, Hamaguchi et al. 1989). This virus, a member of the Paramyxoviridae family, initiates infection by fusion of the viral membrane with host cell plasma membranes (Lamb and Kolakofsky 2001). Virus spread is facilitated by cell-cell membrane fusion. NDV, as well as other paramyxoviruses, encodes two spike glycoproteins, the hemagglutinin-neuraminidase (HN) protein, which mediates attachment of the virion to sialic acid-containing receptors, and the fusion (F) protein, which directly mediates membrane fusion (reviewed in reference (Lamb and Kolakofsky 2001)). Primary sequence and structural analyses indicate that the F protein is a classical type 1 glycoprotein with an amino-terminal signal sequence, a hydrophobic transmembrane domain near the carboxyl terminus, and a 25 to 30-amino-acid cytoplasmic tail domain (CT) (Lamb and Kolakofsky 2001; Morrison 2003). The F protein is synthesized as a precursor, $F_0$, which undergoes proteolytic cleavage to form disulfide-linked amino-terminal $F_2$ and carboxyl-terminal $F_1$ polypeptides, and cleavage is required for fusion activity (reviewed in reference (Lamb and Kolakofsky 2001)).

There are now several examples of both cellular (Dunlop, Jones et al. 1995; Hegde, Mastrianni et al. 1998; Lu, Turnbull et al. 2000) and viral glycoproteins that are found in different topological forms with respect to membranes. Examples of viral
glycoproteins with alternate membrane topologies include the hepatitis B virus L protein (reference (Lambert and Prange 2001) and references therein), the transmissible gastroenteritis virus M protein (Escors, Camafeita et al. 2001), and the hepatitis C virus envelope glycoproteins (Pavio, Taylor et al. 2002; Migliaccio, Follis et al. 2004). We previously reported that synthesis of the NDV F protein in a cell-free protein-synthesizing system containing membranes resulted in at least two topological forms of the protein with respect to membranes (McGinnes, Reitter et al. 2003). The properties of one form were entirely consistent with a type 1 fully glycosylated F protein. The other was a partially translocated or polytopic form in which approximately 200 amino acids of the amino terminus as well as the CT domain of the protein were translocated across membranes (McGinnes, Reitter et al. 2003). Importantly, we detected this second, polytopic form of F protein in COS-7 cells expressing the F protein (McGinnes, Reitter et al. 2003) and provided evidence that it was involved in cell-cell fusion, either directly or indirectly.

Extending these results, we report that the second form of the F protein is also found in F protein-expressing avian cells, which are the natural host cells of NDV. We have detected this second form of the F protein in virions released from both infected COS-7 and avian cells. We report evidence that this second form of the F protein may have a role in virus-cell fusion.
B. Materials and Methods

Cells, virus, and plasmids. COS-7 cells, obtained from the American Type Culture Collection (ATCC), were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with non-essential amino acids, vitamins, penicillin-streptomycin, and 10% fetal calf serum. East Lansing Line (ELL-0) chicken fibroblasts (UMNSAH/DF-1), obtained from ATCC, were maintained in DMEM supplemented with penicillin-streptomycin and 10% fetal calf serum.

NDV strain AV (virulent) and strain B1 (avirulent) stocks (Nagai, Hamaguchi et al. 1989) were prepared by growth in eggs by standard protocols. AV stocks formed plaques in COS-7 cells, while B1 did not, consistent with the expected phenotypes of the two strains of NDV. The F protein gene carried by purified NDV, strain B1, virus was sequenced to verify the absence of a furin recognition sequence. The NDV F and HN genes were expressed in COS-7 and ELL-0 cells using pCAGGS obtained from Common Access to Biotechnological Resources and Information (CABRI) (Niwa, Yamamura et al. 1991).

Infections and virus purification. COS-7 or ELL-0 cells were plated at 6 X 10^5 per 35-mm plate and grown overnight. Cells were then infected with NDV, strain AV, or NDV, strain B1, at a multiplicity of infection of 10. After adsorption, unbound virus was removed and cells were washed in phosphate buffered saline (PBS) and then incubated for 16 or 48 h. Supernatants were clarified by centrifugation at 5,000 rpm for 5 min at 4°C, and virions were pelleted through 20% sucrose by centrifugation at 40,000 rpm for 4 h at 4°C using the SW50.1 rotor (Beckman). Virus was resuspended in 100 μl of PBS.
**Transfection.** Transfections were accomplished using Lipofectamine (Invitrogen) as recommended by the manufacturer. COS-7 or ELL-0 cells were plated at 3 X 10^5 per 35-mm plate. After 20 h, a mixture of DNA (0.5μg) in 0.1 ml OptiMEM (Gibco/Invitrogen) and 5 μl of Lipofectamine in 0.2 ml of OptiMEM was incubated at room temperature for 45 minutes, diluted with 0.7 ml of OptiMEM, and added to a 35-mm plate previously washed with OptiMEM. The cells were incubated for 5 h, the Lipofectamine-DNA complexes were removed, and then 2 ml of supplemented DMEM was added.

**Antibodies.** Anti-NDV is a polyclonal antiserum raised in rabbits against UV-inactivated virions by standard methods. Anti-HR1 was raised against a glutathione S-transferase (GST) fusion protein that contained sequences from amino acid 130 to 173 of the F protein cloned in frame with the carboxyl terminus of GST (McGinnes, Gravel et al. 2002) Anti-Ftail 523-553 was raised against a synthetic peptide with the sequence of the cytoplasmic domain of the fusion protein (amino acids 523-553) as previously described (Wang, Raghu et al. 1992) and prepared by the Peptide Core Facility of the University of Massachusetts Medical School. Anti-F2 was raised against a GST fusion protein containing the sequence between amino acid 96 and 117 cloned in frame with the carboxyl terminus of GST. Anti-Ftail 540-553 was raised against a GST fusion protein containing the sequence between amino acid 540 and 553 cloned in frame with the carboxyl terminus of GST. The GST fusion polypeptides were purified by standard methods recommended in the Novagen Applications Guide. Antibody was raised in rabbits by standard methods by Capralogics, Inc. (Hardwick, MA). Antibodies recognizing GST were removed from the sample by affinity purification using
immobilized GST (Pierce immobilized GST). Antibodies raised against HR1 and Ftail sequences were recovered using Protein A IgG purification (Pierce Immunopure Plus Immobilized Protein A IgG Purification Kit). Actin was detected using phalloidin coupled to Alexa 568 (Invitrogen/Molecular Probes).

**Surface Immunofluorescence.** COS-7 or ELL-0 cells, grown in 35-mm plates containing glass cover slips, were either infected with NDV, strain AV or strain B1, for 12 or 16 h, respectively. COS-7 or ELL-0 cells were transfected with cDNAs as described above for 48 or 24 h, respectively. Cells were washed twice with ice-cold IF buffer (PBS containing 1% bovine serum albumin, 0.02% sodium azide, and 5 mM CaCl₂), blocked with IF buffer for 15 minutes and incubated for 1 hour at 4°C in IF buffer containing specific antibodies, washed three times with ice-cold IF buffer, and incubated for 1 hour on ice with IF buffer containing Alexa 488-labeled anti-rabbit immunoglobulin G (IgG) (Molecular Probes). Cells were washed with ice-cold IF buffer, fixed with 2% paraformaldehyde, and mounted on slides using Vectashield mounting medium (Vector Labs, Inc.) for immunofluorescence microscopy.

Fluorescence images were acquired using a Nikon fluorescence microscope and Openlab software.

**Flow cytometry.** Transfected cells were removed from plates with 0.2 ml trypsin (50 μg/ml) (Sigma Corp.), washed in FACS buffer (PBS containing 1% BSA and 0.02% sodium azide) containing soybean trypsin inhibitor (2 μg/ml), and incubated for 1 h at 4°C with anti-Ftail (540-553) and anti-NDV antibodies diluted in FACS buffer. After three washes with FACS buffer, the cells were incubated for 1 h at 4°C with goat anti-
rabbit IgG coupled to Alexa 488 (Molecular Probes) diluted in FACS buffer. After three washes with FACS buffer, the cells were resuspended in PBS containing 2% paraformaldehyde and subjected to flow cytometry.

**Preparation of extracts and Western analysis.** COS-7 or ELL-0 cells, infected with NDV for 12 and 16 h (multiplicity of infection of 10), were washed in PBS and lysed in RSB buffer (0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl) containing 1% Triton X-100, 0.5% sodium deoxycholate, 2 mg/ml of N-ethylmaleimide, and 0.2 mg/ml of DNase. Freshly made total extracts, diluted in sample buffer (125 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol) with 0.7 M β-mercaptoethanol and incubated at room temperature, were loaded onto 8% or 12% polyacrylamide gels without boiling to avoid protein aggregation. After electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris, pH 8.2, 192 mM glycine, 15% methanol) and transferred to Immobilon-P (Millipore Corp.) membranes. The membranes were blocked overnight at 4°C in PBS containing 0.5% Tween 20 and 10% non-fat milk, washed with PBS-Tween 20, and incubated for 1 h at room temperature with primary antibody diluted to 1:1000 in PBS-Tween 20. Membranes were then washed, incubated for 1 h at room temperature with secondary antibody, goat anti-rabbit IgG coupled to horseradish peroxidase (Amersham Biosciences) diluted to 1:40000 in PBS-Tween 20, and then washed extensively in PBS-Tween 20. Bound antibody was detected using the ECL Western blotting detection reagent system (Amersham Biosciences).

**Mass Spectrometry.** Protein bands were digested “in gel” according to established methods (Lahm and Langen 2000). Eluted peptides were separated on a LC Packings
Ultimate nanoflow HPLC system in the following way. Ten microliters of the peptide digest solution (approximately one half of the total digest) was manually injected onto a micro trap column (LC Packings Precolumn cartridge 0.3mmx5mm C18PM) and the trap column was manually washed with 10 μl of 0.1% formic acid prior to switching in line with the reverse phase separating column (LC Packings 100 μm x 15 cm C18 PepMap). A gradient was developed from 100% solvent A (0.1% formic acid) to 60% solvent B (0.1% formic acid in acetonitrile: water 70:30) in 60 minutes at a flow rate of 500 nanoliters per minute. The outlet of the column was connected to an electrospray needle (New Objective Taper tip 20 μm). Electrospray mass spectrometry was performed on a Thermoelectron Finnigan LCQDeca ion trap mass spectrometer.

Data dependent acquisitions were set up according to a triple play experiment program where full MS scans from 400 Da to 2000 Da were on going until an MS signal grew above a specified threshold upon which a high resolution scan (Zoom Scan) was performed to determine monoisotopic mass and charge state followed by a single MS/MS scan. Dynamic exclusion was applied to prevent repeat scans of the same peptide masses.

The raw data files were converted into mass peak lists using the LCQ_DTA program and then searched against the virus taxonomy of NCBI nr protein database using the Mascot search engine (Matrix Science Ltd.) (www.matrixscience.com) using 1Da mass tolerances for both the parent and fragment masses.

Neutralization assay. Confluent COS-7 or ELL-0 cells in 60mm plates were used for plaque assays. NDV, strain AV, was pre-incubated with anti-NDV, anti-Ftail antibodies,
or pre-immune sera for 1 h at room temperature prior to adsorption. The virus and antibody mixes were then added to cells and incubated for 45 minutes at 37°C. After adsorption, the virus-antibody mixture was removed and cells were washed once in PBS. For plaque assays, cells were then overlaid with agar diluted to 1% in DMEM and supplemented with non-essential amino acids, vitamins, penicillin-streptomycin, sodium bicarbonate and 10% fetal bovine serum. Plaques were counted after 48 h of incubation at 37°C. Cells were fixed in methanol and stained with Giemsa Stain (Sigma) diluted 1:20 in distilled water for photography. For immunofluorescence detection of infected cells, complete media was added after removal of unbound virus and antibody, and cells were incubated for 9 h at 37°C. Surface immunofluorescence was accomplished as described above.
C. Results

Two forms of F Protein Are Expressed in Infected Avian Cells. Because a second form of paramyxovirus F protein has not been previously reported in other systems, it seemed possible that the alternate form of the NDV F protein could be an unusual property of protein expression in COS-7 cells. Thus we asked if this second form of NDV F protein could be detected in avian cells, cells that are the natural host cell for NDV.

Unambiguous detection of the second, partially translocated form of F protein by Western analysis required the expression of an F protein that was not cleaved, since the partially translocated F protein co-migrated on polyacrylamide gels with the F₀ cleavage product, F₁ protein (McGinnes, Reitter et al. 2003). Therefore, we utilized the B1 strain of NDV, a strain that encodes an F protein missing a furin recognition sequence at the cleavage site. The B1 F protein, synthesized in tissue culture cells, is not proteolytically cleaved (Nagai, Hamaguchi et al. 1989; Lamb and Kolakofsky 2001; Morrison 2003). NDV, strain AV, encodes an F protein with a furin recognition site and the F protein present in NDV, strain AV, infected cells is proteolytically cleaved into F₁ and F₂ (Nagai, Hamaguchi et al. 1989; Lamb and Kolakofsky 2001; Morrison 2003).

Avian cells (ELL-0 cells), as well as COS-7 cells, were infected with NDV, strain B1, as well as NDV, strain AV, for 12 and 16h, and F proteins present in the resulting cell extracts were characterized by Western analysis using F protein-specific antibodies. As previously reported (McGinnes, Reitter et al. 2003), two forms of F protein from B1-infected COS-7 cells were detected (Figure 4.1, panel A, lanes 3 and 6), the fully
Figure 4.1. Two forms of F protein are expressed in infected avian and COS-7 cells. COS-7 (panel A) and avian cells (ELL-0) (panel B), infected with NDV strain AV (lanes 2 and 5), strain B1 (lanes 3 and 6), or mock infected (UI)(lanes 1 and 4), for 16 (lanes 1 to 3) and 24 (lanes 4 to 6) hours, were lysed and aliquots of total extract were subjected to Western analysis as described in Materials and Methods. Blots were incubated with anti-HR1. Molecular weight markers were included in each blot and were used to determine sizes of the bands detected. Panel C shows Western analysis of 1X and 2X volume of extracts from COS-7 and avian cells transfected with pCAGGS NP (lanes 2, 3, 5, and 6). Duplicate blots were incubated with anti-NDV or anti-HR1. Untransfected (UT), lane 1.
translocated F₀ protein (68 kDa) and a 59KDa protein. As expected, extracts from AV-infected COS-7 cells contained two polypeptides, a 68 KDa F₀ and a 59 KDa F₁ (Fig 4.1 panel A, lanes 2 and 5). Proteins from ELL-0 cells contained a 68 KDa host band, which obscured the F₀ polypeptide from both B1 and AV infected cells (Figure 4.1, panel B, lanes 1-6). However, ELL-0 cell infection with B1 virus resulted in the synthesis of significant amounts of a 59 KDa polypeptide (Figure 4.1 panel B, lanes 3 and 6), a polypeptide the size of the partially translocated F protein. These results show that the second form of F protein can be detected in B1-infected avian cells.

To eliminate the possibility that anti-F protein antibody cross-reacted with NP, which is also 59 kDa, avian and COS-7 cells were transfected with NP cDNA, and proteins present in the resulting cell extracts were detected by Western analysis using anti-NDV and anti-HR1 antibodies. The results show that NP is only recognized by anti-NDV antibody and not by anti-HR1 antibody (Figure 4.1, panel C). Thus the 59-kDa protein detected with anti-F antibodies in B1 infected cells is not due to a cross reaction of the antibodies with NP.

**Surface Expression of Cytoplasmic Tail Sequences.** Our analysis of the cell free translation products directed by F protein mRNA also showed that the F protein CT domain was translocated across membranes (McGinnes, Reitter et al. 2003) leading to the proposal that the partially translocated F protein may be a polytopic protein with the carboxyl terminus as well as the amino terminus of the protein exposed on cell surfaces. In support of this idea, we found that CT sequences could be detected on surfaces of COS-7 cells expressing the NDV F protein using a polyclonal antibody
Figure 4.2. Detection of cytoplasmic tail sequences on the cell surfaces of transfected and infected avian cells by immunofluorescence. Panels B-E show results with NDV-infected avian cells. Panels F-L show results with transfected avian cells. Uninfected avian (panel A) and avian cells infected with NDV, strain AV (panels B and D), strain B1 (panels C and E) were incubated with anti-NDV (panels A to C) or anti-Ftail540-553 (panels A, D and E). Infected cells incubated with anti-NDV and anti-Ftail antibodies were digitally exposed for 3 sec. Avian cells transfected with vector alone (panel F), transfected with pCAGGS-Fwt (panels G and J), pCAGGS-Fd523-553 (panels H and K), or pCAGGS-Fwt and pCAGGS-HN (panels I and L), were incubated with anti-NDV (panels F - I) and anti-Ftail 540-553 (panels F and J to L). Transfected cells incubated
with anti-NDV and anti-Ftail antibodies were digitally exposed for 3 secs. Images were acquired using a 60x objective and OPEN LAB software.
We asked if F protein CT sequences could be detected on infected or transfected avian cell surfaces (Figure 4.2). We also raised another polyclonal antibody specific for the carboxyl terminal half of the F protein CT domain (amino acids 540 to 553) to eliminate the possibility that our previous results were due to a non-specific effect of the anti-Ftail 523-553 antibody (Figures 4.2 and 4.3).

ELL-0 and COS-7 cells were infected with NDV AV or B1 strains. In addition, ELL-0 and COS-7 cells were transfected with vector alone, wild type F (Fwt) protein cDNA, cDNA encoding an F protein with a cytoplasmic tail deletion (Fd523-553), or a mixture of Fwt protein and HN protein cDNAs. Intact cells were incubated with anti-Ftail 540-553 antibody, and binding of antibody was assessed by cell surface immunofluorescence. Both infected and transfected ELL-0 cells, as well as COS-7 cells, bound this anti-Ftail antibody (Figures 4.2 and 4.3, panels D, E, J, L). In contrast, binding of anti-Ftail antibody to cells expressing Fd523-553 protein was negative (Figure 2 and 3, panel K) although these cells were positive for anti-NDV antibody (Figure 4.2 and 4.3, panel H), which further confirms specificity of the anti-Ftail antibody for the CT sequences. None of the antibodies bound to uninfected or vector transfected cells (Panels A and F). Binding of anti-NDV antibody was detected in parallel cultures (Figures 4.2 and 4.3, panels B, C, G, H, and I).

Detection of CT sequences on surfaces of infected or transfected cells was not due to permeabilization of cells during antibody binding. Using the same conditions,
Figure 4.3. Detection of cytoplasmic tail sequences on the cell surfaces of infected and transfected COS-7 cells by immunofluorescence. Panels B to E show results with NDV-infected COS-7 cells. Panels F to P show results with transfected COS-7 cells. Uninfected COS-7 cells (panel A) and COS-7 cells infected with NDV, strain AV (panels B and D), strain B1 (panels C and E) were incubated with anti-NDV (panels A, B, and C) or anti-Ftail540-553 (panels A, D, and E). Infected cells incubated with anti-NDV and anti-Ftail antibodies were digitally exposed for 3 sec. COS-7 cells transfected with vector alone (panel F), transfected with pCAGGS-Fwt (panels G and J), pCAGGS-Fd523-553 (panels H and K), or pCAGGS-Fwt and pCAGGS-HN (panels I and L, M, N, O, and P), were incubated with anti-NDV (panels F to I) or anti-Ftail540-553 (panels F and J to L). Transfected cells incubated with anti-NDV and anti-Ftail antibodies were digitally exposed for 3 secs using OPEN LAB software. Intact cells expressing HN and F proteins were incubated with anti-NDV and phalloidin-Alexa568 (panels M and N, respectively). Cells expressing HN and F proteins were incubated in 0.1% Triton X-100 and then anti-NDV and phalloidin-Alexa568 (panels O and P, respectively).
fluorescence labeled phalloidin, which binds to actin, did not stain intact cells (Figure 4.3, panel N) although phalloidin readily stained actin after permeabilizing cells in 0.1% Triton X-100 (Figure 4.3, panel P).

To confirm expression of CT sequences on avian cell surfaces, binding of the anti-Ftail 540-553 antibody was also analyzed by flow cytometry. Figure 4.4 shows that cells expressing F protein bound anti-Ftail 540-553 antibody, while cells expressing F_{d523-553} or HN proteins were negative (Figure 4.4, panels B and D). These results show that CT sequences can be detected on the surfaces of both avian and COS-7 cells expressing the NDV F protein using two different anti-Ftail antibodies.

**Two Forms of F Protein are in Virions.** We next asked if the polytopic form of F protein was present in virions. NDV, strain B1, virions were generated from infected ELL-0 or COS-7 cells. NDV, strain AV, virions were also prepared in parallel. Figure 4.5 shows Western analysis of B1 virion proteins, as well as AV virion proteins, using two different F protein specific polyclonal antibodies. F protein specific bands detected were similar to those found in the infected cell extracts. As expected, F_0 and F_1 polypeptides were detected in AV virions using anti-HR1 and anti-Ftail antibodies (Figure 4.5, panel A). Similarly, F_0 was detected in B1 virions grown from both COS-7 and ELL-0 cells using anti-HR1 and anti-Ftail antibodies. Interestingly, a 59 kDa polypeptide was also detected in B1 virions by both anti-HR1 and anti-Ftail antibodies (Figure 4.5, panel A). To determine if the 59 kDa polypeptide was due to unexpected cleavage of F_0 protein, we used anti-F_2 antibody, an antibody specific to F_2 polypeptide.
Figure 4.4. Detection of cytoplasmic tail sequences on the surfaces of cells transfected with Fwt cDNA by flow cytometry. Avian cells (panels A and B) and COS-7 cells (panels C and D) transfected with pCAGGS vector alone, pCAGGS-Fwt, pCAGGS-Fd523-553, or pCAGGS-HN and were incubated with anti-NDV (panels A and C) and anti-Ftail540-553 (panels B and D). FL1-H, fluorescein isothiocyanate (FITC).
Detection of F₂ polypeptide in B1 virions would suggest that the 59 kDa polypeptide was due to cleavage of F₀ polypeptide. Figure 4.5, panel B, shows that while the F protein from the AV virions was cleaved, generating an F₂ polypeptide, F₂ is not present in B1 virions indicating that F protein was not cleaved in these virions.

To verify this conclusion, NDV, strain B1, virions were purified from supernatants of avian infected cells and the polypeptides that migrated with a molecular weight of 59 kDa were analyzed by mass spectrometry. As expected, peptides derived from the viral NP and P proteins were present in the band. In addition, F protein-specific peptides were present (Table 1) supporting the conclusion that a form of the F protein comigrated with the NP and P proteins. Furthermore, peptides from both the F₂ and F₁ regions of the protein were detected. F₁ specific peptides detected were from the CT domain of the F protein and from the HR1 domain. Surprisingly, F₂ peptides detected were from the signal sequence of the F protein. These findings suggest that the p59 F polypeptide contains sequences from the uncleaved F₀ and that the signal sequence was not cleaved from the alternate form of the F protein. These results are consistent with the presence of a second form of F protein, an uncleaved protein with a molecular weight of 59 kDa.

**Effect of Anti-F-tail Antibodies on Plaque Formation.** Since the second form of F protein was detected in virions generated from both infected avian and COS-7 cells, we asked if it has a role in plaque formation. We asked if anti-F-tail antibodies could neutralize virus infectivity in a plaque assay. Egg-grown NDV, strain AV, was incubated
Figure 4.5. Two forms of F protein are present in virions. NDV, strain AV (panel A, lanes 2, 5, 8, and 11) (panel B, lanes 2 and 5) and strain B1 (panel A, lanes 3, 6, 9, and 12) (panel B, lanes 3 and 6), generated from COS-7 (panel A, lanes 2, 3, 8, 9)(panel B, lanes 2,3) and avian cells (panel A, lanes 5, 6, 11, and 12) (panel B, lanes 5, 6) were purified as described in Materials and Methods. Virus particles were subjected to Western analysis using anti-HR1 antibody (panel A, lanes 1 to 6), anti-Ftail antibody (panel A, lanes 7 to 12) or anti-F2 antibody (panel B, lanes 1 to 6) antibodies. Uninfected (UI) supernatant, panel A, lanes 1, 4, 7, and 10, and panel B, lanes 1 and 4.
Virions were purified from the supernatants of NDV strain B1-infected avian cells and proteins in the purified virions electrophoresed on 8% polyacrylamide gels in the presence of a reducing agent. The proteins in the gel were stained with Coomasie blue dye, and the p59-sized band was excised and subjected to analysis by mass spectrometry as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Peptide*</th>
<th>Location in F protein</th>
<th>Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPVPLMLIRVMLILSCIR</td>
<td>F₂</td>
<td>Signal sequence</td>
</tr>
<tr>
<td>SPTNPAPMLMTVR</td>
<td>F₂</td>
<td>Signal sequence</td>
</tr>
<tr>
<td>QNAANILR</td>
<td>F₁</td>
<td>HR1 domain</td>
</tr>
<tr>
<td>LKESSIAATNEAVHEVTNGLSQLAVAVGK</td>
<td>F₁</td>
<td>HR1 domain</td>
</tr>
<tr>
<td>MQQFVNGQFNNTAQELDCIK</td>
<td>F₁</td>
<td>HR1 domain</td>
</tr>
<tr>
<td>TLLWLGNNTLQGDQR</td>
<td>F₁</td>
<td>CT domain</td>
</tr>
</tbody>
</table>

*Virions were purified from the supernatants of NDV strain B1-infected avian cells and proteins in the purified virions electrophoresed on 8% polyacrylamide gels in the presence of a reducing agent. The proteins in the gel were stained with Coomasie blue dye, and the p59-sized band was excised and subjected to analysis by mass spectrometry as described in Materials and Methods.
with pre-immune sera, anti-NDV, or anti-Ftail antibodies (both anti-Ftail 523-553 and anti-Ftail 540-553) and then plated on monolayers of avian or COS-7 cells. After adsorption, unbound virus and antibody was removed and the cells were washed prior to addition of agar overlays. Thus effects of the antibody should reflect effects on the initial virus cell interaction and not subsequence cell-cell fusion. After 40 hours plaques were visible on control plates (Figure 4.6, panels B, C, H, and I). As expected, anti-NDV neutralized virus infectivity and no plaques were observed (Figure 4.6, panels D and J). The anti-Ftail540-553 reduced the titer approximately 2 to 18 fold in COS-7 and 20 fold in ELL-0 cells compared to the pre-immune sera and no antibody controls (Table 2).

Most importantly, however, plaques formed after incubation with both anti-Ftail antibodies were pinpoint (Figure 4.6 panels E, F, K, and L), much smaller than those produced by untreated virus or virus incubated with pre-immune serum (Figure 4.6 panels B, C, H and I). These results suggest that the anti-Ftail antibodies slow plaque formation. This result is not likely due to a nonspecific effect of antibody raised against a GST fusion protein. Incubation of virus with antibody raised against HR2 sequence coupled to GST protein did not inhibit plaque size or number (unpublished observations).

**Effect of Anti-Ftail antisera on a single cycle of infection.** The effect of anti-Ftail antibodies on plaque size suggested that the anti-Ftail antibodies, bound prior to virus attachment, inhibited virus entry. To test this possibility by an alternative approach, effects of anti-Ftail antibody on a single cycle of infection were monitored by immunofluorescence. Virus incubated with anti-Ftail antibody, anti-NDV antibody, anti-β galactosidase, or preimmune sera, as described in Figure 4.6, was then bound to cell
surfaces. After adsorption, unbound virus and antibody was removed and cells were washed and incubated for 9 hours at 37°C. Infected cells were detected by the presence
Figure 4.6. Anti-Ftail antibodies alter plaque morphology. COS-7 (panels A to F) and avian cells (panels G to L) were infected with untreated NDV, strain AV, (panels B and H), or NDV that was pre-incubated with pre-immune sera (panels C and I), anti-NDV (panels D and J), anti-Ftail540-553 (panels E and K) or anti-Ftail523-553 (panels F and L). Uninfected monolayers, panels A and G. Plaques were counted and stained with 1:20 Giemsa and distilled water after 48 hours of incubation at 37°C.
TABLE 4. 2. Effect of anti-NDV and anti-Ftail antibodies on plaque formation in COS-7 and ELL-0 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Titer of virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>COS-7</td>
</tr>
<tr>
<td></td>
<td>Expt 1</td>
</tr>
<tr>
<td>UI</td>
<td>0</td>
</tr>
<tr>
<td>No Ab</td>
<td>$3.45 \times 10^{10}$</td>
</tr>
<tr>
<td>Pre</td>
<td>$3.25 \times 10^{10}$</td>
</tr>
<tr>
<td>Anti-NDV</td>
<td>$&lt;1.0 \times 10^9$</td>
</tr>
<tr>
<td>Anti-Ftail</td>
<td></td>
</tr>
<tr>
<td>540-553$^b$</td>
<td>$5.00 \times 10^8$</td>
</tr>
<tr>
<td>523-553$^b$</td>
<td>$2.15 \times 10^{10}$</td>
</tr>
</tbody>
</table>

$^a$ Dilutions of 10-9 for NDV strain AV were preincubated with anti-NDV and anti-Ftail antibodies for 1 h prior to adsorption. No Ab, without antibody; Pre, preincubated with preimmune sera; UI, mock infected. Antibody was 150 ug protein per ml.

$^b$ Plaques are pinpoint.
Figure 4.7. Anti-Ftail antibodies block infection. COS-7 cells were infected at an MOI of 10 with untreated egg grown NDV, strain B1, (panels C and D), or NDV, strain B1, that was pre-incubated with pre-immune sera (panels E and F), anti-β galactosidase (panels G and H), anti-NDV (panels I and J), or anti-Ftail540-553 (panels K and L) as described in Materials and Methods. Uninfected monolayers are shown in panels A and B. After 9 hours of infection, cells were incubated with Hoechst stain, in order to visualize nuclei of all cells in the monolayer, and then anti-NDV antibody. Binding of anti-NDV antibody was visualized by Alexa 568 coupled to goat anti-rabbit antisera. Images were acquired with a 20X objective using OPEN Lab software. Hoechst stained images were digitally exposed for 0.5 sec and anti-NDV images were digitally exposed for 2.5 sec. Identical results were obtained in two separate experiments.
COS-7 cells were co-transfected with cDNAs encoding the HN and F (HN + F) proteins or with a comparable amount of vector DNA. At 40 h post-transfection, avian red blood cells were incubated with cells in the presence of the indicated antibodies at 4°C for 30 min. Binding was quantified by measuring released hemoglobin after lysis of the attached red blood cells as previously described (24).

<table>
<thead>
<tr>
<th>Cells</th>
<th>Antibody</th>
<th>% RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector-transfected cells</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>HN + F cDNA-transfected cells</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Anti-NDV</td>
<td>10 ± 5</td>
</tr>
<tr>
<td></td>
<td>Anti-HR1</td>
<td>128 ± 50</td>
</tr>
<tr>
<td></td>
<td>Anti-Ftail</td>
<td>108 ± 40</td>
</tr>
</tbody>
</table>

"Effect of anti-Ftail antibodies on attachment activity of HN."
of proteins on cell surfaces that bound anti-NDV antibody. Figure 4.7, panels C and D, show the presence of virus proteins on infected cell surfaces after infection with untreated virus while uninfected cells were negative for anti-NDV staining (panels A and B). Incubation of virus in pre-immune serum or non-specific antiserum had no effect on the infection (panels E, F, G, and H), while incubation with anti-NDV antibody inhibited infection as expected (panels I and J). Incubation of virus with anti-Ftail 540-553 inhibited infection (Figure 4.7, panels K and L) consistent with results of the plaque assay.

This inhibition by anti-Ftail antibody was not likely due to an inhibition of virus binding. Cells co-transfected with HN and F protein cDNAs were incubated with avian red blood cells in the presence or absence of antibody. As expected, anti-NDV blocked the binding of RBCs to these monolayers while binding of RBCs in the presence of anti-Ftail antibodies was unaffected (Table 3).
D. Discussion

We have previously reported that NDV F protein, synthesized in a cell-free protein synthesizing system, containing membranes, inserted into membranes in at least two ways (McGinnes, Reitter et al. 2003). One form of the protein (p68) was typical of a type 1 glycoprotein anchored in membranes by a transmembrane domain located near the carboxyl terminus. The second form (p59) had properties consistent with the partial translocation of the protein, translocation of the F_2 domain and the amino terminus of the F_1 domain. In addition, we reported evidence for the translocation of the CT domain of the protein. Based on these observations, we suggested that the second form of F protein was a polytopic protein that spanned membranes at least twice. We also reported the detection of this second form of F protein in infected and transfected COS-7 cells. A polypeptide consistent with the size of this second form was detected by Western analysis of proteins in extracts of infected cells. In addition, CT sequences were detected on the surfaces of cells expressing the NDV F protein.

Because of the unexpected nature of our findings and because this second form of F protein has not been reported in other paramyxovirus systems, we were concerned that formation of a polytopic NDV F protein in COS-7 cells could be attributed to a translocation defect in primate cells of a glycoprotein normally expressed in avian cells. However, as described above, we detected this second form of F protein in avian cells, both in infected cells as well as cells transfected with F protein cDNA. First, we detected, by Western analysis, a polypeptide the size of the polytopic F protein (p59) as well as the fully translocated F_0 protein (p68) in tissue culture grown avian cells infected...
with an avirulent strain of NDV, strain B1, as well as in purified virions derived from these cells. Cells infected with this virus should express only uncleaved protein (Nagai, Hamaguchi et al. 1989; Nagai 1993; Lamb and Kolakofsky 2001; Morrison 2003) and, indeed, virus released from cells infected with this virus do not form plaques in the absence of trypsin. However, it was possible that the F protein was cleaved accounting for the p59 form of the protein. To verify that the p59 polypeptide was F protein and that it included the full-length polypeptide rather than only a cleaved form of the protein, we analyzed, by mass spectroscopy, the p59 material from tissue culture derived, purified virus particles. As expected, the material contained NP and P protein. Significantly, F protein specific peptides were detected in this band indicating that detection of F protein sequences by Western analysis was not due to a cross reaction of anti-F protein antibodies to another protein. Furthermore, peptides from the F2 region of the protein, the HR1 domain, and the CT domain were detected indicating that sequences from the entire F0 protein were present in this polypeptide.

Interestingly, the F2 sequences detected in the p59 material by mass spectroscopy were from the signal sequence of the protein, suggesting that the signal sequence was not cleaved from this polypeptide. This surprising finding suggests that the second form of F protein is anchored in membranes not only by sequences in the HR1 domain and the transmembrane domain but also the signal sequence. The presence of peptides from the signal sequence also indicates that the material is not from contaminating fully translocated F0 protein (p68) since it has been reported that the signal sequence is cleaved from the fully translocated F protein (Gorman, Nestorowicz et al. 1988). It should be
noted that the two peptides detected from the signal sequence are overlapping but have two amino acid differences. Heterogeneity in the signal sequence is not unexpected since comparisons of signal sequences from F proteins of many different strains of NDV have shown that the sequence of this region of the F protein is highly variable (Toyoda, Sakaguchi et al. 1989).

Another indication for the existence of the alternate form of F protein was the detection, on avian cell surfaces, of sequences from the CT domain of the F protein of both the AV and B1 strains of NDV. To eliminate the possibility that our previous detection of surface expressed CT sequences in COS-7 cells (McGinnes, Reitter et al. 2003) was due to an unusual cross reactivity of that anti-Ftail antibody preparation, we prepared a second polyclonal antibody by different protocols using only the carboxyl terminal half of the F protein CT domain as an immunogen. This antibody also detected surface expressed F protein CT sequences. Furthermore, deletion of the CT domain of F protein eliminated the binding of both antibodies to transfected ELL-0 and COS-7 cell surfaces (these results and (McGinnes, Reitter et al. 2003)) and shows that the antibodies were not binding to an unrelated polypeptide. Thus the existence of an unusual form of the F protein is indicated by Western analysis and mass spectroscopy of proteins in cell extracts and virions and by detection of CT sequences on infected and transfected cell surfaces.

The functional significance of a second form of the F protein is a key question. It is possible that the alternate F protein is an aberrant form that has no direct role in the virus infection. Alternatively, the expression of this form of the F protein may serve to
down regulate the expression of the fully translocated F protein while not having a direct role in infection. However, detection of the second form of the F protein in purified virions released from both infected avian and COS-7 cells led us to explore a role of this polypeptide in membrane fusion.

Current models for type 1 fusion proteins propose that these proteins are initially folded into a metastable conformation (Baker, Dutch et al. 1999; Eckert and Kim 2001; Jardetsky and Lamb 2004). Upon activation of fusion, it is proposed that these proteins undergo a series of conformational changes that involve, first, the insertion of a fusion peptide into the target membrane, followed by the association of heptad repeat domains into a very stable, coiled coil structure. The structure of this coiled coil as well as the anchoring of the protein to both the target and viral membrane by the fusion peptide and the transmembrane domain, respectively, results in pulling the target and viral membrane into close proximity (Baker, Dutch et al. 1999; Eckert and Kim 2001; Jardetsky and Lamb 2004). Membrane merger proceeds first by hemifusion, then pore formation and expansion (Earp, Delos et al. 2005).

We previously reported that antibody specific for CT sequences inhibited red blood cell fusion to cells expressing the NDV HN and F proteins (McGinnes, Reitter et al. 2003). The antibody inhibited hemifusion suggesting that antibody binding interfered with the initial stages in the onset of fusion. Here we explored the effects of anti-Ftail antibodies on virus entry. To limit effects of the antibody to virus-cell fusion and not cell-cell fusion, we removed unbound antibody after virus binding. We showed that two different antibodies specific for CT sequences considerably slowed plaque formation
suggesting a role of surface exposed CT sequences in virus-cell fusion. Furthermore, in a single cycle of infection, pre-incubation of anti-Ftail antibody with virions eliminated infection. These combined results suggest that antibody bound to the surface exposed CT sequences inhibited both virus-cell fusion (Figures 6 and 7) as well as cell-cell fusion (McGinnes, Reitter et al. 2003).

Since the antibody inhibited hemifusion, initial steps of fusion are affected. It is possible that binding of the antibody to CT sequences sterically inhibits conformational changes in the fully translocated F protein required for initiation of fusion and that the CT domain has no direct role in the fusion process. Alternatively, the CT domain could be involved in the formation of the metastable form of the fully translocated F protein or the activation of that form required for fusion. The presence of the anti-Ftail antibody could inhibit this activation.

Previous reports indicate that CT domains of paramyxovirus F proteins do have a role in fusion (Sergel and Morrison 1995; Bagai and Lamb 1996; Dutch and Lamb 2001; Tong, Li et al. 2002; Seth, Goodman et al. 2004; Waning, Russell et al. 2004). Deletion of the CT domain of the NDV F protein interferes with complete fusion (Sergel and Morrison 1995). It has been proposed that this domain affects the conformation of the ectodomain (Sergel and Morrison 1995; Waning, Russell et al. 2004). Surface exposed CT domains may also have additional roles. Clarification of the role of surface expressed CT domain in fusion as well as potential roles of the amino terminal regions of the alternate F protein requires further investigation.
CHAPTER V

NDV VIRUS-LIKE PARTICLES AS VACCINES

A. Introduction

Viral pathogens that cause serious public health concerns and significant economic disruption pose a challenge for us to develop innovative, safe and effective vaccines. The goal of this part of this thesis is to develop NDV VLPs as a vaccine for Newcastle disease or as a framework for generation of a vaccine vector for a wide variety of human pathogens including emerging and re-emerging pathogens.

Commonly used vaccines are either live, attenuated virus or inactivated, virulent virus. Live, attenuated vaccines provide long-lasting, protective immunity and are considered the most effective type of vaccine. However, these vaccines are not without their drawbacks. First, live attenuated vaccine viruses may lead to the development of disease in immunocompromised individuals. Another is that vaccine viruses have the potential for reversion from attenuated vaccine strain to a more virulent form, which can lead to the development of disease in vaccinated individuals. In addition, teratogenic effects in vaccinated individuals have been associated with vaccination using live attenuated strains. For example, vaccination with live attenuated blue tongue virus (BTV) in cattle has been reported to be teratogenic (reviewed in (Murray and Eaton 1996)).

Inactivated vaccines are safer but only offer a short-lived immune response compared to live attenuated virus. The potential for incomplete inactivation of the virus could also lead to the development of disease in vaccinated animals. Inactivation may
also result in alteration of epitopes, which will affect the immunogenicity of the protein. In addition, vaccination with some inactivated viruses, notably measles virus (Kapikian, Bell et al. 1962; Kapikian, Bell et al. 1966) and respiratory syncytial virus (Dittman 1981; Graham 1995; Artimos de Oliveira, Jin et al. 2000), exacerbated the disease upon subsequent exposure to the live virus.

Subunit vaccines based on recombinant proteins offer an effective alternative to live, attenuated vaccines. However, vaccinations based on subunits are more costly because they require multiple doses of higher concentrations of immunogen. In addition, recombinant proteins may be less immunogenic due to incorrect folding.

DNA vaccinations are based on the transfer of genetic material, encoding an antigen, to the cells of the vaccine recipient. Despite their great potential as vaccines, their clinical utility remains unproven (Schalk, Mooi et al. 2006). Thus, there is still a need to develop vaccines that do not have or at least have minimal adverse effects while preserving their efficacy in protecting individuals from the disease.

Virus-like particles (VLPs) are increasingly being considered as potential vaccines (Grgacic and Anderson 2006). VLPs are multiprotein structures that mimic the overall morphology of the authentic virus. VLPs are non-infectious because they assemble without incorporating the infectious genetic material. VLPs offer a promising approach to the production of vaccines against many viral diseases for several important reasons. First, VLPs have a characteristic repetitive and high density display of proteins that is similar to the authentic virus (Noad and Roy 2003). In addition, VLPs contain surface glycoproteins that are properly folded and inserted into membranes. Moreover,
VLPs provide the spatial structure for display of conformational epitopes. Thus, the antigenicity of the assembled viral components is likely similar to the authentic virus. These properties will likely make VLPs very effective in eliciting strong immune responses. Vaccines based on VLPs have been generated for a variety of viruses including human papilloma virus (HPV), hepatitis B virus (HBV), Ebola virus and Marburg virus demonstrate great potential for use in prevention of infectious diseases (reviewed in (Grgacic and Anderson 2006)).

VLPs have also been exploited as a framework for displaying foreign epitopes by generating chimeric VLPs. Chimeric VLPs are important in producing “marker” vaccines and vaccine vectors. Marker vaccines are essential in diagnostics where it is important to differentiate antibody production due to natural infection or vaccination. Chimeric VLPs as vaccine vectors are important for the delivery of foreign epitopes specific for other important pathogens. Generation of chimeric VLPs can be achieved by making modifications in the gene sequences of the viral components necessary for VLP formation without altering their capacity to assemble into particles. VLPs that have an insertion or fusion of foreign antigenic sequences into the VLP structural components have been proven to be effective in inducing a strong immune response against the foreign protein (Huang, Liang et al. 2005; Woo, Doan et al. 2006). For example, VP21 from foot and mouth disease virus (FMDV) fused with the Hepatitis B core antigen (HBcAg) induced immune responses to both VP21 and HBcAg after intraperitoneal injection in mice (Huang, Liang et al. 2005).
The goal of experiments described in this part of the thesis was to develop NDV VLPs as a vaccine vector for important human diseases as well as a vaccine for Newcastle disease. Specifically, we would like to accomplish the following:

(i) To develop a protocol for a scaled-up production of VLPs;

(ii) To apply and extend the rules for incorporation of viral components into VLPs to inclusion of foreign sequences and to find permissive sites for incorporation of foreign sequences;

(iii) To investigate the immunogenicity of NDV VLPs or chimeric VLPs.

The results of this thesis accomplished the first and second goals. Our results showed that production of VLPs from tissue culture can be scaled up, producing particles that are as pure as virus generated from eggs. We also demonstrated that a functional property of one of the structural components of VLPs is intact. In addition, we showed that fusion of a foreign peptide to the HN protein did not inhibit incorporation of HN protein into VLPs. Moreover, we found that the cytoplasmic domain of the F protein is necessary for its incorporation into VLPs.
B. Materials and Methods

Cells and virus. A spontaneously transformed fibroblast cell line derived from the East Lansing strain (ELL-0) of chicken embryos (UMNSAH/DF-1) was obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS). NDV, strain B1, was propagated in embryonated chicken eggs by standard protocols (McGinnes, Pantua et al. 2006).

Plasmids. Expression vector, pCAGGS (Miyazaki, Takaki et al. 1989; Niwa, Yamamura et al. 1991), containing NDV cDNA sequences encoding NP, M, HN, and uncleaved F (F-K115Q) proteins were used to express NDV proteins in avian cells (Pantua, McGinnes et al. 2006).

Transfection, infection and metabolic labeling. For large scale production of VLPs, avian cells were grown in T150 flasks. Cells were transfected with 8 μg each of pCAGGS-NP, pCAGGS-M, pCAGGS-F-K115Q and pCAGGS-HN according to previously described protocols (Pantua, McGinnes et al. 2006). After 5 h of transfection, culture media was replaced with supplemented DMEM. Supernatants were collected at 24 and 48 h post-transfection.

Transfection of ELL-0 cells to generate VLPs containing radioactively labeled NP, M, F and HN proteins were done as described previously (Pantua, McGinnes et al. 2006). In some experiments, pCAGGS vector containing HA-tagged HN, FLAG-tagged-F-K115Q, B1 strain F, or B1 strain HN were used in the co-transfection when indicated. A pulse–chase protocol using [35S] methionine and [35S] cysteine mixture (NEG-772
EASYTAG™ Express Protein Labeling Mix, $^{[35}S$, Perkin Elmer Life Sciences Inc.) was accomplished as described previously (Pantua, McGinnes et al. 2006). Cells were harvested and homogenized for immunoprecipitation assays.

**Virus and VLP purification.** To purify VLPs from avian cells grown in T150 flasks, supernatants were initially clarified by centrifugation at 5,000 rpm for 5 min. VLPs in supernatants were pelleted by ultracentrifugation at 18,000 rpm for 12 h at 4°C (Type 19 rotor). Pelleted VLPs were concentrated into the interface of 20% and 65% sucrose gradient by ultracentrifugation at 24,000 rpm for 12 h at 4°C (SW 28 large buckets). Particles were further purified by floating into a step gradient, as described in Materials and Methods of Chapter II). The upper fraction, containing a visible particle band, was collected. Finally, VLPs were diluted in TNE buffer (25mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA) and pelleted by ultracentrifugation at 40,000 rpm for 4 h at 4°C. Pelleted VLPs were resuspended in an appropriate volume of TNE buffer.

Virus particles from amniotic fluid of virus infected embryonated eggs were purified using the same protocol except virus was not pelleted after flotation (McGinnes, Pantua et al. 2006).

Radioactively labeled VLPs were purified as described previously in Chapter II.

**Antibodies.** Rabbit polyclonal antibody, raised against UV inactivated NDV (anti-NDV) by standard protocols, was used to precipitate NP. Anti-NDV also contained antibodies specific for HN, F and M proteins. Antisera used to precipitate F protein were raised against glutathione S-transferase (GST) fusion proteins that contained amino acid sequences 130 to 173 (anti-HR1) (McGinnes, Gravel et al. 2002), 470 to 500 (anti-HR2)
(Dolganiuc, McGinnes et al. 2003), or 96 to 117 (anti-F₂-96). Antiserum used to precipitate HN protein was raised against HN protein sequences from amino acid 96 to 117 (anti-A) (McGinnes and Morrison 1998). Antibody used to precipitate M protein was a mouse monoclonal antibody raised against purified M protein (Faeberg and Peeples 1988). Antibody used to precipitate HA-tagged HN protein was anti-HA conjugated to agarose beads (Sigma). Antibody used to precipitate FLAG-tagged F protein was anti-FLAG antibody (Sigma).

VLP binding assay. Radioactively labeled VLPs were separated into three different aliquots. Anti-NDV antibody was added to one VLP aliquot and incubated at room temperature for 1 h along with the untreated VLPs. VLPs were added to avian cells grown in 12 well plates and incubated at 4°C on ice for 30 min. Cells were then washed twice with cold TNE buffer and lysed in 0.1 ml lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH7.4) containing Triton-DOC (1% Triton X-100, 1% sodium deoxycholate) and 1.25 mg N-ethylmaleimide (NEM). The resulting lysates were homogenized and used for immunoprecipitation protocols.

Immunoprecipitation, polyacrylamide gel electrophoresis and silver stain. Immunoprecipitation of radioactively labeled proteins was accomplished by combining one volume of cell lysate or sucrose gradient fraction with two volumes of TNE buffer containing 1% Triton X-100. Samples were incubated with specific antibodies for 16 hours at 4°C. Immune complexes (ICs) were adsorbed to Protein A (Pansorbin Cells, CALBIOCHEM) for 2 hours at 4°C, pelleted, and then washed three times in immunoprecipitation (IP) wash buffer (phosphate buffer saline (PBS) containing 0.5%
Tween-20 and 0.4% sodium dodecyl sulfate (SDS)). ICs were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer (125 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.4% bromphenol blue) with 1 M β-mercaptoethanol (BME) and boiled. Immunoprecipitated proteins were separated on 8% polyacrylamide-SDS gels and subjected to autoradiography.

Proteins in purified VLPs and virus were separated on 8% polyacrylamide-SDS gels and detected by silver stain using SilverSNAP® Stain Kit II as recommended by the manufacturer (Pierce). Briefly, the gel was washed twice in ultrapure water and fixed in solution containing 30% ethanol and 10% acetic acid. The gel was washed in ultrapure water twice prior to addition of essential working solutions. In order, the gel was incubated in Sensitizer (1 min), Stain (30 min), and Developer Working Solutions. The gel was incubated in Developer Working Solution until bands appeared (about 2 to 3 min). The reaction was stopped by replacing the Developer with 5% acetic acid for 10 min.

Quantification of resulting autoradiographs and silver stained gels were accomplished using a Fluor-STM™ MultiImager (BioRad).
C. Results

**VLPs generated from tissue culture were as pure as virus produced from eggs.** To determine the purity of VLPs produced from tissue culture, proteins in both strain B1 virus particles and VLPs were resolved in 8% SDS-PAGE gels. Silver staining was used to visualize the proteins present in both virus and VLPs. Figure 5.1 shows that VLPs and virus have a similar protein profile. This result indicates that particles released from cells expressing the four major structural proteins are as pure as virions propagated from embryonated eggs.

The amounts of protein in VLPs were quantified from the silver stained gel using the FluorS Imager. Known amounts of bovine serum albumin (BSA) were correlated with its densitometer units. These correlations were used to determine the amounts of each protein in VLPs. Quantification of the amounts of protein in VLPs produced from tissue culture are shown in Table 5.1. Avian cells expressing the NDV NP, M, F-K115Q and HN proteins efficiently produced VLPs with a concentration of 178.43 μg total protein per 1.0 X 10^8 cells. NDV, strain B1, virus harvested from 3.3 dozen eggs contained 209.54 μg total protein. Protein ratios in VLPs and virus were slightly different, which may be due to over-expression of proteins in tissue culture or differences in assembly of NDV strains B1 and AV. These results suggest that VLPs can be generated in significant quantities from tissue culture using standard transfection protocols.
Figure 5.1. **Purity of VLPs from tissue culture.** VLPs were generated from avian cells grown in T150 flasks and transfected with NDV NP, M, F and HN cDNAs in pCAGGS vector. NDV, strain B1 virus was propagated from embryonated eggs. Virus and VLPs were pelleted from the supernatant (Type 19 rotor, 18,000 rpm, 4°C, 16 h). Particles were isolated by concentrating into the interface of 20% and 65% sucrose solution (SW 28 rotor, 24,000 rpm, 4°C, 12h). Particles from the interface were floated into a step gradient. Particles were pelleted from the flotation fractions. Proteins in egg-grown NDV strain B1 virus and VLPs were separated by SDS-gel electrophoresis. Proteins in the gel were silver stained as described in Materials and Methods.
Table 5.1 Comparison of the amounts of VLPs and virus produced

<table>
<thead>
<tr>
<th>Particle</th>
<th>Protein</th>
<th>Conc (ng/ml)</th>
<th>Total volume</th>
<th>Total protein (μg)</th>
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<tr>
<td><strong>Strain B1 virus</strong></td>
<td></td>
<td></td>
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<tr>
<td>(from 3.3 dozen eggs)</td>
<td>H</td>
<td>23.05</td>
<td>1 ml</td>
<td>23.05</td>
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<tr>
<td></td>
<td>F</td>
<td>11.09</td>
<td></td>
<td>11.09</td>
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<tr>
<td></td>
<td>NP</td>
<td>100.32</td>
<td></td>
<td>100.09</td>
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<tr>
<td></td>
<td>M</td>
<td>75.08</td>
<td></td>
<td>75.08</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td>209.54</td>
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<tr>
<td><strong>VLP</strong></td>
<td></td>
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<tr>
<td>(from 1.0 X 10^8 cells)</td>
<td>HN</td>
<td>109.70</td>
<td>0.5 ml</td>
<td>54.85</td>
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<tr>
<td></td>
<td>F</td>
<td>85.42</td>
<td></td>
<td>42.71</td>
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<tr>
<td></td>
<td>NP</td>
<td>98.24</td>
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<td>49.71</td>
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<td></td>
<td>M</td>
<td>63.50</td>
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<td>31.75</td>
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<td></td>
<td>Total</td>
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<td>178.43</td>
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</table>
**VLPs bound to host cells.** To characterize a functional property of the structural components of VLPs, we initially asked whether VLPs could bind to host cells. To answer this question, we performed a binding assay. Different amounts of radioactively labeled VLPs were allowed to bind to avian cells at 4°C. A polyclonal anti-NDV antibody was used as a control to block binding. In addition, supernatant from cells transfected with vector alone was used as a negative control. Figure 5.2 shows that VLPs attached to cells and the binding was inhibited by the addition of anti-NDV to VLPs prior to incubation with cells (compare lanes 3 to 5). No signal was observed in the vector supernatant incubated cells (lane 2). Moreover, by comparing the input VLPs to the maximum amounts bound to cells, we found that the majority of added VLPs bound to the cells (compare lanes 1 and 4). In addition, the binding of VLPs was concentration dependent (lanes 4, 5 and 6). This result clearly demonstrates that the attachment function of the HN protein in VLPs is intact.

**HA tagged HN glycoprotein incorporated into VLPs.** One of the new strategies in designing a vaccine is to incorporate a foreign peptide into one of the structural components of the vaccine. To determine if there are permissive sites in the two viral glycoproteins for foreign epitope insertion, we generated HN and F proteins with HA and FLAG tags fused to their carboxyl terminus, respectively (HN-HA and F-FLAG). The HN-HA and F-FLAG were shown to mediate normal attachment and membrane fusion, respectively (McGinnes, unpublished observation). We asked if the HN-HA and F-FLAG could be incorporated into VLPs. Figure 5.3 shows that VLPs were assembled and released from cells that expressed either HN-HA or F-FLAG proteins along with the
Figure 5.2. VLPs bound to host cells. Different amounts (3x, 2x and 1x) of radioactively labeled VLPs were allowed to bind to avian cells on ice at 4°C for 30 min. Negative controls were supernatant from vector transfected cells (lane 2) and VLPs treated with anti-NDV for 1 h (lane 3). Input lane is 3x concentration. Cells-VLPs were lysed and proteins were resolved by SDS-PAGE and visualized by autoradiography.
**Figure 5.3. HN-HA protein is incorporated into particles.** FLAG tagged F protein (F-FLAG) or HA tagged HN protein (HN-HA) were co-expressed with NP, M and a glycoprotein and radioactively labeled in a pulse–chase protocol as described in Materials and Methods. Panels show, pulse (left) and chase (middle) lysates and VLPs (right).

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<tbody>
<tr>
<td>AV-NP</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>AV-M</td>
<td>-</td>
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<tr>
<td>AV-F-K115Q</td>
<td>-</td>
<td>+</td>
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<tr>
<td>AV-HN</td>
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<td>AV-F-Flag</td>
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<td>AV-HN-HA</td>
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- HN
- F₀
- NP
- M
other three wild type proteins. However, F-FLAG protein was not incorporated into VLPs, while HA-tagged HN was incorporated into VLPs. The amounts of VLPs released from cells expressing the HN-HA (with NP+M+ F or F-FLAG) were lower compared to cells expressing NP, M, F and HN without HA tag. This result is likely due to the absence of a spacer sequence between the HN sequence and the HA tag. Nevertheless, this result suggests that foreign peptides can be fused to the carboxyl terminus of HN protein without inhibiting incorporation into VLPs.

**F protein cytoplasmic (CT) domain is necessary for its incorporation into VLPs.**

The formation and release of VLPs without incorporation of F-FLAG is consistent with our previous results that particles are released in the presence of M protein and at least two other proteins (NP and HN proteins) (Pantua, McGinnes et al. 2006). The observation that appending a FLAG tag on the CT of F protein inhibited its incorporation into particles suggests that the CT domain is necessary for F protein incorporation into VLPs. To define the specific requirements for incorporation of NDV F protein into particles, we asked whether the CT domain of the NDV F protein plays a role in the assembly of VLPs. Using different CT truncation mutants of F protein, we asked if any region of the CT domain of F protein was necessary for its incorporation into particles. Figure 5.4, panel A, shows a linear diagram of a full length F protein and different CT truncation mutants. Deletion of the carboxy-terminal 13 amino acid residues (F-Gterm) inhibited incorporation of the F protein into VLPs (compare lanes 16 and 18). In addition, deletion of the carboxyl-terminal 6 amino acid residues (F-Mterm) slightly
Figure 5.4. CT domain of F protein is necessary for incorporation into VLPs. 
A, linear diagram of full length F-K115Q and CT (cytoplasmic tail) truncation mutants. 
B, Full length and truncation mutants were co-expressed with NP, M and HN proteins in 
a pulse – chase protocol as described in Materials and Methods. Panels show, pulse (left) 
and chase (middle) lysates and VLPs (right). C, amount of F protein incorporated into 
particles (values were normalized to the amount of NP). Identical results were observed 
from two separate experiments.
Figure 5.5. Heterologous NDV glycoproteins are incorporated into VLPs. NDV strain B1 F or HN proteins were co-expressed with NP, M and a glycoprotein from NDV strain AV (lanes 3 and 4, 8 and 9). B1 HN and F proteins were co-expressed with AV NP and M proteins (lanes 5 and 10). Cells were subjected to a pulse-chase protocol as described in Materials and Methods. Panels show, pulse lysates (left) and VLPs (right).
decreased incorporation of the F protein into VLPs (compare lanes 16 and 19; Figure 5.4, panel C). In contrast, an internal deletion (F-delK) and deletions from amino acid residues 550 to 553 (F-Tterm) did not affect F protein incorporation into VLPs (compare lanes 16 to 20 and 21). These results demonstrate that the CT of F protein is important for its incorporation into VLPs. Moreover, these results also suggest that amino acid residues from position 523 to 550 are necessary for F protein incorporation into VLPs.

**Heterologous NDV glycoproteins incorporated into VLPs.** We also asked if glycoproteins from a different strain of NDV could be incorporated into VLPs. We co-expressed F or HN glycoproteins from NDV, strain B1, with NP, M and HN or F glycoprotein, respectively, from NDV strain AV. We also expressed both the B1 F and HN proteins with AV M and NP proteins. We found that either F or HN glycoprotein or both from NDV strain B1 were incorporated into VLPs with strain AV viral proteins (Figure 5.5). However, the amounts of VLPs from cells expressing B1 HN protein were slightly lower compared to amounts released from cells expressing the AV HN protein (compare lanes 7, 8 and 10). The B1 F protein appeared to have no effect on VLP release and it was normally incorporated into VLPs (compare lanes 7 and 9 in the right panel). These results indicate that glycoproteins from a heterologous strain of NDV would incorporate into AV VLPs.
D. Summary and Conclusion

To develop NDV VLPs as a vaccine for Newcastle disease or as a vaccine vector, we scaled up the production of VLPs, characterized the binding activity of HN protein, and defined the rules for incorporation of viral components into particles. The results described above demonstrate that production of VLPs from tissue culture using a transfection protocol can be scaled up to produce microgram quantities of VLPs that are as pure as virus generated from eggs. However, this production protocol is costly due to the large quantities of lipofectamine required for the transfection. Thus it is still necessary to develop new ways of producing NDV VLPs. One alternative is to use a baculovirus multigene expression vector system. An advantage of this system is that proteins have been shown to be folded correctly (Possee 1997), thus producing VLPs with components that are still similar to the authentic virus. Another approach is to establish stable cell lines that co-express NDV NP, M, F and HN proteins. This approach would eliminate the need for transfection.

We also showed that NDV VLPs can normally bind to host cells. The binding activity of VLPs strongly suggests that the HN protein is in its normal conformation, which allows attachment to the receptor.

In addition, we also showed that a foreign peptide sequence could be fused to the HN protein without inhibiting the incorporation of HN protein into VLPs. However, the amounts of VLPs released were decreased. It is possible that the HA tag interferes or blocks sites for interaction with other viral proteins, which are necessary for assembly and release of particles. Thus, there is a need to identify other sites in the HN protein for
insertion of foreign epitopes. Nevertheless, these findings are important in identifying permissive sites for the design of a chimeric VLP vaccine.

We also found that at least part of the CT domain of F protein is necessary for its incorporation. This result suggests that a specific sequence in the F protein CT domain is essential for its incorporation into VLPs. Altogether, these results contribute significantly to the establishment of NDV VLPs as a framework for a vaccine vector for many human viral pathogens.

In the future we intend to define the CT sequence of the HN protein that is necessary for incorporation of the HN protein. We also plan to further define the necessary regions in the F protein CT domain. By identifying specific regions in the F protein necessary for assembly, we can generate chimeric glycoproteins that contain the necessary elements for assembly and the foreign sequences that are necessary for the induction of immune responses. For example, we can fuse the NDV F protein or HN protein CT domains to the ectodomain and transmembrane domain (TM) of a type 1 glycoprotein or type 2 glycoprotein, respectively, and ask if the CT domains of NDV F and HN proteins could support incorporation of the chimeric protein into VLPs.

We also found that heterologous NDV F and HN proteins were incorporated into AV VLPs. The decreased amounts of VLPs released from cells expressing the B1 HN protein and AV viral proteins may be due to differences in assembly of AV and B1 particles. Nevertheless, these results are important in developing a multivalent vaccine for Newcastle disease.
It is also imperative to examine the immune response induced by NDV VLPs. We plan to determine if NDV VLPs can elicit both humoral and cell-mediated immune responses. These issues are currently under investigation using a murine model.
CHAPTER VI
GENERAL DISCUSSION

The mechanisms of paramyxovirus assembly and budding have been investigated in this dissertation with the underlying goal of determining the viral and host protein requirements for assembly and release of Newcastle disease virus. We have established a VLP system for NDV and we have used this system in answering important questions in assembly and budding of this virus. The key player in the assembly and release of particles was defined here in terms of the minimum viral protein requirements for the formation and release of NDV VLPs. The network of interactions between viral proteins involved in assembly of complete VLPs was also defined. The involvement of cellular VPS machinery in virus budding was explored. In addition, the potential involvement of the alternate form of NDV F protein was examined. The potential of NDV VLPs as a vaccine for Newcastle disease and as a vaccine vector for important human pathogens was investigated.

Taken together, the data presented in this dissertation provide a better understanding of the requirements and mechanisms of assembly and budding of Newcastle disease virus. A better understanding of this process could contribute to the design of antiviral drugs that inhibit virus release. More importantly, the data will contribute to the development of safe, economical and effective vaccines for Newcastle disease as well as human pathogens. Since an in depth discussion has been provided in Chapters II, III, IV and V, this chapter provides a brief overview of the major points
made in this thesis and highlights the relevant questions that remain to be answered in future work.

A. **Cell sites of viral protein-protein interactions**

In this dissertation, I have shown that co-expression of viral structural components resulted in the formation and release of particles (Figure 6.1). One major point is that these viral components interact with each other in an orderly way to initiate the assembly and budding process. In this thesis, we showed that M protein-HN protein, M protein-nucleocapsid protein, F protein-nucleocapsid protein, and F protein-HN protein interactions are crucial to the assembly of particles. An important issue in the virus assembly process is the site or cellular location where viral protein-protein interactions occur to initiate particle assembly. Some of the important questions are the following: do NDV viral proteins interact strictly in the assembly site in the plasma membrane; alternatively, do specific interactions between these proteins occur during transport through the secretory pathway; lastly, is there a sequential order in viral protein-protein interactions required for assembly and release?

It has been widely accepted that paramyxoviruses assemble at the plasma membrane of infected cells (Lamb and Kolakofsky 2001). However, there is also evidence that suggests that viral proteins interact while being transported to the cell plasma membrane (reviewed in (Schmitt and Lamb 2004; Takimoto and Portner 2004)). It has been reported that Sendai virus M protein accumulated at the Golgi membrane when transport of glycoproteins was restricted by low-temperature inhibition or by the addition of ionophore monensin (Sanderson, McQueen et al. 1993). This result suggests
Figure 6.1. Model of NDV assembly and budding. This figure shows the network of protein interactions necessary for particle formation. The YANL sequence serves as a late domain in the NDV M protein. The late domain and the VPS machinery are both essential for particle budding. 1 to 4 are some of the unanswered questions in NDV assembly and budding: 1 - Do viral proteins form a complex while in transit to the secretory pathway?; 2 – Does the alternate form of F protein play a role in particle formation?; 3 – Does actin play a role in particle assembly and release?; and 4 – Does the YANL sequence in NDV M protein mediate interaction with the VPS machinery? And which VPS molecule interacts with the M protein?
that the interaction between the M protein and glycoproteins occurs during their transit through the secretory pathway. But if M protein transits through the MVBs, then monensin or low temperature incubation would directly inhibit M protein transport to the plasma membrane.

It has also been shown that the Sendai virus M protein was recruited to the cytoplasm by the viral nucleocapsid (Stricker, Mottet et al. 1994). This result suggests that M protein-nucleocapsid complexes are formed prior to assembly of the virus particle in the plasma membrane.

Based on the studies presented above, we hypothesized that NDV viral proteins form complexes during transport to the plasma membrane (Figure 6.1). Based on our results that indicate interactions between M-HN proteins and F-NP proteins, this hypothesis can be tested by inhibiting the transport of NDV F or HN proteins and determining the site of accumulation of M protein or NP. Accumulation of NDV M or NP in the Golgi membranes would suggest that complexes are formed while NDV F and HN proteins are in transit through the secretory pathway. The formation of M-NP complexes prior to assembly in the plasma membrane could be examined by performing co-immunoprecipitation assays using cytosolic fraction of cells expressing M protein and NP. To further determine where in the cell the NDV viral proteins interact, viral proteins can be visualized by electron or confocal microscopy along with markers of the secretory compartments and other relevant cell compartments.

Another issue in the assembly of virus particles is the order of assembly of components. This process is poorly understood and the relevant questions that are
worthy of future investigation are as follows: Is there a sequential order of viral protein interactions in the formation of a complete particle; Do M protein-NP complexes form first before interacting with HN (with M) or F (with NP) proteins to initiate assembly; Are complexes formed randomly to initiate assembly? Answers to these questions would provide a better understanding of the mechanisms of assembly of paramyxoviruses.

In addition to the points presented above, we have shown evidence that the F protein exist in two forms, the classical type 1 glycoprotein and an alternate form, in virus particles. However, the role of the alternate form of F protein in virus assembly is not known (Figure 6.1). We hypothesize that the alternate form of F protein may be involved in the assembly process. Using the NDV VLP system, we plan to investigate if the alternate form of F protein is involved in any viral protein-protein interactions that are necessary for particle formation. Specifically, the localization of the alternate form of F protein relative to the M protein could be explored by immunofluorescence microscopy of cells co-expressing NP, M, F and HN proteins. Another approach is to determine whether the alternate form of F would co-precipitate with HN protein or NP.

Lastly, it is also important to confirm the cellular localization of the NDV M protein by confocal microscopy since there is evidence that the M protein is localized in the nucleus (Peeples, Wang et al. 1992). The implication of this finding on virus assembly and budding should be explored in the future.

B. Involvement of cytoskeleton in virus budding

Cytoskeletal proteins such as actin and tubulin are implicated in the life cycle of paramyxoviruses. Previous studies indicated that cellular actin was incorporated into SV,
mumps virus, and measles virus particles (Lamb, Mahy et al. 1976; Orvell 1978; Tyrrell and Norrby 1978). It has also been shown that the M protein of NDV and SV interacts with actin as shown by chromatography and cosedimentation assays (Giuffre, Tovell et al. 1982). In addition, it has been shown previously by McGinness et al that cytochalasin D, an inhibitor of actin polymerization, significantly accelerated the release of radioactively labeled virions (Morrison and McGinnes 1985). We have consistently observed the incorporation of actin into both NDV virions and VLPs by Western analysis (also in Figures containing gels of VLPs and virus, and unpublished observation). We have also found that M protein and NP specifically co-precipitate from VLPs with anti-actin antibody (unpublished observation). We also observed that actin co-precipitated with M protein from VLPs formed from M, F and HN proteins (Figures in chapter 2) indicating that the M protein may bind to actin. Immunoprecipitation with NP-specific antibody would show whether actin interacts with NP. All these findings suggest a role for actin in the process of NDV budding (Figure 6.1).

Our lab has shown evidence which suggest that lipid rafts serve as platform for assembly and budding of NDV (Laliberte, McGinnes et al. 2006). These findings and the report that show evidence that actin associates with lipid rafts (Nebl, Pestonjamasp et al. 2002) lead us to hypothesize that actin rearrangement in lipid rafts play a role in assembling the viral components and in the release of virus particles. To further explore the involvement of cytoskeleton in NDV budding, drugs that disrupt the microfilaments and microtubules may be used and examine their effects on VLP release.
C. Mechanisms by which viruses use the VPS system

The second major point in this thesis is that intact VPS machinery is essential for budding of NDV VLPs. Many different studies have shown that a variety of enveloped RNA viruses depend on the recruitment of cellular VPS machinery to assist in the late stages of the virus budding process (reviewed in (Pornillos, Garrus et al. 2002; Bieniasz 2006)). Although we showed that the VPS system is involved in NDV VLP budding, the fundamental role of the VPS machinery in NDV budding remains to be determined. Important questions relevant to this issue are the following; first, does virus infection affect the cellular localization of the VPS machinery; second, how does NDV use the VPS machinery? The answers to these questions would help define the steps that are necessary for virus assembly and release.

The first basic question is whether virus infection changes the localization of the VPS machinery. In other words, is the VPS machinery recruited to the site of NDV assembly and budding? It is thought that the VPS machinery is recruited by the retrovirus Gag protein to the sites of assembly in the plasma membrane for virus egress (reviewed in (Pornillos, Garrus et al. 2002; Morita and Sundquist 2004)). In contrast, there is also evidence that intracellular budding of progeny Marburg virus occured in MVBs (Kolesnikova, Berghofer et al. 2004).

These results suggest that viruses may use the VPS pathway in two different ways. One is by recruiting the machinery to the plasma membrane where assembly and budding occurs and second is by using the machinery for particle budding intracellularly through the MVBs (Figure 6.1). To discriminate between the two possibilities, the cell
site where there are interactions between the viral matrix protein and molecules of the VPS machinery has to be defined. Confocal microscopy and ultrastructural analysis of the localization of viral proteins and MVBs could be performed to define the site of M protein-VPS molecule interaction. This issue has not been explored for any paramyxovirus and should be a subject for future investigation.

Another unanswered question that we plan to address in the future is the effect of dominant negative mutant proteins of the VPS machinery on the release of particles from infected cells. Does expression of dn mutant proteins of the VPS pathway result in the accumulation of the M protein as well as other viral proteins in infected cells? We predict that an intact VPS pathway is also necessary for release of virus particles from NDV-infected cells.

Viruses that co-opt the VPS machinery have been shown to contain L domains in their matrix-like proteins (reviewed in (Pornillos, Garrus et al. 2002; Bieniasz 2006)). L domains are necessary to mediate interactions between matrix-like proteins and molecules of the VPS machinery (reviewed in (Freed 2002; Bieniasz 2006)). We have shown that the YANL sequence in the NDV M protein has L domain properties. One fundamental question is how the YANL sequence in the NDV M protein mediates interactions with the VPS machinery (Figure 6.1). With which particular molecule of the VPS machinery does M protein interact? It has been shown that YXXL motifs mediate interactions with the endocytic proteins, AP2 (Chen, Vincent et al. 2005) and AIP1 (Strack, Calistri et al. 2003), respectively. We have shown that AIP1, a protein that connects ESCRT I and ESCRT III, is incorporated into VLPs. However, we did not
observe an interaction between AIP1 and M protein in the extracts using a co-immunoprecipitation assay. It is possible that a second protein mediates the interaction between M protein and AIP1. Alternatively, it is possible that another cellular protein of the VPS machinery, and not AIP1, interacts with the M protein to facilitate virus budding. Further screening for interacting proteins has to be done to identify the interacting partner of NDV M protein.

D. VLPs as a vaccine for NDV

There is increasing attention and effort in developing VLPs as vaccines. VLP systems have been established for several different viruses (Noad and Roy 2003). For example, VLPs composed of papilloma virus capsid proteins have been approved by the FDA as a vaccine for cervical carcinoma (FDA 2006). It has been reported that Ebola VLPs produced from insect cells can stimulate neutralizing antibody production and induce cytokine secretion from human dendritic cells (Ye, Lin et al. 2006). VLPs composed of the HIV gag and env proteins induced significant levels of both systemic and mucosal neutralizing antibodies, a proliferative response of T helper cells, and cytolytic activity of cytotoxic T cells (Buonaguro, Racioppi et al. 2002; Jaffray, Shephard et al. 2004; Buonaguro, Visciano et al. 2005; Buonaguro, Tornesello et al. 2006). Collectively, these results suggest that VLPs can induce a wide range of immune responses. However, the full potential of VLPs, which mimic enveloped viruses, as vaccines has not been fully exploited despite their many advantages over commonly used vaccines. In the future we plan to exploit the potential of NDV VLPs as a vaccine for Newcastle disease.
We plan to develop a multivalent vaccine for NDV. Swenson, et al, have shown that expression of glycoprotein (GP) and VP40 from a single filovirus, either Ebola or Marburg virus, resulted in the formation of VLPs from mammalian cells (Swenson, Warfield et al. 2004). When a mixture of Ebola and Marburg VLPs was used as vaccine, these VLPs stimulated a strong immune response to both viruses in guinea pigs (Swenson, Warfield et al. 2005). Neutralizing antibodies were produced and animals were protected from live virus challenge (Warfield, Swenson et al. 2004; Swenson, Warfield et al. 2005). These results suggest that combinations of Ebola and Marburg VLPs could be used as effective multivalent vaccine for filoviruses. With our goal of developing a multivalent vaccine for NDV, we examined the incorporation of glycoproteins from a heterologous strain of NDV into VLPs. We showed that F and HN proteins from the B1 strain of NDV were incorporated into VLPs. However, it is important to further compare and characterize the efficiency of assembly and release of AV and B1 VLPs.

It is also important to determine if AV and B1 VLPs will elicit an immune response in a murine model. Furthermore, it will be important to compare the immune responses to AV and B1 VLP vaccinations with vaccine virus vaccinations. Currently, we are conducting experiments to compare the immune responses to VLPs and UV inactivated B1 strain virus in a murine model.

**E. VLPs as vaccine vectors for human pathogens**

Recombinant viruses expressing foreign proteins or epitopes have been produced with the aim of developing multivalent vaccines that are capable of stimulating both
humoral and cellular immune responses against more than one pathogen (Bukreyev, Huang et al. 2005; Park, Steel et al. 2006). We propose to develop NDV VLPs as vaccine for different human pathogens. To this end, we have started to define rules for incorporation of NDV glycoproteins into VLPs in order to guide the development of NDV VLPs as a vaccine for human pathogens. We showed that the CT domain of the F protein is necessary for F protein incorporation into VLPs. To make chimeric NDV VLPs as vaccine for human pathogens, we will fuse the CT domain of F protein to a type 1 glycoprotein ectodomain and transmembrane (TM) domain from other pathogens to determine if there is an active incorporation of the chimeric protein into VLPs. Alternatively, it may be necessary to fuse both the CT and TM domains of F protein to the ectodomain of the foreign protein. Sequences necessary for NDV HN protein incorporation into VLPs have yet to be defined. By doing so, we can also use regions of the HN protein to create a chimera of NDV HN protein and type 2 glycoproteins from other pathogens.

VLPs have also been exploited as carriers for foreign epitopes (reviewed in (Grgacic and Anderson 2006)). For example, VLPs derived from the small envelope protein (HBsAg-S) of hepatitis B virus (HBV) containing the immunodominant hypervariable region of the hepatitis C virus (HCV) E2 protein have been reported to induce the production of antibodies to HCV E2 in a murine model. In addition, it has also been reported that mice immunized with VLPs derived from HBV core antigen (HBVcAg) containing a VP1 epitope from foot and mouth disease virus (FMDV) were protected from FMDV challenge (Huang, Liang et al. 2005). Similarly, VLPs derived
from HBVcAg containing T and B cell epitopes from HCV NS3 protein induced high levels of anti-NS3 antibodies (Mihailova, Boos et al. 2006). Collectively, these results suggest chimeric VLPs have great potential as vaccines.

We have started to define permissive sites for addition of foreign epitopes into VLPs with the underlying goal of maintaining the efficient incorporation of a protein into VLPs as well as efficient release of particles. We demonstrated that NDV-HN protein fused with an HA tag was incorporated into VLPs. However, the amount of VLPs released was slightly decreased. It is possible that the HA tag interfered with interactions of HN protein with other viral proteins, interactions necessary for efficient incorporation of HN protein into VLPs. It is possible that the absence of a linker in between the HN sequence and the HA tag influenced the decreased incorporation of the HN-HA protein. It has been shown that insertion of a linker sequence between the measles virus hemagglutinin (HA) protein and a single chain antibody resulted in a chimera protein that was biologically active and was assembled into recombinant measles virus (Hammond, Plemper et al. 2001; Peng, Donovan et al. 2003).

A key objective in developing VLPs as a vaccine is to design strategies that would facilitate safe and economical methods for producing large quantities of VLPs. Although we have generated significant quantities of NDV VLPs, other methods should be explored to scale up the production of VLPs. An alternative to our protocol is the use of cells stably expressing the NDV structural proteins. Another is to generate VLPs using a multigene baculovirus vector system. Although these approaches would support a scaled
up production of VLPs, they are not without their drawbacks. Stable cell lines may be
difficult to maintain. Use of baculovirus risks contamination with baculoviruses.

The ability of NDV VLPs to elicit an immune response in a murine model is
currently being characterized in our laboratory. The potential of chimeric NDV VLPs as
a vaccine vector will be pursued in future work.

F. Summary

In summary, the results described in this thesis indicate that the budding of
paramyxoviruses, specifically NDV, involves interactions between viral proteins and
interplay of viral and cellular proteins. The results in this thesis further support the
conclusion that the M protein is the central organizer of the virus budding process. We
also provided evidence that the VPS pathway is involved in NDV VLP budding.
Moreover, the NDV M protein contains a sequence that has L domain properties.

Finally, through this work, we have taken a significant stride towards a clearer
understanding of the steps in the assembly and budding of paramyxoviruses, which
would contribute to the design of better strategies for therapeutic intervention and to
development of safe, economical and effective vaccines.
REFERENCES


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