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VIRAL PROTEIN REQUIREMENTS FOR EFFICIENT HUMAN PARAINFLUENZA VIRUS 3 VIRUS-LIKE PARTICLE FORMATION

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VIRAL PROTEIN REQUIREMENTS FOR EFFICIENT HUMAN PARAINFLUENZA VIRUS 3 VIRUS-LIKE PARTICLE FORMATION

By Megan Bracken

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ABSTRACT

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Enveloped viruses exit infected cells after the genome and specific viral proteins assemble at the cell membrane and bud from the host cell as infectious virions. To investigate the process by which human parainfluenza virus 3 (HPIV-3) proteins assemble and release, viral proteins were expressed in 293T cells individually or in combination to produce virus-like particles (VLPs). When the HPIV-3 matrix (M) protein was expressed individually, enveloped VLPs containing M protein were released from cells, indicating that M protein is critical for virus particle budding. When viral envelope proteins hemagglutinin-neuraminidase (HN) and fusion (F), as well as RNA-binding nucleoprotein (N), were expressed individually, these proteins could not trigger their own release from cells. However, when M protein was coexpressed with HN, F, or N, VLPs that contained M and HN, M and F, and M and N, respectively, released from cells. M protein release was not enhanced by coexpression of other viral proteins. Electron microscopy analysis verified that VLPs were morphologically similar to HPIV-3 virions. These findings suggest that M protein interacts with the HN, F, and N proteins and plays a coordinating role in HPIV-3 virus particle assembly and release.

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INTRODUCTION

Background

The four serotypes of human parainfluenza virus (HPIV) were discovered between 1956 and 1960 and were grouped in the *Paramyxoviridae* virus family (34, 47). The first three serotypes known, (HPIV-1, HPIV-2, and HPIV-3), were recovered from infants and children with lower respiratory tract infections (LRIs) (47). These viruses were called "parainfluenza" viruses due to the flu-like respiratory symptoms present during infection, as well as for having structural similarities to the influenza virus (34, 47). Some of these shared properties include an envelope, a hemagglutinin protein, and a neuraminidase protein (47). After this group of viruses was discovered, HPIV types 1 and 2 were recognized to be important causes of croup among children, while HPIV-3 in particular was determined to be a major cause of pneumonia and bronchiolitis in infants. Human parainfluenza virus 4 (HPIV-4) was later isolated from children and young adults infected with minor upper respiratory tract disease. HPIV-4 is less common and usually not as severe as the other human parainfluenza viruses (17, 47). Although there are several serotypes of HPIVs, the focus of this study is HPIV-3.

Despite the fact that HPIV-3 is best known for the disease it causes in infants and young children, it also causes pneumonia among immunosuppressed adults, such as bone marrow transplant patients, where it can reach mortality rates of 30-50% (53, 88). Only respiratory syncytial virus (RSV), another member of the *Paramyxoviridae*, causes more cases of serious respiratory tract disease in infants and children in the United States than

HPIV 1, 2, and 3 (17, 28, 34). In the United States, there are about 500,000 to 800,000 cases of lower respiratory infection (LRI) hospitalizations each year in patients under age 18. Of these patients, 12% are infected with HPIV1, HPIV-2, or HPIV-3 (34). In children with positive viral cultures, HPIV 1, 2, or 3 were recovered from 18% of outpatients with upper respiratory infections, 22% with lower respiratory illness, and 64% with croup (34).

HPIV-3 Classification

HPIV-3 is a member of the large order of non-segmented, negative-sense RNA viruses named the *Mononegavirales*. Many medically important viruses of both humans and animals are grouped within this order, including rabies virus in the *Rhabdoviridae*, and Ebola virus (EbV) in the *Filoviridae*. The human parainfluenza viruses belong to a third family within the *Mononegavirales* called the *Paramyxoviridae* (Fig. 1). In this diverse family, there are numerous clinically significant viruses, including RSV, measles, mumps, canine distemper virus, the highly lethal emerging Nipah virus (NiV), and Newcastle disease virus (NDV), which can cause high mortality rates in birds (13, 33). HPIV-3 and its bovine counterpart, BPIV-3, are members of the genus *Respirovirus*, along with HPIV-1 and Sendai virus (SeV) (34, 47).

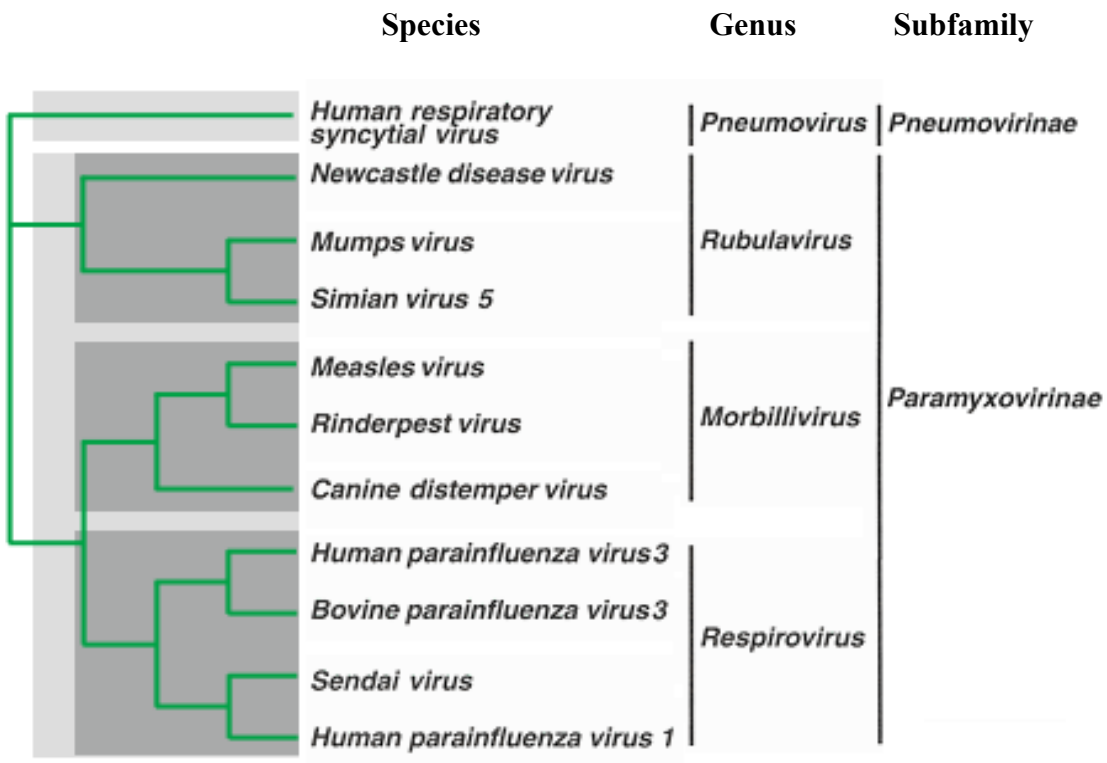


Figure 1. Phylogenetic tree of relevant members of the *Paramyxoviridae*. Not all genera are incorporated into the figure. Divergences are not to scale. (Courtesy of Christine Hulseberg)

Clinical Importance

The family *Paramyxoviridae* is one of the most significant families of viruses in regard to mortality, morbidity, and economic cost (34). In 2000, approximately 1.9 million children worldwide died due to acute respiratory infections caused by respiratory pathogens such as RSV and the human parainfluenza viruses 1-3, among others (86). In the United States, lower respiratory tract infections cause 19-27% of hospitalizations in children under the age of five years (35). Human parainfluenza viruses 1-3 are an important cause of these LRIs among infants, young children, the elderly, and even adults who are immunocompromised (34, 53, 84, 88).

As a group, human parainfluenza viruses can cause a wide range of respiratory tract diseases from asymptomatic infections and upper respiratory illness to more serious

lower respiratory conditions, such as croup, bronchiolitis, and pneumonia, which can be life threatening (34-35, 84). Human parainfluenza virus infections can also be accompanied by complications such as otitis media, pharyngitis, and conjunctivitis.

Among all of the human parainfluenza viruses, HPIV-3 is considered to be the most common and the most severe, causing the hospitalization of about 18,000 infants and children each year in the United States. Despite the fact that extensive epidemiological surveillance on HPIV-3 is lacking and such studies can yield differing results, data indicate that HPIV-3 is responsible for approximately 4,500 croup cases among children less than five years where the causative agent has been identified, and can also cause severe croup in adults. HPIV-3 has been identified as the cause of 10-15% of bronchiolitis cases in non-hospitalized children and approximately 40-60% of cases of bronchiolitis in hospitalized children. Similar to bronchiolitis, HPIV-3 causes about 10% of outpatient pneumonias, but causes three to four times as many cases among hospitalized patients. Among cases of bronchiolitis and pneumonia in infants, HPIV-3 is the second largest contributor after RSV (34).

Infections with HPIV-3 also result in longer and more expensive hospital stays than do other human parainfluenza viruses (28, 34, 53, 84, 88). The virus remains endemic year round to some extent; especially in immunocompromised populations, but most infections in the United States occur with yearly outbreaks peaking in the spring and summer (34, 53, 88). For about the first four months of life, infants are protected from HPIV-3 infection by maternal antibodies, but once these antibody levels begin to decline, the rate of infection rises until cresting at about 18 months of age. HPIV-3 infection is so common that by the age of three, most children show serologic memory when challenged

by HPIV-3 antigen (34, 88). Approximately 40% of HPIV-3 infections occur in the first year of life with about 60% of children infected by the age of two and 80% infected by the age of four (17, 34). Immunity following acute infection is not lasting and reinfection is common throughout life.

Despite the fact that acquired immunity is incomplete, it appears that at least some protection is achieved once primary infection has occurred. Typically, reinfection with HPIV-3 results in less serious disease, with the clinical presentation more likely to be an upper respiratory infection than a more serious lower respiratory tract illness (17, 28, 42, 84, 88). Still, as the immune system ages, elderly individuals can once again become susceptible to severe HPIV-3 infection. In nursing homes, HPIVs cause 4-14% of all respiratory illnesses, with some infections progressing to fatal broncho-pneumonia (19). However, even mild cases of reinfection with HPIV-3 among healthy adults are important, as these serve to further spread the virus to high risk groups, such as the immunosuppressed, the elderly, and infants (28, 53, 88).

Outbreaks of HPIV-3 in hospitals have been reported, especially among pediatric patients and immunosuppressed patients. In these cases, infection rates can be managed by adhering to infection control measures such as hand washing, wearing gloves and gowns, decontaminating surfaces, and even placing sick patients in isolation (88).

Transmission of HPIV-3 is likely achieved by large droplet aerosolization during coughing or sneezing and contamination of surfaces and fomites. The virus itself can survive up to 10 hours on nonporous surfaces, but rapidly loses its infectivity on skin. Therefore, it is not likely that the virus is efficiently spread person-person through direct hand contact (34, 88). The virus is more easily spread when contaminated surfaces or

objects are touched and then introduced to the host's respiratory system by contact with the eyes, nose, or mouth. The major site for virus binding and initial infection is likely respiratory epithelium. After an incubation period of two to eight days, symptoms become apparent (10, 28, 34). One to three days later, the virus may spread to the lower respiratory epithelium (28).

Because there are no specific treatments proven effective for HPIV-3 infections, treatment for mild cases is usually restricted to supportive therapy. There have been attempts to produce antiviral treatments for severe HPIV-3 infections, but most efforts to combat HPIV-3 have focused on developing an effective HPIV-3 vaccine (55, 81). These vaccines would probably not prevent infection completely, but would instead prevent serious lower respiratory illness and complications by acting as the "primary infection" so that subsequent HPIV-3 infections are less severe. The most effective vaccine for HPIV-3 would likely be a live attenuated vaccine. Currently, a temperature sensitive HPIV-3 vaccine candidate is in phase I/II clinical trials (17). At this time however, there are no FDA-approved vaccines for HPIV-3 available in the United States due to the difficulty in striking the appropriate balance between attenuation and immunogenicity (17). Continued study of HPIV-3 and related viruses is imperative for production of useful antiviral therapies and vaccines.

Structure and Organization of HPIV-3

Human parainfluenza virus 3 is a pleomorphic, medium-sized virus approximately 150-200 nm in diameter (10, 34). In the center of a virus particle, the viral genome consists of a single-stranded negative-sense RNA of 15,462 nucleotides that encodes six

viral structural proteins (Fig. 2). The exterior of the virion consists of a lipid envelope acquired by budding from the infected host cell membrane during viral release (10, 34). Embedded within this envelope are two surface glycoproteins (10). These proteins, hemagglutinin-neuraminidase (HN) and fusion (F), are secured by a hydrophobic transmembrane domain in an orientation in which most of each protein is outside the virus particle, except for small interior tails. The matrix (M) protein is located just beneath the membrane of the virus particle and is believed to act as a bridge between the membrane with its surface glycoproteins (HN and F) and the ribonucleoprotein (RNP) core of the virion. The RNP includes the viral genome bound by the nucleocapsid (N) protein (forming a helical nucleocapsid) as well as two other viral proteins, the phosphoprotein (P) and the large (L) polymerase protein (34).

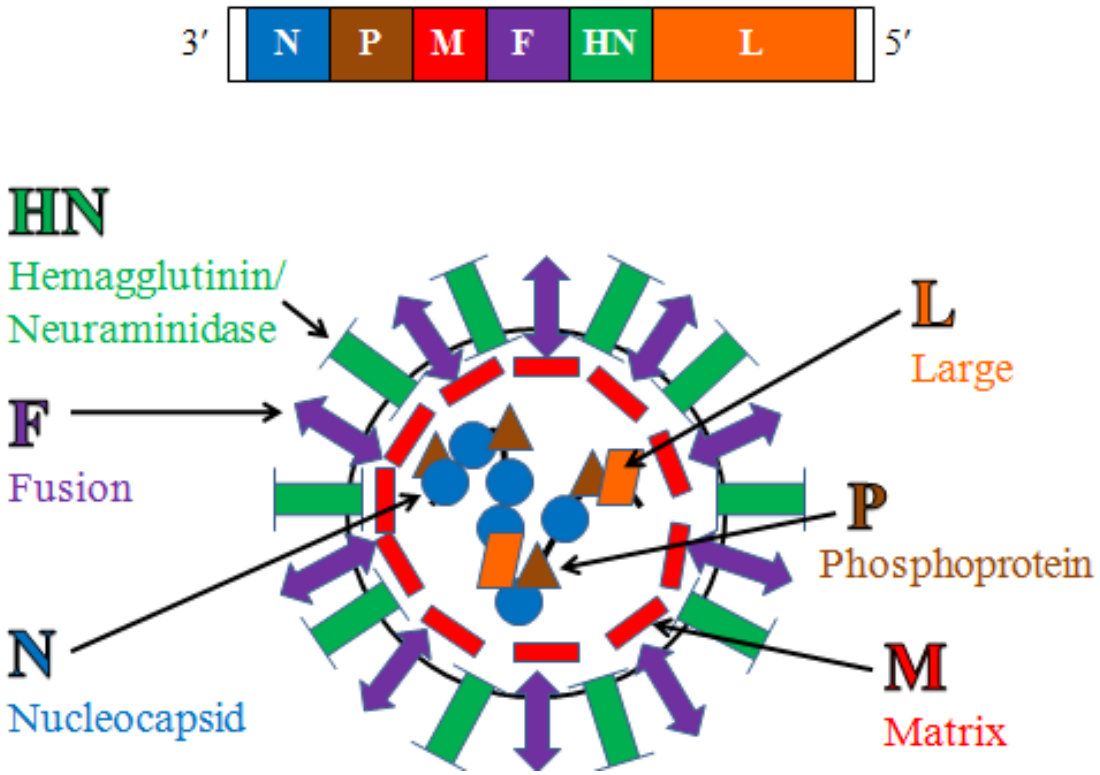


Figure 2. The structure and genome of HPIV-3.

HPIV-3 Life Cycle

Attachment and Entry

The HPIV-3 life cycle begins with virus attachment to a host cell. Infection is initiated by the tetrameric hemagglutinin-neuraminidase (HN) protein, which mediates adsorption of the virus to the host cell through its hemagglutination property of binding sialic acid residues present on carbohydrate side chains of proteins found on the surface of the host cell plasma membrane (10, 47, 58). Attachment then triggers the trimeric fusion (F) protein to insert into the host cell membrane. The F protein then mediates fusion of the viral membrane with the host cell plasma membrane, allowing the viral RNP access to the host cell cytoplasm (10, 18, 47, 58).

Transcription, Protein Synthesis, and Genome Replication

After the viral RNP enters the cytoplasm, transcription begins. Transcription is initiated when the phosphoprotein (P) and large (L) protein associate to form the viral RNA-dependent RNA polymerase and bind the 3′ end of the genome. This complex transcribes the N-encapsidated RNA genome by a sequential start-stop mechanism (10, 47). Because there is only a single promoter, located at the 3′ end of the viral genome, viral transcription always initiates with transcription of the 3′ most gene, the N gene. As it moves 3′ to 5′ along the viral RNA, the polymerase comes across transcription stop and transcription start signals at gene junctions which direct transcription termination of the upstream gene and transcription initiation of the downstream gene. However, after encountering a gene stop signal, the polymerase sometimes falls off the template. Because of this, more mRNA is made from genes situated close to the promoter (3′ end), and less mRNA is made from genes situated far from the promoter (5′ end). By this

method, the viral RNA is transcribed into six mRNAs which encode for the viral N, P, M, F, HN, and L proteins. During transcription the 5′ end of each transcript is capped and methylated and the 3′ ends of the mRNAs are polyadenylated. The mRNAs are then translated by host cell ribosomes to generate the final viral proteins, which collect in the cells. Once enough viral structural proteins have amassed from the initial transcription/translation cycle, replication of the viral genome can begin (47). Replication of a negative-sense RNA genome, facilitated by the viral RNA-dependent RNA polymerase, occurs by first synthesizing a positive-sense RNA intermediate, termed the antigenomic RNA. This antigenomic positive-sense RNA serves as a template for synthesis of genomic negative-sense RNA, which can then be further replicated, transcribed, or packaged into progeny virus particles (47).

Assembly and Release

As genome replication proceeds, viral proteins are positioned at various locations in the cell. Progeny genomes, in the form of RNP complexes, stay in the cytoplasm while the F and HN glycoproteins are directed to the endoplasmic reticulum for additional processing, and then are transported to the plasma membrane of the cell (27, 80). Although the mechanism for viral assembly at the plasma membrane is not fully understood, assembly of new virus particles is thought to be mediated largely by the matrix protein, which presumably brings the RNP core to the transmembrane F and HN glycoproteins located in the cellular plasma membrane (2). In an infected cell, the cytoplasmic tails of the glycoproteins likely make contact with the matrix protein, which is also associated with the RNP (2, 15, 18, 80).

Once the glycoprotein-M-RNP complex is formed, the virus is released from the host cell by budding. In this complex process, the virus pushes through the membrane of the host cell until membrane fission occurs, resulting in the release of an enveloped, mature virus particle (18, 85). The neuraminidase activity removes sialic acid residues from the HN protein and other cell membrane proteins, preventing progeny virus particles from self-aggregating as well as reattaching to the host cell. This enables the virus particles to efficiently disperse and infect new host cells, completing the viral life cycle (10, 47) (Fig. 3).

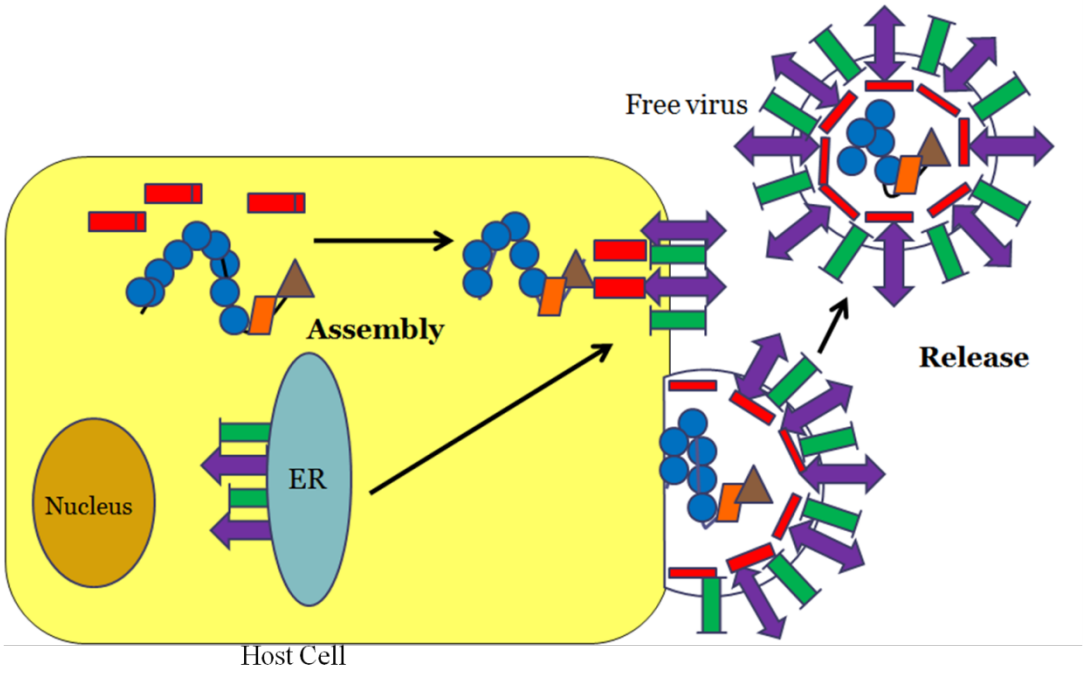


Figure 3. Assembly and release of HPIV-3.

The Matrix Protein of the *Mononegavirales*

The matrix protein in many viruses of the *Mononegavirales* coordinates both the localization of viral proteins during assembly and also the release of virus particles (3, 11, 16). The foundation for the hypothesis that M is the director of viral assembly has to do

with the protein's location in virus particles and its ability to associate with surface glycoproteins, the viral core RNP, as well as the plasma membrane (47). Structurally, the M proteins are highly basic, moderately hydrophobic, and likely contain amphipathic α-helices that enable them to integrate into the lipid bilayer (47). While there has been little research on the HPIV-3 M protein specifically, we know the HPIV-3 M protein is relatively conserved in sequence within the genus *Respirovirus*, and M protein function is expected to be conserved within the order *Mononegavirales*. However, the precise function of M protein has yet to be determined for HPIV-3.

Matrix proteins from different members of the *Mononegavirales* have been demonstrated to play an integral role in viral assembly interactions. For example, in both rhabdoviruses and filoviruses it has been shown that the matrix protein associates with the N protein in the RNP core of the virus, directing the RNP to the cytoplasmic surface of the plasma membrane where the cytoplasmic tails of the surface glycoproteins are located (29, 41, 73). The association of M and N proteins has been documented in many other members of the *Mononegavirales* such as HPIV-1, SeV, NDV, Ebola virus (EbV), Vesicular stomatitis virus (VSV), and measles virus (12, 15-16, 21-22, 34, 36-37, 44, 63-65, 77). Interactions between M and the two membrane glycoproteins, HN and F, have been demonstrated in SeV, EbV, VSV, and measles virus (2, 4, 9, 34, 54, 61, 71-72, 76). In SeV, EbV, VSV, and measles virus, an interaction between the M protein and the cell membrane has been observed (40, 48, 60, 64, 69-70, 75, 77). Besides associations with the viral proteins N, HN, and F, as well as the plasma membrane, it has been shown that the matrix protein also self-associates to form multimers in SeV, EbV, and VSV (24, 56, 70). The M protein in EbV, which has been particularly well studied, produces

monomeric, hexameric, and octameric forms (29). It has been shown that these different conformations have been tied to different functions in the viral life cycle (29). For example, the monomer is necessary for membrane interaction as well as oligomerization (the formation of the other protein conformations, the hexameric and octameric ring structures.) The hexamers, which are found in virus particles, seem to be stabilized by binding nucleic acids and are involved in membrane interactions. The octameric form consists of four dimers, which form pockets which bind single-stranded RNA molecules (29, 39). The role of EbV octamers is unclear, but it is possible that the octameric form may interact with viral nucleocapsids and aid in RNP formation or help control transcription or translation.

In support of a role for M proteins in binding RNA, the M proteins in the rhabdoviruses VSV and rabies virus have been shown to contribute to the regulation of viral RNA synthesis (8, 14, 38, 87). Additionally, VSV-M protein has been shown to arrest cellular transcription, although the mechanism for this inhibition is not known (5-6). However, RNA interactions do not appear to be a universal property of M proteins. In SeV (closely related to HPIV-3) the M protein does not seem to have a role in the control of viral RNA synthesis (59).

Besides being an integral force in the process of virus assembly, the M protein is also essential for advancing virus particle release from host cells. Most significantly, the majority of M proteins from members of the *Mononegavirales* facilitate their own release from host cells. When M proteins of SeV, HPIV-1, Nipah virus, VSV, and EbV were expressed individually in eukaryotic cells, virus-like particles (VLPs) comprised of M protein surrounded by an envelope were released into the surrounding media (13, 15, 30-

31, 43, 50, 79). These are unique properties for a single protein to possess, as outward release of a vesicle from a cell is rare and, in general, vesicle formation is usually a complex process involving interactions between multiple cellular proteins.

For some mononegaviruses, the surface glycoproteins also appear to have roles in viral release. For example, the paramyxovirus SV5 needs coexpression of M, N, and one of the surface glycoproteins (F or HN) for efficient VLP release (74). In SeV and EbV, the membrane glycoproteins, when expressed independently, do stimulate virus-like particle (VLP) release into the medium with low efficiency. However, in cells in which the SeV or EbV M proteins are coexpressed along with the respective glycoproteins, VLP release is more efficient (63, 79).

The HPIV-3 Matrix Protein

The HPIV-3 matrix protein is a 40 kDa, basic, slightly hydrophobic protein composed of 354 amino acids, similar to the matrix proteins from related viruses within the *Mononegavirales* (47). To identify and characterize interactions between the HPIV-3 M protein and other viral proteins that may be important in virus assembly and release, our lab previously performed a series of co-immunoprecipitation experiments. To do this, a vaccinia virus-T7 RNA polymerase expression system was used to express different plasmid- encoded HPIV-3 proteins in HeLa cells. A version of the M protein with a C-terminal FLAG tag (DYKDDDDK) was created to allow for immunoprecipitation purification with an anti-FLAG antibody. In this procedure, proteins were radiolabeled with [^{35}S]Met/Cys and then immunoprecipitated from cell lysates using the anti-FLAG antibody. During the immunoprecipitation, the FLAG-tagged matrix protein (M_f) was precipitated, as well as any other proteins that M_f bound.

SDS-PAGE and autoradiography were then performed to visualize precipitated proteins bands (45) (Fig. 4).

When M_f was coexpressed with wild type HPIV-3 proteins M, N, F, HN, P, and L proteins, the M, N, and P proteins were found to co-immunoprecipitate with M_f, indicating that the M protein self-associates and interacts directly with the HPIV-3 N and P proteins (45). Importantly, immunoprecipitation of cells expressing M alone, N alone, or P alone were not immunoprecipitated with the α-FLAG antibody (data not shown). The lack of interactions between M and F or HN may be due to the detergent and high-salt conditions of the co-immunoprecipitation procedure.

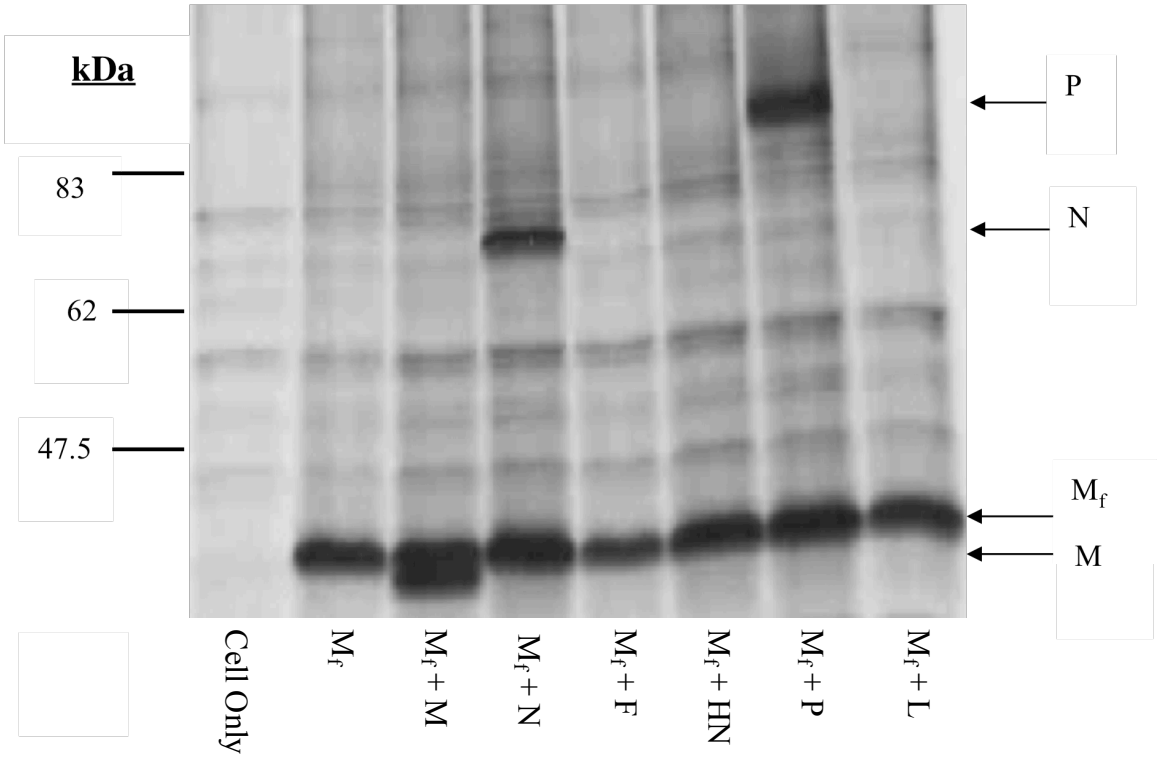


Figure 4. Protein interactions between FLAG-tagged matrix protein (M_f) and wild-type HPIV-3 proteins. (Christin Kiesner, unpublished data).

To investigate if the HPIV-3 matrix protein was involved in the budding process as in related viruses, M protein was expressed alone in eukaryotic cells and observed for

lipid vesicle release. To express the HPIV-3 M protein, the M gene was cloned into the pCAGGS eukaryotic expression vector and the resulting pCAGGS-M plasmid was transfected into 293T cells, a human cell line derived from embryonic kidney cells. Cells were then radiolabeled with [^{35}S] Met/Cys and allowed to incubate 18 hours until the media was collected and the cells lysed. The media was then clarified by centrifugation and treated with a detergent to solubilize the membranes of any VLPs. Then, both lysate and media samples were incubated with an anti-HPIV-3 polyclonal antibody and protein A agarose beads for immunoprecipitation. Proteins were then separated by SDS-PAGE and visualized by phosphorimager analysis. Quantification of protein levels were determined by ImageQuant software. M protein was identified from the media of the transfected cells, indicating that the matrix protein alone triggers the release of VLPs (45). Approximately 25% of the total HPIV-3 M protein was released into the media, similar to Ebola virus VP40 and natural HPIV-3 infection (data not shown).

To verify that the released M protein was truly in the form of membrane-enveloped VLPs, media from pCAGGS-M transfected 293T cells was treated with either trypsin, 1% Triton X-100, or both trypsin and 1% Triton X-100. After treatment, trypsin inhibitor was added and the M protein immunoprecipitated. M protein only became vulnerable to digestion when Triton X-100 was added in addition to trypsin. This indicates that M protein was protected from digestion by the lipid envelope of the VLP and was only susceptible to digest when the envelope was solubilized by the detergent (Fig. 5).

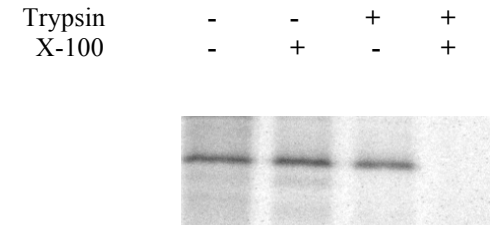


Figure 5. Trypsin digestion of M protein from the media occurs only when added with the detergent Triton X-100. (Michael Hoffman, unpublished data).

RESEARCH OBJECTIVES

Based on the HPIV-3 M protein's homology with the matrix proteins of related viruses, its interaction with other HPIV-3 proteins, and its release in the form of membrane-enveloped VLPs even when expressed alone, the matrix protein of HPIV-3 appears to play an important role in HPIV-3 assembly and release. While it has been shown that the M protein is all that is needed for release of HPIV-3 virus-like particles, it is still unclear as to whether other HPIV-3 proteins can aid in the assembly and budding processes. Investigating this possibility is the central objective of my research.

Currently, it is not known if any other viral proteins besides M are able to initiate HPIV-3 virus particle assembly and release. Similar to the preliminary findings regarding HPIV-3, the M protein of SeV is all that is required for efficient budding. However, there is also evidence from SeV that the envelope protein F, when expressed individually, can stimulate budding of VLPs, although less efficiently than SeV M protein (63, 79). In addition, when SeV M and F are coexpressed, VLPs are released at a higher efficiency than when either protein is expressed alone, indicating cooperative interactions between the proteins. Also, when SeV M, N, HN, and F were expressed together, it resulted in the release of particles containing these proteins (78). Thus, based on findings from SeV and other related viruses, it is believed that HPIV-3 N, F, and HN proteins, when coexpressed with M, will be able to assemble and then exit the cell together in VLPs (23, 25, 43, 63, 74, 79, 83). Also, it is possible that coexpressing additional viral proteins with M may result in an enhancement of virus particle budding.

Therefore, the main goal of this research is to determine the contribution of HPIV-3 M, N, HN, F, and P proteins in stimulating virus particle assembly and release. Doing so would result in the development of a multi-HPIV-3 protein assembly and budding assay. Development of an HPIV-3 assembly and budding assay would help to better understand these processes in HPIV3, and also help establish or refute generalizations with regard to paramyxovirus assembly and budding. This functional assay would enable the study of protein structure, function, and their relationships to viral assembly and release. For example, in the future we plan to do investigations in which protein-protein interaction domains are mapped by mutating HPIV-3 proteins and assaying in co-immunoprecipitation experiments. The assembly and budding assay could then be used to determine if these mutated proteins also lose the ability to assemble together into VLPs.

Generation of multi-protein VLPs has the potential for use in vaccine development. The Hepatitis B and human papilloma virus vaccines are composed of non-enveloped VLPs, while enveloped Ebola virus multi-protein VLPs show promise as a vaccine (1, 7, 20, 89).

Two main objectives for this research have been established to determine HPIV-3 viral protein requirements for assembly and budding:

1. Determine the budding capabilities of individual HPIV-3 proteins in promoting virus particle assembly and release.
2. Determine the contributions of the N, HN, F, and P proteins to M protein-driven VLP formation.

MATERIALS AND METHODS

Cells

293T cells (human renal epithelial cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and Penicillin-Streptomycin. HeLa cells were grown in DMEM supplemented with 5% FBS, L-glutamine, and Pen-Strep. Both cell lines were grown at 37°C under 5% CO_2.

Plasmid Construction

HPIV-3 DNA sequences corresponding to M, N, F, and HN proteins were previously cloned into the pCAGGS eukaryotic expression vector system to generate pCAGGS-M, pCAGGS-N, pCAGGS-F, and pCAGGS-HN, respectively. A C-terminal FLAG tag was cloned into pCAGGS-F by PCR-directed mutagenesis for detection by anti-FLAG antibody during immunoprecipitation assays. This plasmid was designated pCAGGS-F_f. All clones were validated by sequencing by Eton Biosciences, San Diego, CA.

Transfection and Metabolic labeling

293T cells were propagated to subconfluent levels (70-80%) in standard 6-well plates. Transfections were carried out in Opti-MEM using Lipofectamine LTX and PLUS reagents (Invitrogen by Life Technologies, Carlsbad, CA) as per manufacturer's instructions. Plasmid quantities per well were as follows unless otherwise indicated: pCAGGS-M, 0.8µg; pCAGGS-N, 0.1 µg; pCAGGS-HN, 0.18µg; pCAGGS-F_f, 0.18µg.

Variances in plasmid amounts were due to differences in protein expression from the plasmids. When needed, transfections were supplemented with empty pCAGGS vector to equalize amounts of overall plasmid DNA. At 24 hr post transfection, the DMEM supplemented with 10% FBS was removed and replaced with 1.2 mL methionine and cysteine-free DMEM to deplete cells of the supply of methionine and cysteine for protein synthesis. After 30 min incubation at 37°C, 40 μCi of [^{35}S] Met/Cys (Amersham Biosciences) was added and then incubated an additional 20 hr.

Generation of VLPs and Immunoprecipitation

After radiolabeling, the media was collected and the cells lysed with 400 μL cell lysis buffer (0.5% NP-40, 20 mM Tris-HCl [pH 7.4], 150 mM NaCl; Promega, Madison, WI). Both media and cell lysate samples were centrifuged for 2 min at 2000 × *g* in a microcentrifuge to remove cell debris. Supernatants from the lysate samples were incubated at room temperature with either anti-HPIV-3 whole virus antibody (used to detect M, N, HN and P proteins) and Protein A beads (Thermo Scientific) or anti-FLAG antibody (Sigma, used to detect F_f) and Protein G beads (Thermo Scientific). After incubation with the appropriate antibodies, proteins were immunoprecipitated by washing the pellets four times with 1X radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Thermo Scientific) and once with water. Pellets were resuspended with 30 μL 2X SDS running buffer (125 mM Tris-HCl [pH 6.8], 4.6% SDS, 20% glycerol, 10% 2-mercaptoethanol) and heated to 95°C for five minutes. Proteins were separated by SDS-PAGE on 0.75 mm 10% acrylamide gels and then dried. Proteins were visualized using a Storm Imager 860 phosphorimager and ImageQuant software. Instead of

immunoprecipitation, supernatants from media samples were layered onto 3.2 mL 20% sucrose in PBS and centrifuged at 186,000 × *g* for two hours at 4°C. Pellets were then resuspended in 2X SDS running buffer, separated by SDS-PAGE, and visualized as above.

Sucrose Floatations

To verify proteins were being released from cells in the form of enveloped VLPs, culture media suspected to contain VLPs from duplicate wells were subjected to slow speed centrifugation to clarify from cell debris and then layered onto 2.5 mL 20% sucrose in NTE (0.1 M NaCl; 0.01 M Tris-HCl, pH 7.4; 0.001 M EDTA) and centrifuged at 140,000 × *g* for 1.5 hours at 4°C. Pellets were resuspended in 0.5 mL NTE, mixed with 1.3 mL 80% sucrose in NTE (final concentration 58%), and then placed at the bottom of another ultracentrifugation tube for floatation analysis. Additional layers of 50% sucrose (1.8 mL) and 10% sucrose (0.6 mL) were layered on top of the sample. Samples were then centrifuged at 110,000 × *g* for 3 hours at 4°C. A 2.1 mL volume was collected off the top of the gradient after centrifugation. This 2.1 mL fraction was layered over a 20% sucrose cushion and centrifuged at 140,000 × *g* for an additional 1.5 hours at 4°C. Pellets were resuspended in 2X SDS loading buffer and the samples were run on SDS-PAGE and visualized as described above.

Electron Microscopy

To generate concentrated virions for electron microscopy, 150 mm dishes of HeLa cells were infected with HPIV-3 at an MOI of 0.1. At 48 h p.i., media from the dishes was harvested and centrifuged at 1500 × *g* for 15 minutes at 4°C to clarify cellular debris. The supernatant was then centrifuged at 131,000 × g for 1 hour 15 minutes and

pellets were resuspended in 250 µL serum-free DMEM. To generate VLPs for electron microscopy, 293T cells grown in 150 mm dishes to 70-80% confluency were transfected with plasmids to produce HPIV-3 M protein, HPIV-3 M and N proteins, HPIV-3 M and HN proteins, HPIV-3 M and F proteins, HPIV-3 M, N, and HN proteins, or HPIV-3 M, N, HN, and F proteins. At 40 hours post transfection, the culture medium was harvested. Cellular debris was removed by centrifugation at 1500 × *g* for 15 minutes at 4°C. The supernatant was then layered onto sucrose cushions (10 ml of 20% sucrose in NTE) and centrifuged at 131,000 × g for 3 hours at 4°C. VLP pellets were resuspended in 75 µl of NTE. Samples were adsorbed onto 200 mesh formvar-coated copper grids and then stained with 2% phosphotungstic acid and examined using a Zeiss EM-900 microscope, operated at 50 kV.

RESULTS

Aim 1: Determine the budding capabilities of individual HPIV-3 proteins in promoting virus particle assembly and release

To determine the ability of individual HPIV-3 proteins in contributing to virus particle assembly and release, 293T cells were transfected with plasmids encoding each of the viral proteins. The plasmid amounts used resulted in expression levels similar to those seen in HPIV-3 infections. The cells were then metabolically labeled with [^{35} S] Met/Cys for 20 hours, after which both culture media and cell lysates were harvested. After clarification of the media, particles were pelleted through 20% sucrose, resuspended, and then analyzed by SDS-PAGE. Cell lysates were analyzed by immunoprecipitation with the appropriate antibody and SDS-PAGE.

As expected, HPIV-3 M protein was detected in the culture media when the M protein was expressed individually in cells (Fig 6, lane 7, all panels). When the N protein was expressed individually, there was no release of N protein into the culture medium (Fig. 6A, lanes 8 and 9). The HPIV-3 envelope proteins, HN and F, were also expressed individually in cells. No HN protein was detected in the culture medium (Fig. 6B, lane 8) indicating that HN was not able to trigger release of viral protein by itself. The HPIV-3 F protein was expressed alone in cells using pCAGGS-F_f and again, although there was expression of F protein in the cell lysates, no F_f protein was released into the culture medium (Fig. 6C, lane 8). The HPIV-3 F protein is initially made as a precursor protein, F_0, which is incapable of mediating fusion, but may still function in assembly (65). This

precursor protein is cleaved intracellularly by a trypsin-like protease to produce the two "active" subunits of the F protein, F_1 and F_2, which contribute to the fusion activity and infectivity of the virus (46). Both F_0 and F_1 are capable of assembly and release in other paramyxoviruses, such as SeV and NDV (49, 65-66, 68, 78). Finally, P protein was analyzed by the same budding assay to determine if it could be involved in the assembly process (Fig. 6D). As with the other viral proteins, P protein was unable to prompt its own release from host cells (Fig. 6D, lane 8). Thus, based on these findings, none of the tested HPIV-3 proteins (N, HN, F, and P), when expressed alone, were able to promote release of viral proteins, unlike the HPIV-3 M protein.

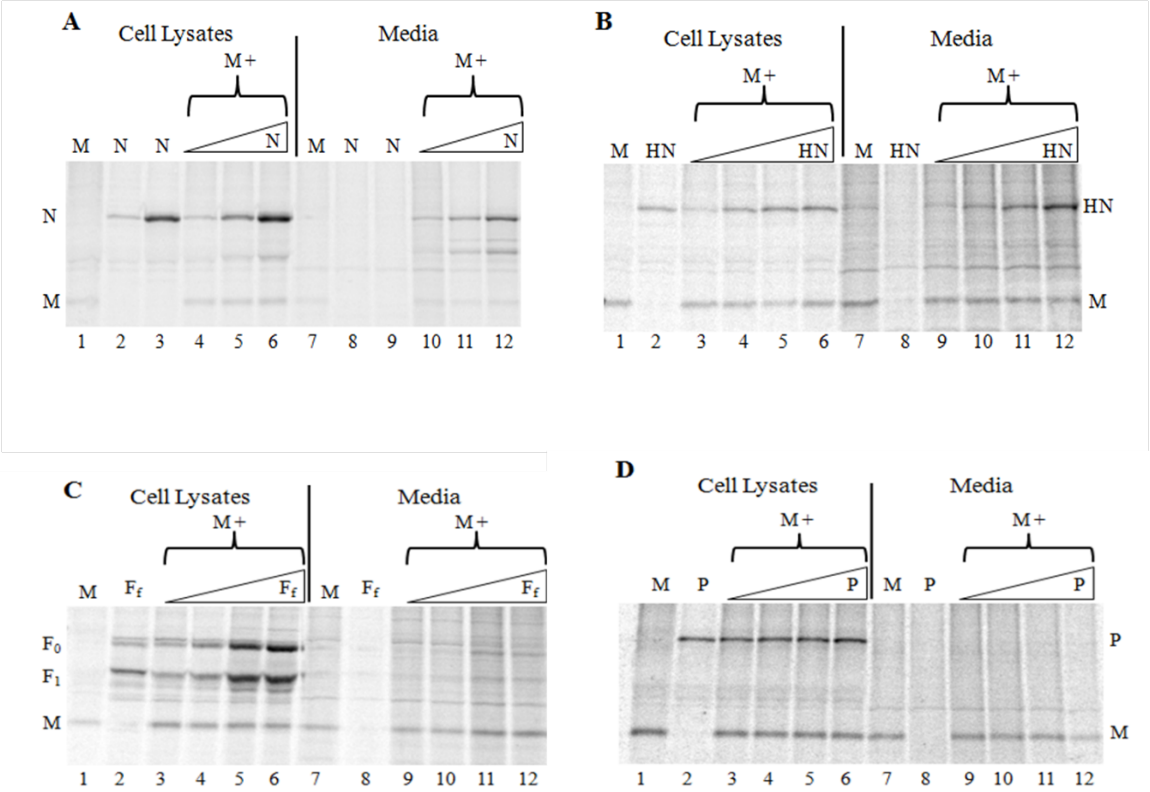


Figure 6. Abilities of individual HPIV-3 proteins to exit from cells when expressed alone and with M protein. M protein was expressed with 0.8 µg plasmid in all panels. (A) Cells transfected with plasmid encoding HPIV-3 N protein. (B) Cells transfected with plasmid encoding HPIV-3 HN protein. (C) Cells transfected with plasmid encoding HPIV-3 F_f protein. (D) Cells transfected with plasmid encoding HPIV-3 P protein.

Aim 2: Determine the contributions of the N, HN, F, and P protein to VLP formation

To determine whether N, HN, F, and P structural proteins release from host cells when coexpressed with the HPIV-3 M protein and possibly increase the efficiency of budding, similar budding assays were used. First, N and M proteins were coexpressed in cells at levels comparable to those found in cells infected with HPIV-3 (Fig.6A). Unlike when N was expressed alone, when N was coexpressed with M, both N and M proteins were released into the culture medium, indicating that the M protein interacts with N to

facilitate the release of N protein from host cells. This procedure was also used with proteins HN (Fig 6B), F_f (Fig. 6C), and P (Fig. 6D). As seen with N, HN was only secreted when coexpressed with M, indicating that M interacts with HN to facilitate the release of HN protein. In analyzing the HPIV-3 F protein, results were somewhat ambiguous. There was good expression of the F_f protein in cell lysates, but when the culture media from cells transfected with M and F_f were examined, a band corresponding to F_0 appeared to be present (lanes 11 and 12). However, the F_0 band was weak and difficult to detect on the gels, and the F_1 band was not detectable. This may be due, in part, to background bands (particularly with the F_1 protein) which may be obscuring visualization of the F_f bands from the media. These results did not give definitive data as to whether F_f exited host cells with the assistance of M protein. Finally, the P protein, even with the coexpression of M, was not released into the culture medium. This suggests that viral proteins besides M protein must be present to allow for P to be successfully assembled into the virus particle and then released.

It is possible that different combinations of viral proteins could result in the packaging of several proteins into virus-like particles. To determine if this was achievable with HPIV-3, budding assays similar to those already described were performed; except three or more viral proteins were coexpressed in cells together. Lysates for the M, N, HN and M, N, F groupings were again immunoprecipitated, while the media was centrifuged through 20% sucrose cushions. When M, N, and HN were coexpressed, all three proteins were successfully released from the cells into the culture media (Fig 7A, lane 12). However, when M, N, and F were expressed in cells together, F_f was not visible and only M and N were clearly being released (Fig. 7B, lane 12).

However, there is a fairly strong background band visible (a possible breakdown product of N) in lane 11 at the location of F_0, which could be obscuring the F_0 band.

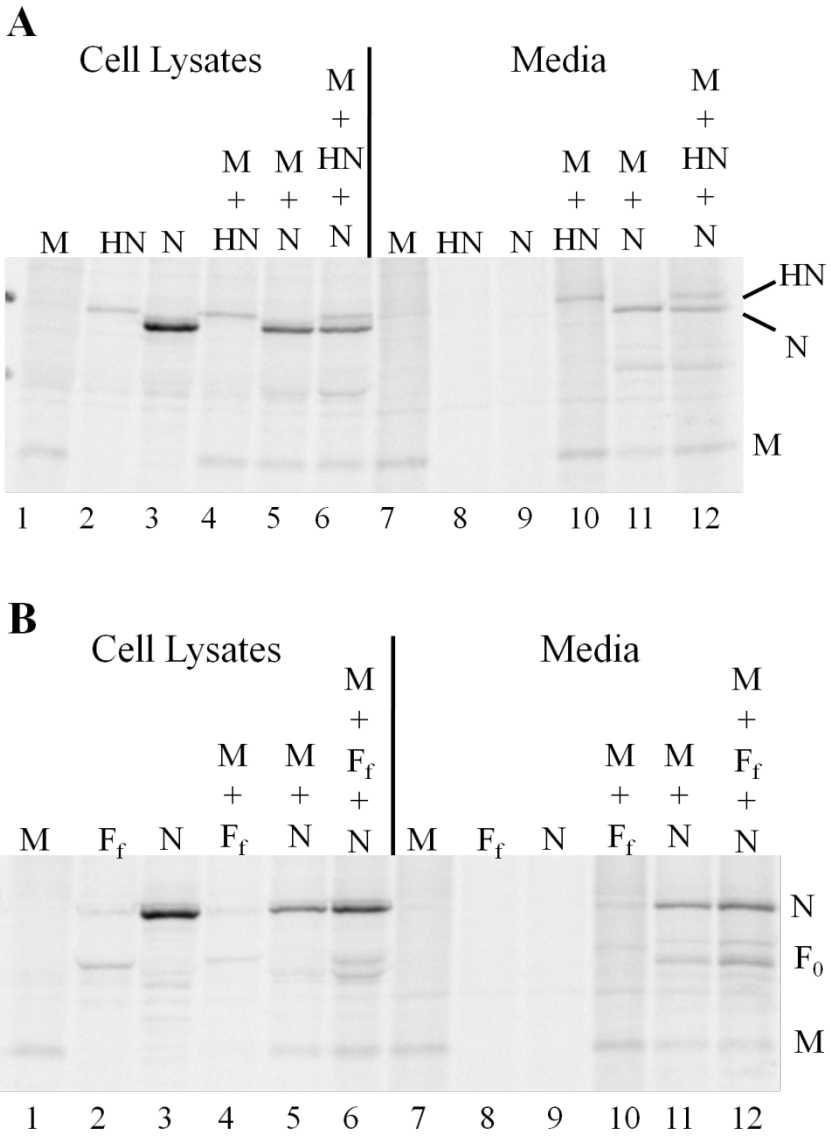


Figure 7. Multiple HPIV-3 proteins assemble and release from 293T cells. Plasmid amounts used are indicated in Materials and Methods. Lysate samples were immunoprecipitated using either α-RNP Ab, α-FLAG Ab, or both (lanes 4 and 6). Media samples were layered onto 20% sucrose cushions and ultracentrifuged. (A) 293T cells were transfected to produce particles containing M, HN, and N. (B) Cells transfected with plasmids encoding M, N, and F proteins.

To verify that viral proteins detected in the media by budding assays were being released in the form of true enveloped virus-like particles, a sucrose floatation was performed. The released proteins, if in fact surrounded by an envelope, should float toward the top of the gradient above the 50% sucrose layer due to the lipid content of the envelope. A protein that is not enveloped should remain near the bottom of the gradient due to its higher density. After floatation centrifugation the top 2.1 ml was collected and then pelleted by ultracentrifugation through a 20% sucrose cushion. Proteins were then separated by SDS-PAGE and analyzed using a phosphorimager. Combinations including M and N; M and HN; M and F; M, N, and HN; M, N, and F; and M, N, HN, and F were transfected into 293T cells. Successful release and floatation of VLPs occurred with all combinations (Fig. 8). However, similar to past experimental results, no evidence showed that F_f was being released in the form of a VLP with M protein. When M, N, and F_f were coexpressed, the M and N proteins clearly floated, but the F_f protein did not. A similar result also occurred with coexpression of M, N, HN, and F_f proteins, with F_f not floating, despite the successful floatation of the M, N, and HN proteins. It is possible that due to the extra floatation step during this procedure, additional material was lost, which could account for faint bands for all combinations of viral proteins.

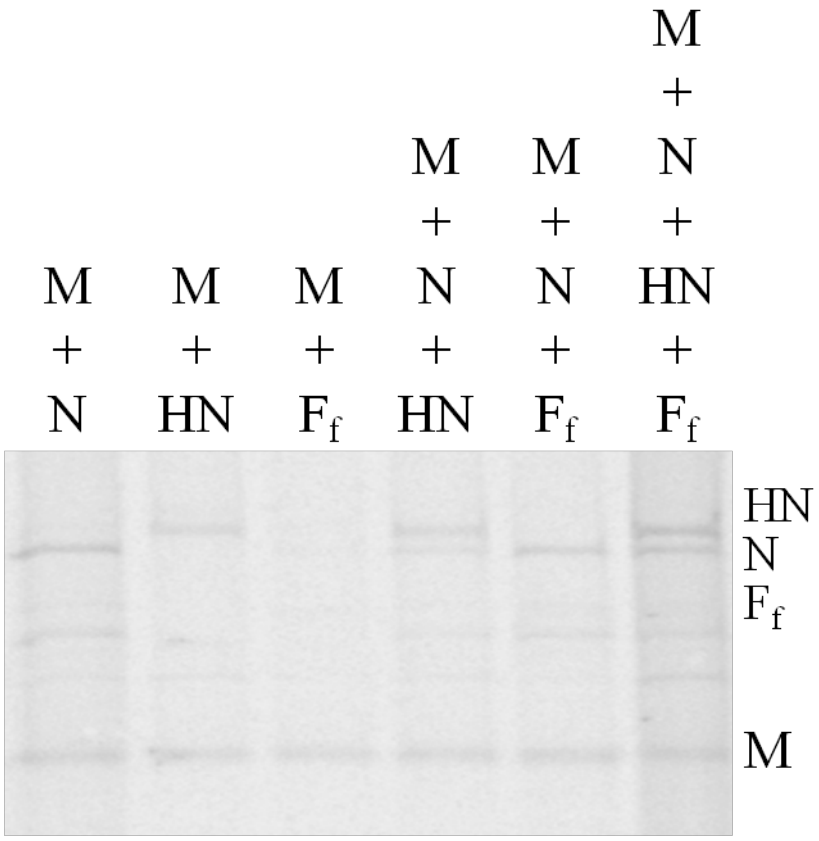


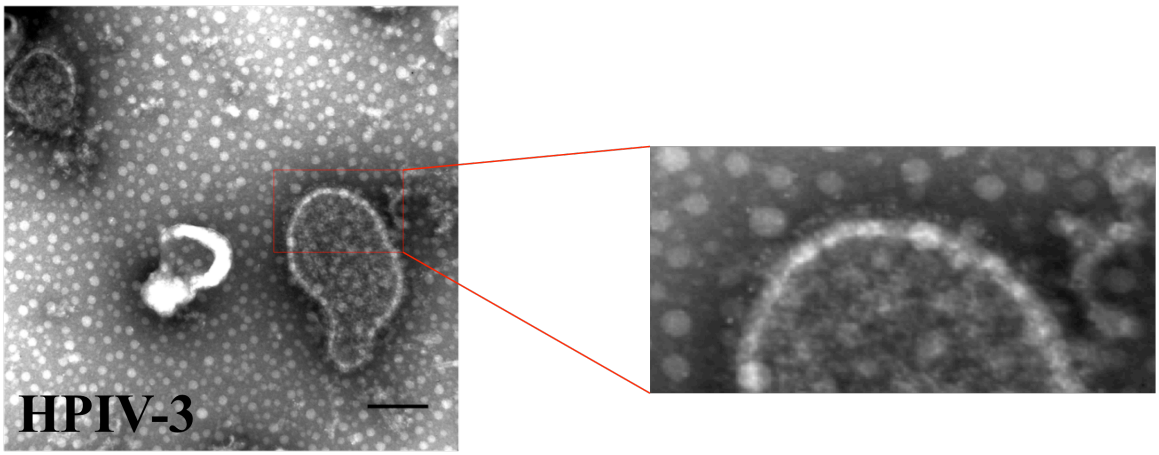
Figure 8. Verification of VLP formation by sucrose floatation. 293T cells were transfected with the indicated plasmids in amounts described in the Materials and Methods. At 24 h p.t. cells were radiolabeled with [^{35}S] Met/Cys and then the media was harvested 48 h p.t. Media samples were pelleted through 20% sucrose cushions, resuspended, and separated on sucrose floatation gradients. The top 2.1 ml was collected and pelleted through a 20% sucrose cushion by ultracentrifugation. Pellets were resuspended and analyzed by SDS-PAGE.

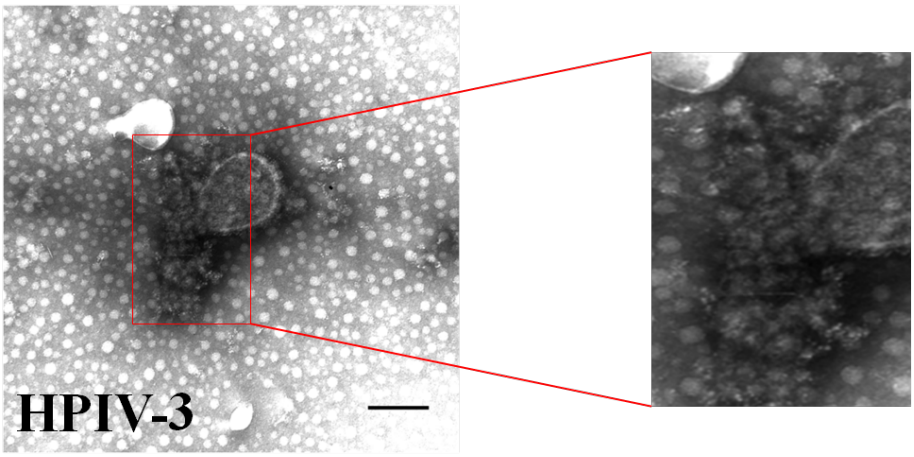
Electron microscopy was used to further confirm that these proteins were being released into the culture medium in the form of enveloped, virus-like particles (Fig. 9). HPIV-3 grown in tissue culture cells forms particles which are roughly spherical in shape and about 150 nm in diameter. Viral HN and F proteins form projections from the lipid envelope that can be visualized by electron microscopy as glycoprotein spikes. In this experiment, HPIV-3 virions visualized by EM were larger than expected, with diameters ranging from about 200 nm to 300 nm. However, paramyxovirus virions in general have

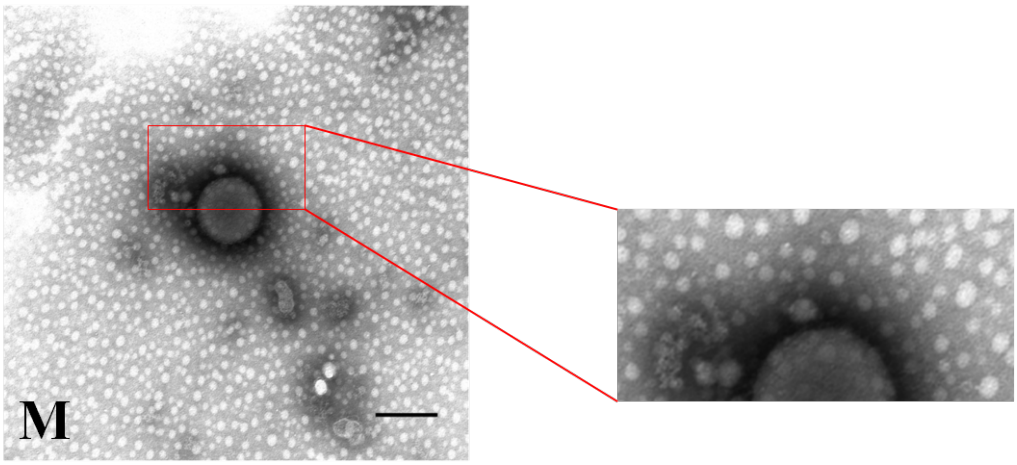
demonstrated to be extremely pleomorphic, with diameters ranging from 120 to 450 nm (82). For example, a study on SeV revealed virions and VLPs that were highly variable in size, ranging from 110 nm to 540 nm in diameter (51). Viral glycoproteins were visible as a spike fringe on the exterior of the virus particle. To determine if virus-like particles released from transfected cells were similar in morphology and size to infectious HPIV-3 virions, concentrated portions of media were examined by electron microscopy.

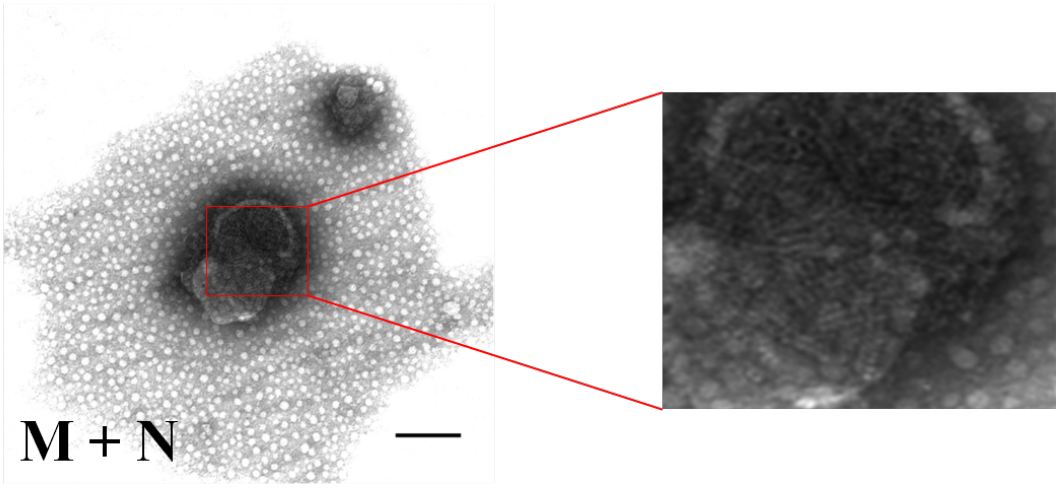
Virus-like particles from cells expressing only M protein were visualized by electron microscopy as smooth, roughly spherical vesicles approximately 100 nm in diameter (Fig. 9, M). M and N proteins were also coexpressed together to be imaged by electron microscopy (Fig. 9, M+N). Results showed enveloped particles about 150 nm in diameter with N-RNA "herring bone" structures within and spilling out of the particles. These "zipper-like" structures are often seen when N protein binds cellular RNA to from a helical N-RNA complex. Images from cells transfected with plasmids encoding M and HN were also indicative of both M and HN proteins releasing in the form of virus-like particles, in agreement with my previous budding assay results (Fig. 9, M+HN). These particles had the characteristic protein spike fringe on the outer surface of the envelope, which is consistent with true virus particle appearance, with a diameter of about 120 nm. Interestingly, when specimens were processed for electron microscopy using cells transfected with M and F_f, despite inconclusive results for F_f protein being released from cells, it appeared that F_f protein was being packaged and released with M protein in the form of virus-like particles (Fig. 9, M+F). In these images, envelope protein spikes were visible on the surface of virus-like particles, indicating that F_f is in fact capable of successful assembly and release from host cells when coexpressed with M. Notably,

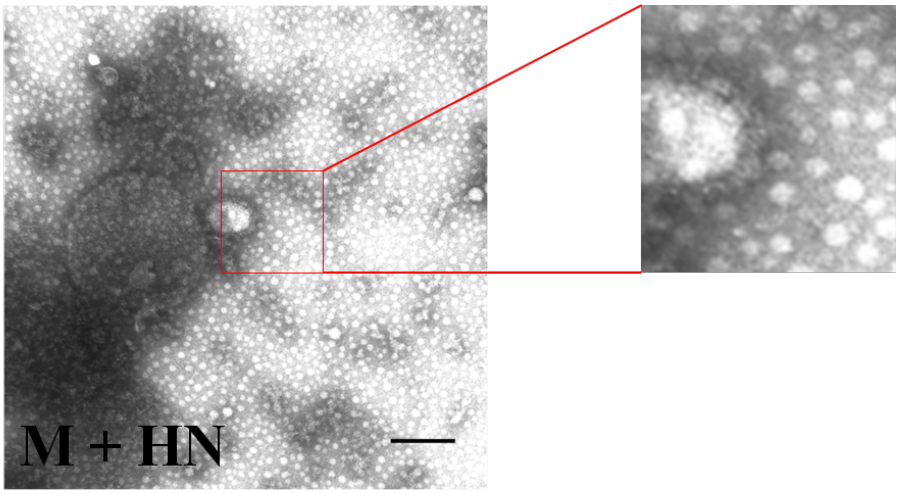
these particles were larger than expected, measuring approximately 220 nm in diameter, with one particle about 450 nm in diameter. Finally, VLPs from cells that expressed multiple HPIV-3 viral structural proteins (M, N, HN, and F) were similar in morphology to true HPIV-3 virions, indicating that these viral proteins are able to assemble and release from cells as enveloped VLPs, although they were larger than average virions with diameters that ranged from 275 nm to 400 nm (Fig. 9, M+N+HN+F). While virions and certain VLPs visualized by electron microscopy were larger than typical HPIV-3 particles, diameters seen were still within the range documented for paramyxoviruses (51, 82). N-RNA "herring-bone" structures were also visible on the inside of the particles. Although a protein fringe was visible on the exterior of the particle, it was not possible to distinguish between the two glycoprotein spikes when they were coexpressed. Cells transfected with plasmids encoding for M, N, and HN proteins did not yield particle production under electron microscopy, despite the fact that previous budding assays suggest that these proteins are able to successfully assemble and release the host cell as VLPs. In the future, this combination of proteins could be coexpressed again, perhaps with larger amounts of concentrated particles, as well as the combination of M, N, and F, which has been difficult to detect using budding assays alone. Another sample combination that may be interesting to view under electron microscopy is M, HN, and F proteins, which should again result in particles with glycoprotein spikes visible protruding from the envelope.

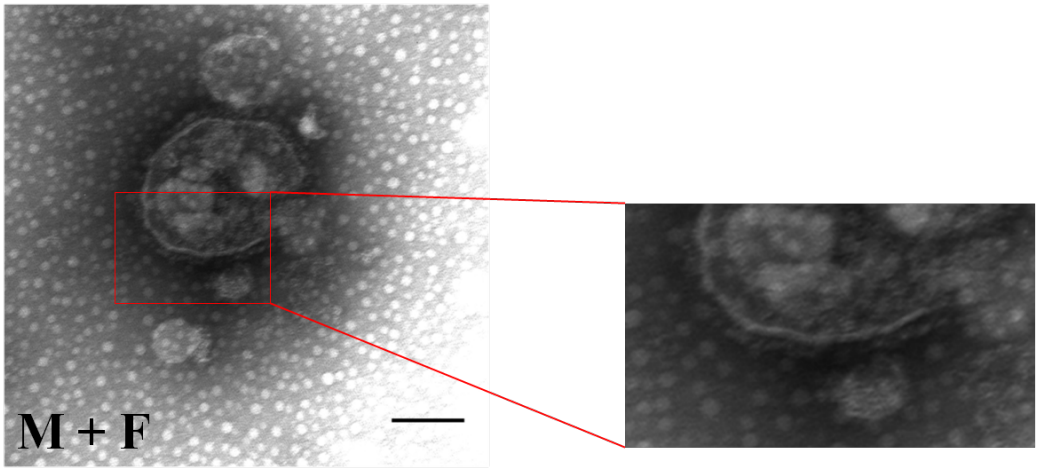

HPIV-3


HPIV-3


M


M + N


M + HN


M + F

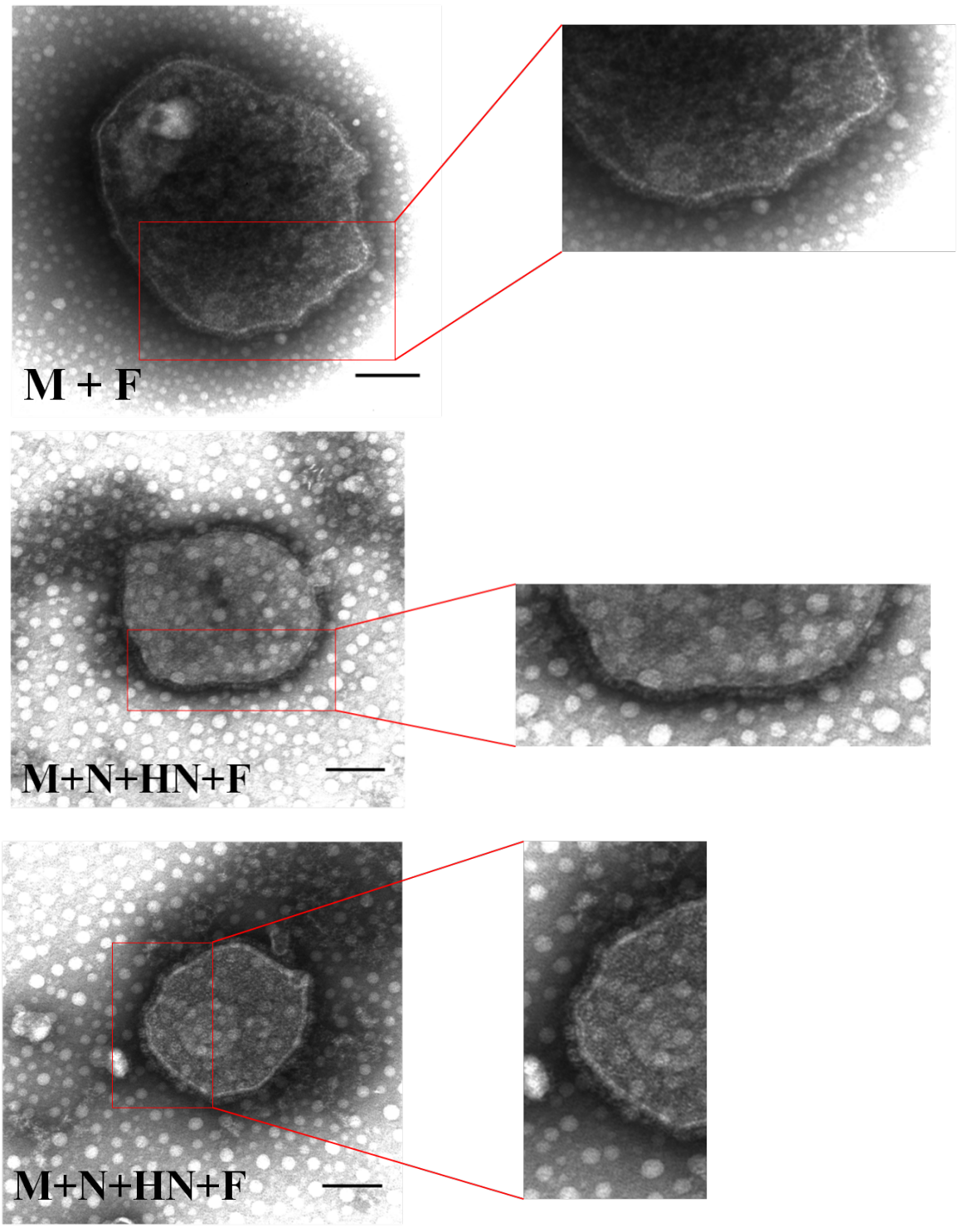


Figure 9. Transmission electron microscopy images of HPIV-3 virion and virus-like particles. HPIV-3, M, M+N, and M+HN panels were imaged at 150,000 X. M+F and M+N+HN+F were imaged at 185,000 X. Scale bars represent 100 nm.

DISCUSSION

Virus-like particle production assays have been valuable tools in studying viral assembly and release from host cells. I have established a system for generating HPIV-3 VLPs containing multiple viral proteins from transfected cells. In this study, I first determined the budding capabilities of individual HPIV-3 proteins in promoting virus particle assembly and release. HPIV-3 VLP creation required expression of the viral M protein. This is in agreement with the M protein's perceived role as the primary coordinator of viral assembly, and is consistent with results from a number of different negative-stranded RNA viruses where M protein is sufficient for release, including SeV and HPIV-1 (15, 78-79).

HPIV-3 M protein is also required for release of any other HPIV-3 proteins. Without expression of M protein, other HPIV-3 viral proteins are not capable of inducing particle release. When expressed alone, HPIV-3 N, HN, and P proteins are unable to trigger budding from host cells. Additionally, despite issues with F protein detection, HPIV-3 F protein does not seem to be able to prompt its own release. These findings contrast studies with SeV, where it has been shown that SeV F protein is able to trigger the release of F-containing particles when expressed alone, although release efficiency is lower (78-79).

HPIV-3 proteins, while unable to trigger their own release, successfully bud from host cells once coexpressed with HPIV- M protein, as expected. Pair-wise expression of N and HN protein with M results in particles containing N and M or HN and M,

respectively. These results indicate that the M protein must interact with these proteins, thereby facilitating the assembly and release of VLPs. Incorporation of N protein into released VLPs has been shown previously in HPIV-1, SeV, Nipah virus, PIV-5, and mumps virus (16, 49, 66, 74, 78). In some cases (PIV-5 and mumps virus) the inclusion of N protein increased efficiency of budding, although this is not the case with HPIV-3 (49, 74). Coexpression of M and P does not result in the release of particles containing both proteins. These results contradict an earlier co-immunoprecipitation experiment done in the Hoffman lab which indicated a possible interaction between M and P (Fig. 4). However, it is possible that the interaction between M and P may be involved in replication or transcription control rather than in facilitating assembly and release. The P protein evidently requires additional factors besides just the expression of M protein to successfully bud from host cells. It is possible that coexpression of M and N protein with P may allow for P to release as a VLP.

It is still somewhat unclear at this point whether F protein is indeed exiting the cell in VLPs with M. The electron micrographs are indicative of successful egress, but using sucrose cushions and floatation experiments, I was unable to clearly visualize released F protein. This could be due to background bands at the approximate location of the F protein bands. Alternatively, the C-terminal FLAG tag could be impeding its assembly and subsequent release from cells, making it more difficult to visualize an F_f band. In a study on NDV, a C-terminal FLAG tag was fused to the NDV F protein, and the FLAG-tagged F protein was not incorporated into particles. In this case, the authors surmised the tag sequence inhibited assembly of F protein into particles (57). It is possible that immunoprecipitating after pelleting the VLPs through sucrose cushions or

sucrose floatations may be a way to reduce background interference and clearly detect and define the F protein band on SDS-PAGE gels.

After these proteins were expressed pair-wise with M protein, different combinations of multiple proteins were studied to confirm that true VLPs were being produced and to characterize these VLPs. As expected, VLP production was confirmed by sucrose floatation and VLPs were characterized by electron microscopy. Using the same budding assay procedure as well as additional sucrose floatation gradients, it is possible to generate VLPs containing M, N, and HN proteins. This method was attempted with M, N, and F, but again, it was difficult to detect the F protein being released into the media, although M and N were visualized easily. A budding assay with all four proteins (M, N, HN, and F) was also completed to determine if other factors besides M would induce more efficient budding of F, but I obtained similar results and difficulty detecting F protein, although the other three proteins released into the media efficiently. In this case, as above, it may be possible to better distinguish the HPIV-3 F protein by additional immunoprecipitation after pelleting by sucrose cushion and floatation on sucrose gradients.

Electron microscopy reveals that particles produced from the transfected cells were VLPs with morphology similar to that of true virus particles. HPIV-3 virus particles are roughly spherical in shape and also exhibit glycoprotein spikes protruding from the envelope of the virus. When cells were transfected with plasmid encoding for just M protein, VLPs imaged by electron microscopy revealed roughly spherical enveloped shapes which were lacking the surrounding protein spikes, which is what was anticipated. Also as expected, once cells were transfected with both M and HN or M and

F, these virus-like particles were now exiting cells complete with protein spikes protruding from the particle membrane. Similar results have been seen in several related viruses such as NDV, Nipah virus, PIV5, and mumps virus (13, 49, 65-66, 74). When HPIV-3 M and N were expressed, smooth, spherical particles were visible and it was possible to discern what appeared to be nucleocapsid protein in its characteristic herringbone shape, as seen previously in HPIV-1, SeV, and Nipah virus VLPs (15-16, 66, 78).

Interestingly, coexpression of any other HPIV-3 viral proteins does not seem to enhance the release efficiency of HPIV-3 M protein as has been seen in related viruses. When M protein was expressed alone, M protein-containing particles were released at an efficiency similar to that seen when M was expressed with multiple combinations of HPIV-3 proteins, indicating that no other protein is needed for successful particle budding. In contrast, SeV F protein contributes to improved VLP production when coexpressed with SeV M (78-79). Similarly, mumps virus and PIV-5 require additional viral protein expression before efficient VLP release occurs (49, 74). Mumps virus and PIV-5 M proteins, when expressed alone, result in poor VLP production. Coexpression of M with F protein enhances VLP production, but it is only when PIV-5 M, N, and F or HN are coexpressed that full budding efficiency is achieved (49). However, with NDV, measles virus, and Nipah virus, coexpression of other viral proteins with M do not contribute to an increased production of virus-like particles, as was observed for HPIV-3 (13, 65-67).

The development of this assembly and budding assay can be used to further investigate HPIV-3 protein-protein interactions in the future. This functional assay will

allow for the study of protein structure and its role in viral assembly and release. By mutating various regions of HPIV-3 proteins, it may be possible to determine which regions of a given protein are required for protein-protein interactions involved in assembly and release from host cells. This assay could be used to verify if these mutated proteins are still able to successfully assemble into virus-like particles. In the future, this may be useful information in the creation of antiviral drugs, which could target these specific protein regions.

Additionally, the role of the HPIV-3 C protein in budding will be investigated. The C protein is a non-structural protein expressed from an alternate reading of the P mRNA. In SeV, expression of the C protein with M, N, HN, and F proteins increased VLP release by more than two-fold (78). Additionally, a mutant form of SeV, which was unable to produce C protein, had less efficient assembly and release, resulting in titers approximately two logs less than wild-type (32). However, another group was unable to confirm a role for C protein in virus assembly. Using a different experimental system where C protein expression was depleted by siRNA, the lack of C protein expression had no measurable effect on virus particle synthesis (26). Any findings regarding C protein and HPIV-3 may help to further clarify the role of C protein in viral assembly and release.

VLPs comprised of HPIV-3 proteins could create an avenue for the development of an HPIV-3 vaccine. The ability to produce an HPIV-3 VLP vaccine should be effective and safe because the VLPs mimic the overall structure of infectious virions without being infectious themselves. These VLPs are more easily recognized by the host's immune system than traditional subunit vaccines, and thus are more immunogenic

and more effective in the long term. While VLP vaccines elicit a strong immune response, they are also a safer alternative to attenuated vaccines; which though effective, carry some risk due to low level of viral replication in the host and the potential for reversion of a vaccine strain back to its original virulent form. Because VLPs do not contain viral genetic information, viral replication would not take place in the patient (52, 62).

To synthesize a VLP vaccine for HPIV-3, a suitable expression system that is safe and is also capable of large scale production must be developed. Some examples of efficient VLP production methods include infection of insect cells with baculovirus, and transfection of genetic material into mammalian cells (52, 62). In the case of HPIV-3, a stable tissue culture cell line expressing HPIV-3 M, HN, and F genes could be used. This method would give long term expression and would be capable of large scale production. Of course, an HPIV-3 VLP vaccine must be tested in animal models successfully before progressing to clinical vaccine trials.

In conclusion, the HPIV-3 M protein is the only HPIV-3 protein tested that is able to trigger its own release from host cells. HPIV-3 proteins N and HN are able to exit as virus-like particles once coexpressed with M. Coexpression of several HPIV-3 viral proteins leads to the formation of multiple protein-containing VLPs that are morphologically similar to true virus particles. This assembly and budding assay can be used in the future to better clarify protein-protein interactions involved in the viral life cycle which could be targeted by anti-viral drugs, and be used for the development of a HPIV-3 VLP vaccine.

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