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Viral Fusion Protein TM-TM Interactions: Modulators of Protein Function and Potential Antiviral Targets

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VIRAL FUSION PROTEIN TM-TM INTERACTIONS: MODULATORS OF PROTEIN FUNCTION AND POTENTIAL ANTIVIRAL TARGETS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Stacy R. Webb

Lexington, Kentucky

Director: Dr. Rebecca Ellis Dutch, Professor of Molecular and Cellular Biochemistry

Lexington, Kentucky

2017

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VIRAL FUSION PROTEIN TM-TM INTERACTIONS: MODULATORS OF PROTEIN FUNCTION AND POTENTIAL ANTIVIRAL TARGETS

Enveloped viruses, such as HIV, influenza, and Ebola, utilize surface glycoproteins to bind and fuse with a target cell membrane. This fusion event is necessary for release of viral genomic material so the virus can ultimately reproduce and spread. The recently emerged Hendra virus (HeV) is a negative-sense, single-stranded RNA paramyxovirus that presents a considerable threat to human health as there are currently no human vaccines or antivirals available. The HeV utilizes two surface glycoproteins, the fusion protein (F) and the attachment protein (G), to drive membrane fusion. Through this process, the F protein undergoes an irreversible conformational change, transitioning from a meta-stable pre-fusion conformation to a more thermodynamically stable post-fusion structure. Understanding the elements which control stability of the pre-fusion state and triggering to the post-fusion conformation is important for understanding F protein function. Studies that replace or mutate the TM domain of the F protein of several viruses implicated the TM domain in the fusion process, but the structural and molecular details in fusion remain unclear. Previously, analytical ultracentrifugation was used to demonstrate that isolated TM domains of HeV F protein associate in a monomer-trimer equilibrium. To determine factors driving this association, we analyzed the sequence of several paramyxovirus F protein TM domains and found a heptad repeat of β-branched residues. Analysis of the HeV F TM domain specifically revealed a heptad repeat leucine-isoleucine zipper motif (LIZ). Replacement of the LIZ with alanine resulted in dramatically reduced TM-TM association. Mutation of the LIZ in the whole protein resulted in decreased protein expression and pre-fusion conformation. To further understand the role of the TM domain, the TM domain was targeted as a potential modulator of F protein stability and function. Exogenous HeV F TM constructs were co-expressed with the full length F protein in Vero cells to analyze the effects on protein expression. Co-expression of the exogenous HeV F TM constructs dramatically reduced the expression of HeV F. However, the co-expression of exogenous HeV F TM constructs with a different paramyxovirus F protein, PIV5 F, did not strongly affect PIV5 F expression levels, suggesting that the interaction of the exogenous TM constructs is specific. Fusion assays revealed that HeV F TM constructs dramatically
reduced HeV F, but not PIV5 F fusion activity. We hypothesize that the short exogenous HeV TM constructs associate with the TM domain from full-length HeV F, resulting in pre-mature triggering or protein misfolding. The work presented here demonstrates that specific elements in the TM domain contribute to TM association and pre-fusion protein stability. Furthermore, targeting these interactions may be a viable approach for antiviral development against this important pathogen.

KEYWORDS: Membrane Fusion, Viral Fusion Protein, Paramyxovirus, Transmembrane Domain

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VIRAL FUSION PROTEIN TM-TM INTERACTIONS: MODULATORS OF PROTEIN FUNCTION AND POTENTIAL ANTIVIRAL TARGETS

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“I want to stand as close to the edge as I can without going over. Out on the edge you see all kinds of things you can’t see from the center.” – Kurt Vonnegut
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Chapter 1: INTRODUCTION

MEMBRANE PROTEINS

A search of the protein data bank (PDB) for membrane proteins results in a hit number that represents less than 3% of total reported structures. This is an incredible short-coming in the field considering that estimates suggest 20-30% of all open reading frames encode for membrane proteins. More importantly, almost 50% of drugs currently target membrane proteins (1). This large gap in the field is primarily the result of the difficulty encountered when studying hydrophobic domains of membrane proteins, and impacts research areas such as cell signaling and viral infection. Enveloped viruses, including members of the paramyxovirus family, such as measles virus, mumps virus, and the zoonotic Hendra virus (HeV), utilize surface membrane proteins to promote the vital steps of attachment and membrane fusion. Membrane fusion of the viral envelope and target cell membrane is a critical early step in infection. Though there have been many advances in the field, the details of the mechanism that the viral envelope proteins utilize to drive fusion remains to be clearly understood.

PARAMYXOVIRUSES

The Paramyxoviridae family is a family of enveloped, negative-strand RNA viruses composed of several human pathogens, such as measles virus, parainfluenza virus (1, 2, 3, 4a and 4b), and mumps virus (2). Within the paramyxoviridae family, there are five genera: ferlavirus, Henipavirus, morbillivirus, respirovirus, and rubulavirus. Viruses of the paramyxoviridae family are spread through the respiratory route with many being highly contagious. Members of this family include mumps, human parainfluenza viruses, and measles, which caused over 134,000 deaths in 2015 and remains one of the leading causes of death for young children. Paramyxoviruses contain a 15 to 19 kB non-segmented genome that produces six to ten gene products, which are important for virus replication and entry. The proteins encoded include the large polymerase (L), nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F) and attachment protein (HN/H/G) (Fig 1.1). The L protein is the largest viral protein and is responsible for initiation, elongation, and termination of mRNA transcription and genome
replication. The N protein encapsulates the viral genome. The P protein is necessary for genome replication and makes up the replication complex with N and L. Virion structure is maintained by the M protein, which is found on the inner surface of the viral envelope (3). Entry of paramyxoviruses is mediated by two glycoproteins studding the surface of the virion. The attachment protein (HN/H/G) is generally important for the role of adsorption to the target cell by binding to a cellular receptor protein. The fusion protein (F) is necessary for fusion of the viral membrane with the target cell membrane (4). The fusion process requires energy to merge the two membranes, which is thought to be provided by the large conformational change that the fusion protein undergoes upon some triggering event. The fusion protein is initially found in a meta-stable conformation and, upon triggering, refolds to the lower energy post-fusion conformation, which produces energy that aids in membrane fusion (5). Triggering of the fusion protein to undergo the conformational changes needed to form the post-fusion structures is not fully understood, with some fusion proteins triggering under acidic conditions, while others are thought to trigger as a result of an interaction with the attachment protein. Ultimately, the fusion of the two membranes results in release of the viral genome within the target cell, where the genome can undergo replication and transcription for further virus amplification.

The newest human pathogens in this family include the closely related Hendra and Nipah virus, two highly pathogenic, zoonotic viruses. Because of their genetic similarity, high virulence, and broad host range, Hendra and Nipah were classified into their own genus, *Henipavirus* (6). The Hendra virus was first identified in 1994 when an outbreak in Queensland, Australia resulted in the death of several horses and one trainer. The infection presented as an acute respiratory syndrome and resulted in a second human fatality a year later resulting from viral meningoencephalitis. Transmission of Hendra virus has been reported from bat to horse, and horse to human, with a total of 55 occasions of Hendra virus detected in Eastern Australia as of 2015 (7, 8). These incidents resulted in the death or euthanasia of 97 horses, two Hendra virus antibody positive and euthanized dogs, and four fatalities out of seven human cases. The closely related Nipah virus was identified a few years after Hendra virus in 1998 when there was a large encephalitis outbreak in pig farmers in Malaysia (9). The newly discovered Nipah virus was found to cross-react with antibodies against Hendra virus. Nipah has been found to
transmit from bat to pig, pig to human, bat to human, and human to human directly. The most recent incidence of human infection occurred in 2015 resulting in nine human fatalities (7). The high mortality rates, suspected human to human transmission and lack of currently available human vaccines for Hendra or Nipah virus resulted in classification of these viruses as biosafety level 4 (BSL-4) pathogens (10).

Identification of these zoonotic viruses reinforces the premise that newly emergent viruses have the potential to dramatically affect public health. The WHO identified Nipah virus as one of the top emergent pathogens, alongside viruses such as Ebola, severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS) (11). Hendra virus infection generally presents as systemic infection, especially resulting in neurological and/or respiratory tropism with the potential for encephalitis to relapse or become chronic in nature. Henipavirus has a broad geographical distribution in humans and animals throughout Malaysia, Bangladesh, Singapore, and Australia (12). The natural host is likely the Pteropus bat, also known as the flying fox. The large habitat range and more than 60 species of Pteropus bat indicate the potential for disastrous Henipavirus outbreaks (13, 14). Together, the characteristics of the host and the Henipavirus call for the development of a human vaccine, as there is none currently available.

SURFACE GLYCOPROTEINS

Paramyxoviruses are enveloped viruses with two major glycoproteins studding the surface of the virus. These proteins are important for the initial interaction of the virus with its target cell; they mediate receptor binding and membrane fusion. There is an additional third glycoprotein, the small hydrophobic protein (SH) that is expressed by members of the Rubulavirus genus, such as PIV5. The attachment protein is designated as hemagglutinin (H), hemagglutinin neuraminidase (HN), or glycoprotein (G) depending on the virus. The fusion protein (F) drives membrane fusion via dramatic conformational changes (15-17). Together the attachment and fusion protein are ultimately responsible for viral entry into the host cell.
**Paramyxoviridae attachment protein**

Paramyxovirus attachment proteins are structurally similar and all mediate attachment to the target cell, but can vary in specific function. The attachment proteins are classified by their ability to bind sialic acid as a receptor and the ability to cleave sialic acid. The attachment protein HN (hemagglutinin-neuraminidase) is able to bind and cleave sialic acid. Viruses of the *Avulavirus, Respirovirus, and Rubulavirus* genera utilize HN proteins as their attachment protein. The attachment protein H (hemagglutinin) is only able to bind sialic acid, lacking neuraminidase activity. Canine distemper virus, a *Morbillivirus*, expresses the H attachment protein (18-23). Interestingly, measles virus also utilizes the H attachment protein, how it uses a protein receptor, CD46 (24). Lastly, the attachment protein G, utilized by the *Henipaviruses*, maintains neither sialic acid binding nor neuraminidase activity, but is able to bind protein receptors. Specifically, the Hendra virus G protein has been found to bind to the cellular proteins, ephrin B2 and B3 (25-27). Though the mechanism of attachment varies, the attachment proteins of the paramyxovirus family are structurally similar; they are type II transmembrane proteins composed of an N-terminal cytoplasmic tail, a single-pass transmembrane domain, a four helix bundle stalk, and a globular head. The attachment glycoproteins are found as a dimer of dimers, resulting in a di-sulfide linked tetramer. Additionally, it has been found that most *Paramyxoviridae* require their homotypic attachment protein for membrane fusion (2, 28-31). The closely related Hendra and Nipah viruses can utilize either *Henipavirus* attachment protein (32-34). This necessity is likely the result of the attachment protein’s proposed dual functionality. The attachment protein is required for attachment to the target cell, and it has also been proposed that it is involved in fusion protein triggering. Though the mechanistic details are not clear, it is evident that the fusion protein is unable to drive membrane fusion without expression of the corresponding attachment protein in vitro.

**Paramyxoviridae fusion protein**

Paramyxovirus F proteins are synthesized as non-fusogenic homo-trimers, referred to as F0. This type-I integral membrane protein must undergo proteolytic processing to its active form as a disulfide linked heterodimer, F1+F2. The paramyxovirus
F protein has a domain structure, consisting of a hydrophobic fusion peptide (FP), two heptad repeat regions (HRA and HRB), a single-pass transmembrane domain (TM), and a C-terminal cytoplasmic tail (CT) (Fig 1.2A) (18, 35). The F protein is inserted into the endoplasmic reticulum (ER), where it is co-translationally folded into its tertiary trimeric structure (Fig 1.2B). Proper folding and glycosylation is necessary for proper F protein function. N-linked glycosylation sites can be found within the F protein ectodomain, though the specific sites vary among viruses in the *Paramyxoviridae* family. The Hendra virus F protein contains six sites (N-X-S/T) for N-linked glycosylation, with three in the F2 subunit and the other three upstream of the TM domain. Four sites (N67, N99, N414 and N 464) were found to be important for F protein folding and fusion activity (36).

Cleavage of the F protein from its inactive state, F₀, to the active disulfide linked heterodimer is important for the pathogenicity of the virus. Upon cleavage, a new N-terminus is exposed in the F₁ subunit, the fusion peptide, which is a highly hydrophobic domain that is responsible for insertion into the target membrane (37). Though cleavage is necessary for all paramyxovirus F proteins, the protease necessary for cleavage may vary between viruses. For most F proteins, the cleavage event takes place as the F protein traffics through the *trans*-Golgi network and is mediated by furin, a ubiquitous subtilisin-like cellular protease (38, 39). Furin recognizes the sequence R-X-K/R-R and has been found to be responsible for the cleavage of several paramyxovirus F proteins, including PIV5 and mumps virus (39). Hendra and Nipah virus do not contain the R-X-K/R-R sequence for furin cleavage, but instead are cleaved by cathepsin L, an endosomal/lysosomal cysteine protease that recognizes a single basic residue in the N-terminal sequences VGDVK₁₀⁹ and VGDVR₁₀⁹, respectively (40, 41). Since cleavage occurs in the endosome, the Nipah virus contains a tyrosine-based endocytosis signal (Y₅₂⁵RSL) in the C-tail that is essential for efficient cleavage (42). Currently, the known paramyxoviruses only require a single cleavage event for activation. Some viruses, including Sendai virus and human parainfluenza virus 1 (hPIV1) utilize tissue-specific extracellular proteases (38, 43). Respiratory syncytial virus (RSV), a pneumovirus, requires two cleavage events for fusion (44). The cleavage step is essential for not only the function of the fusion protein, but also for pathogenicity and virulence.
VIRAL MEMBRANE FUSION

Enveloped viruses must overcome a challenging barrier in order to mediate entry into its target cell. The envelope of the viral particle must fuse with the cell membrane to facilitate the release of viral components and ultimately function intracellularly. Fusion of two membranes is thermodynamically favorable; thus a kinetic barrier must be overcome. As the membranes come into close contact, the repulsive hydration force of the polar head groups must be overcome (Fig 1.3A). The energy required to drive membrane fusion is thought to be provided by the conformational change of the fusion protein. The most widely accepted model of viral membrane fusion proposes that fusion occurs through a hemi-fusion state, where only the outer leaflet of the bilayers is merged. Upon completion of fusion protein refolding, the membranes fuse completely, resulting in the formation of a fusion pore (Fig 1.3B). This model is supported by studies in which hemi-fusion was observed with wild-type fusion proteins by slowing the fusion reaction with inhibitors, or decreasing fusion protein density on the surface (5, 45, 46). Although there is little sequence homology, an assorted group of viral fusion proteins including HIV env, influenza HA, and paramyxovirus F proteins drive membrane fusion through conformational changes in the fusion protein. These diverse fusion proteins can be divided into three classes to more specifically describe their mechanism of fusion, though there may be some viral fusion proteins that do not fit into any of these classes. While the fusion proteins are fairly diverse, they ultimately must drive membrane fusion for entry into the target cell.

Class I fusion proteins

Several important viral families utilize class I fusion proteins to mediate membrane fusion; these viral fusion proteins include Hendra virus F, HIV env, and influenza HA. Crystallography, NMR, and cryo-electron microscopy have provided great insights into the structure of the pre- and post-fusion conformations of several fusion proteins, including parainfluenza virus 5 (PIV5) F, Hendra virus F, and HIV env (47-50). Class I fusion proteins are present on the viral surface as trimers in a meta-stable pre-fusion conformation. Some class I fusion proteins require proteolytic cleavage to prime the fusion protein for membrane fusion. Influenza hemagglutinin (HA), one of the most
well studied class I fusion proteins, requires cleavage to expose the fusion peptide domain. Upon some triggering event, a series of dramatic conformational changes result in the formation of a more stable post fusion conformation, in a process that is essentially irreversible. The energy released during this conformational rearrangement of the protein is thought to drive the fusion process. The triggering event can vary for different viral fusion proteins. For example, influenza HA is triggered upon exposure to low pH, whereas the HIV env protein initiates its conformational change upon receptor binding (51, 52). Class I fusion proteins exhibit a characteristic post-fusion conformation in which the protein is found as a trimer of α-helical hairpins with a central coiled-coil.

Paramyxovirus fusion proteins are categorized as class I fusogens and exhibit many of the characteristic features of class I fusion. The fusion protein is initially found in a meta-stable pre-fusion conformation. This conformation has a globular head, in which the highly hydrophobic fusion peptide is buried, and a coiled-coil stalk domain comprised of the HRB domain that is anchored in the viral membrane by a hydrophobic transmembrane domain. Proteolytic cleavage is required for activation of paramyxovirus fusion proteins, though the enzymes used for cleavage vary. Hendra F protein is cleaved by cathepsin L, whereas Newcastle disease virus fusion protein is cleaved by furin (41, 53-55). Upon triggering, the fusion peptide is released for insertion into the target cell membrane, while the HRA and HRB domains fold into a stable six-helix bundle. The triggering event is thought to be initiated by receptor binding of the attachment protein, though there are several proposed models, which will be discussed in detail later. The formation of the six-helix bundle is thought to provide some of the energy needed for merging the two lipid bilayers.

Key conserved residues have been implicated in the fusion process for several paramyxovirus fusion proteins. Previously, alignment of a set of paramyxovirus F proteins identified a conserved region between the two heptad repeat regions in the F₁ segment. Residues in this conserved region were mutated in the Hendra and PIV5 F protein, resulting in proteins that were defective in proteolytic processing, trafficking, and oligomerization of the F protein (56). Fusion activation of PIV5 F was found to be enhanced when co-expressed with a headless attachment protein, implicating interaction with the attachment protein stalk domain in triggering (57). Additional studies with PIV5
F found that multiple hydrophobic residues toward the lower part of the pre-fusion F globular head were important for F-HN interaction (58). The measles virus F protein was also shown to receive a triggering signal at the base of its F protein head. Six residues were found to be important for fusion with four of the identified residues positioned near the base of the F protein head (59). NiV F protein was shown to prematurely trigger when co-expressed with a headless form of the G protein, again suggesting the importance of fusion protein-attachment protein interactions in triggering. It has been proposed that the G protein head conceals the residues in the stalk domain that are important for triggering until receptor binding has occurred (60). In addition, residues within the C-tail of the NiV F protein were shown to be important for membrane fusion. A KKR motif was found to be involved in inside-out signaling; truncation of the C-tail reduced cell-cell fusion and mutation of the KKR motif altered fusion activity (61). Despite these advancements, significant questions remain regarding intermediates and maintenance of the pre-fusion conformation.

**Class II fusion proteins**

The dimer-to-trimer transition is the hallmark characteristic of class II viral membrane fusion. Despite this difference, the overall mechanisms of membrane fusion follow similar structural themes as class I. Several important pathogens, including Dengue, Semliki Forest, and West Nile virus, express class II fusion proteins. The fusion proteins in this class are initially folded as hetero-dimers that are maintained in the pre-fusion conformation by chaperone proteins (62-67). Unlike the largely alpha-helical class I fusion proteins, class II fusion proteins are primarily composed of β-sheet. After receptor binding and triggering by low pH, the fusion protein must dissociate, allowing insertion of the fusion peptide into the target cell membrane. Upon insertion, the fusion protein oligomerizes as a homo-trimer, folds back on itself and mediates hemi-fusion, ultimately producing a viral pore via fusion. In the post-fusion conformation, the fusion protein differs from the class I fusion protein in that the final conformation is a trimer of hairpins, instead of the six helix bundle found in class I proteins (5). Regardless of this difference, it is thought that the transition to the hairpin structure is able to drive membrane fusion.
**Class III fusion proteins**

Class III fusion proteins are utilized by several viruses, including Epstein-Barr virus, herpes simplex virus, and vesicular stomatitis virus (VSV) (68, 69). The most well studied class III fusion protein is the VSV G glycoprotein. Although both class I and II fusion proteins undergo irreversible conformational changes, class III fusion proteins are unique in that some members can reverse the conformational change, such as VSV-G, depending on pH conditions. This distinctive characteristic is possible because the fusion protein does not require priming, such as proteolytic cleavage as seen with class I fusion proteins. In the pre-fusion conformation, like class I members, the fusion protein is a mix of α-helical and β-sheet secondary structure, and is co-translationally folded as a trimer. Like members of class II, the hydrophobic fusion loops are located at the tip of the ectodomain and come into close proximity with the target membrane upon conformational changes in the ectodomain upon receptor binding and exposure to a low pH environment. The post-fusion conformation of VSV G is trimeric with the TM domain and FP on the same side of the new structure of the protein (70, 71). Ultimately, the post-fusion conformation can be described as a trimer of hairpins, similar to class II fusion proteins. The theme that can be applied to all three classes seems to be a requirement for protein oligomerization and conformational change upon specific triggering conditions.

**Triggering and Stability of the Pre-fusion F Conformation**

As described for class I fusion proteins, paramyxovirus F proteins require a triggering event to undergo conformational changes and drive membrane fusion. The F protein is initially found in a meta-stable conformation, with an energy barrier between the pre-fusion conformation and the post-fusion. The triggering event destabilizes the pre-fusion conformation so the F protein can refold into the low energy post-fusion structure. Since the post-fusion conformation is no longer able to drive membrane fusion and the conformational change is considered essentially irreversible, it is important for the triggering process to be carefully regulated, spatially and temporally. Three types of fusion triggers have been described for viral fusion proteins: exposure to low pH,
receptor binding, and a combination of receptor binding followed by exposure to a low pH environment. Viruses that enter the cell via endocytosis, such as orthomyxoviruses, utilize low pH to drive the F protein triggering event. Receptor binding as a triggering event can occur via different mechanisms. The viral fusion protein may directly bind a receptor, an additional viral glycoprotein may be responsible for interacting with a receptor, and, in the case of HIV, the fusion protein can require multiple receptors. Members of the paramyxo-, retro-, and herpesvirus families employ receptor binding for triggering. Avian retroviruses are unique in that they utilize a two-step fusion activation mechanism that requires receptor binding followed by exposure to a low pH environment (72-77).

Paramyxoviruses differ from other class I fusion proteins, which have F proteins that are capable of binding the target cell receptor and driving membrane fusion. Members of the paramyxovirus family require two surface glycoproteins to perform these functions: the attachment and fusion protein. Generally, it is thought that the two glycoproteins interact and that the F protein is triggered as a result of some conformational change that occurs when the attachment protein binds its receptor. The details of this mechanism of activation are still unclear, though there are several models proposed. The first model, the sliding model, hypothesizes that the attachment protein head slides from a planar configuration to a staggered structure (Fig 1.4A). The change in conformation then results in dissociation from the F protein, allowing triggering to occur. The stalk-exposure model suggests that the attachment protein undergoes a conformational change upon receptor binding that allows the F protein to interact with the newly exposed attachment protein stalk (Fig 1.4B). Experiments utilizing headless attachment proteins of PIV5 or Measles virus to trigger the F protein support this model (57, 59, 78). However, there are several viruses that exhibit F-attachment protein interactions before the receptor binding event. A third model, the safety-catch model, suggests that the attachment protein is found in a heads down conformation to prevent F protein triggering, essentially locking the F protein (Fig 1.4C) (75). Upon receptor binding, the head domain of the attachment protein changes conformation which frees the F protein for triggering. The Nipah virus has been proposed to use yet another model, the bi-dentate model of triggering (79). This model suggests that the F protein interacts with
the attachment protein head until receptor binding, at which point the F protein associates with the stalk of the attachment protein. Nipah virus F and its attachment protein, G, have been shown to interact before receptor binding and the attachment protein has been shown to undergo conformational changes in the head and stalk domain after receptor binding, supporting the bi-dentate model (Fig 1.4D). There are some redundancies in these models of triggering, and more than one may appropriately describe the triggering mechanism for one virus, but not another. From these proposed models, it becomes clear that the interface at which the F and attachment protein interact is important for the F protein triggering process (80).

In addition to these protein-protein interactions, there are several regions of paramyxovirus F proteins that have been implicated in triggering. Most integral membrane proteins in the exocytic pathway are modified by oligosaccharides, such as N-linked glycans. Previous studies have shown that N-linked glycans are important for proper protein folding, protein stability, and maintenance of specific conformations. Not surprisingly, many viral F proteins are modified by oligosaccharides, especially N-linked glycans. The Influenza virus HA protein contains two conserved glycosylation sites that are necessary for stabilizing the HA trimer (81, 82). Mutation of these glycosylation sites resulted in reduced cleavage, and therefore fusion activity, of the HA protein (82). The fusion peptide domain is highly conserved across class I viral fusion proteins and, as expected, is important for F protein function. There are often conserved glycine residues within the FP domain that are thought to be important for membrane perturbation. Interestingly, mutation of conserved glycine residues in the PIV5 F protein resulted in a hyper-fusogenic protein that could trigger even in the absence of its homotypic attachment protein (83). These results implicate the FP and these specific residues in the triggering process of the F protein. Additional regions of conservation, the heptad repeat domains A and B, have also been implicated in the triggering process. The Sendai virus has previously been targeted by peptides designed based on the HRB domain to inhibit membrane fusion by binding to the HRA domain in a pre-hairpin intermediate, ultimately preventing formation of the coiled-coil structure in the post-fusion conformation. Mutation of residues in the heptad repeat of the HRA domain of Sendai virus modulated the activity of the F protein, with some mutations resulting in hyper-fusogenic proteins.
The mutations that resulted in a hyper-fusogenic protein were hypothesized to enable the F protein to trigger more readily than the wild-type protein. On the other hand, stabilizing the pre-fusion conformation can also prevent fusion activity.

**Modulating the F Protein via the Transmembrane Domain**

Though regions within the soluble ectodomain have been shown to be important in stability and function of the F protein, the hydrophobic anchor of class I viral F proteins, the transmembrane domain, has also been implicated in the fusion process. The transmembrane domain is essential for anchoring the protein to the viral membrane, but studies regarding additional roles of this domain have been limited due to the difficulty in working with such hydrophobic domains. Cellular proteins, such as receptor tyrosine kinases, have been shown to utilize their transmembrane domains for signaling. Neuropilin-1, a receptor tyrosine kinase that is involved in vascularization, requires a GxxxG motif within its transmembrane domain to promote dimerization for downstream signaling (85). To determine its role in the fusion protein function, the TM domain of influenza virus HA was replaced with the lipid anchor, glycosylphosphatidylinositol (GPI), and analyzed for protein folding and function. The GPI-anchored HA protein was able to form stable trimers, but exhibited additional oligosaccharide modifications that altered the ability of GPI-HA to bind to a target cell (86). An additional study with GPI-HA that was synthesized under conditions that resulted in glycosylation similar to that of the wild-type protein demonstrated that the lipid-anchored F protein was only able to promote hemi-fusion, not full fusion, suggesting that the TM domain is more than an anchor (87). A similar study was performed with the fusion protein (G) of vesicular stomatitis virus (VSV), in which replacement of the TM domain with GPI resulted in a non-fusogenic fusion protein (88). The TM domain of VSV G was suggested to be important for initial pore formation and content mixing, as TM peptide of the G protein was able to drive vesicle fusion (89). The TM domain was thought to disrupt the membrane by inducing positive lipid curvature, aiding in membrane fusion.

Class I fusion proteins are ultimately folded as a trimer in a meta-stable conformation. The spring loaded conformational change must be spatially and temporally regulated. The cleavage activation step is one means of regulation. The TM domain has
been proposed as an additional region of pre-fusion maintenance. The F protein of Newcastle disease virus (NDV) was mutated by replacing with the TM domain of Sendai virus (SeV), measles virus (MV), or VSV to test for sequence specificity of the TM domain. The TM domain of SeV and MV resulted in a protein that was expressed and trafficked appropriately, however membrane fusion was defective (90). Conformation sensitive antibodies were used to determine that the TM domain replacement resulted in conformational changes in the ectodomain, implicating the TM domain in maintenance of the pre-fusion conformation. Since the first pre-fusion crystal structure of influenza HA was determined in 1981, there have only been eight unique pre-fusion structures determined. These include fusion proteins from different viral families, such as VSV G (rhabdovirus), HIV env (retrovirus), and HeV F (paramyxovirus). Structural data has been difficult to obtain for the pre-fusion conformation, as most often the protein triggers during sample preparation. Most of the structures reported to date have been modified to remove the transmembrane domain and C-tail, and replace them with a trimeric coiled coil segment, such as GCNt or foldon (the natural trimerization domain of T4 fibritin). Though it is evident that the TM domain of the fusion protein is important for the overall function of the protein, the mechanism by which the TM functions in fusion remains unclear.

Previous work from our research group by Clint Smith and Stacy Smith have demonstrated that the TM domain of HeV associates in a monomer-trimer equilibrium in isolation as determined by analytical ultracentrifugation, and that alteration of the length or sequence of the TM domain results in changes in protein expression and activity. Given these data in combination with requirement of a trimeric tag for pre-fusion protein crystallization, I hypothesized that the TM domain of the Hendra F protein was important for pre-fusion protein stability. Andreea Popa previously identified a leucine/isoleucine zipper (LIZ) that continued in frame from the HRB domain into the TM domain of Hendra F. I utilized sedimentation equilibrium AUC to determine that the LIZ was important for TM-TM association, as mutation of this motif resulted in a shift in equilibrium toward a larger monomeric population. Though mutation of the LIZ resulted in reduced expression in the full length F protein, I utilized a thermal triggering assay to demonstrate that reduced TM-TM association resulted in a protein that triggers more
readily than the wild type protein. Additionally, sedimentation equilibrium AUC was used to analyze the oligomeric state of four additional fusion protein TM domains, including Ebola GP, SARS CoV S, Rabies GP, and Influenza HA, representing four additional viral families and class I and III viral fusion proteins. The TM domains exhibited monomer-trimer or monomer-trimer-hexamer equilibrium. The ability of TM domains of these fusion proteins to oligomerize may suggest that TM-TM association is important for pre-fusion stability of the glycoprotein, as well. Further studies would be necessary to determine whether the TM domain of these proteins does contribute to pre-fusion protein stability. Ultimately, these data provide a strong argument for the importance of TM-TM association in fusion protein function, specifically maintenance of the pre-fusion conformation.

**TARGETING THE F PROTEIN TM DOMAIN TO INHIBIT MEMBRANE FUSION**

Functional data described here implicate the TM domain in membrane fusion, as alteration in various systems results in changes in fusion activity. The role the TM domain plays in the fusion protein function implicates the domain as a potential druggable target. With our data that demonstrate TM-TM association, I hypothesized that disruption of TM-TM interactions could prevent fusion by either prematurely triggering the fusion protein, or causing the protein to misfold. Previously, a study with Epstein-Barr virus latent membrane protein 1 (LMP1) demonstrated that small molecule inhibitors could be used to target TM-TM interactions and prevent signaling (91). This concept can be applied beyond targeting viruses, as well. A study with the receptor tyrosine kinase, ErbB2/Neu, found that TM peptide mimics could prevent downstream signaling. The authors hypothesized that the TM peptide prevented ErbB2/Neu TM oligomerization, which is necessary for signaling (92). To determine whether the TM domain of the Hendra F protein was targetable, I designed homologous TM constructs for co-expression with the F protein in cell culture. I found that the presence of the exogenous TM protein resulted in a dramatic reduction in expression and fusion activity. Additionally, TM peptides were utilized to determine whether viral infection could be inhibited. PIV5-GFP virus treated with PIV5 F TM peptide exhibited a reduction in infected cells, while HMPV treated with the same peptide maintained infection. Thus,
this proof of concept experiment suggests that the TM domain is a potential target, and the interaction of exogenous TM is protein specific.
Figure 1.1. Schematic of paramyxovirus virion. The negative sense single stranded RNA genome (black line) is encapsulated by the nucleocapsid protein (purple). The phosphoprotein (light blue) and large polymerase (navy blue) are also found associated with the coated RNA genome. The matrix protein is found under the lipid bilayer and helps to provide structure to the virion. Two surface glycoproteins stud the lipid envelope, the attachment protein (peach) and the fusion protein (green).
Figure 1.2. Hendra F protein processing. A. The Hendra virus fusion protein (F) has a domain structure consisting of the highly hydrophobic fusion peptide (FP), two heptad repeat domains (HRA and HRB), a large ectodomain, a transmembrane domain (TM) and a C-tail domain (CT). B. F is synthesized as an inactive protein (F₀) that requires cleavage by cathepsin L to a disulfide linked heterodimer (F₁+F₂). Cleavage occurs after the protein is trafficked to the plasma membrane and endocytosed. Once cleaved, the F is trafficked back to the plasma membrane.
Figure 1.3. Membrane fusion. A. Membrane fusion of two lipid bilayers with the intermediates depicted. B. A proposed model for fusion protein mediated membrane fusion that results in the fusion protein in a six helix bundle conformation.
Figure 1.4. Proposed mechanisms of paramyxovirus fusion protein triggering. Attachment protein (left, navy blue stalk and grey head) and fusion protein (right). Different head group conformational changes and interactions between the attachment protein are shown as possible mechanisms for fusion protein triggering. The light blue circles on the attachment protein stalk indicate a conformational change. The yellow star indicates the fusion protein is active.
Chapter 2: MATERIALS AND METHODS

CELL LINES AND CULTURE
Vero cells, kindly provided by Robert Lamb, HHMI/Northwestern University and 293T cells, generously provided by Judith White, University of Virginia, were maintained in Dulbecco’s modified Eagle’s media (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS).

PLASMIDS AND ANTIBODIES
Plasmids containing Hendra F or G in the pGEM vector were generously provided by Dr. Lin-Fa Wang (Australian Animal Health Laboratory). Plasmids containing PIV5 F or HN were kindly provided by Robert Lamb, HHMI/Northwestern University. All Hendra F mutants were made in pGEM using the QuikChange site-directed mutagenesis kit (Stratagene) and subcloned into the eukaryotic expression vector pCAGGS (93). The short (D482-T546), long (Q478-T546) and HRB (I460-T546) linker TM genes were ordered from GenScript. The cut sites ClaI and XhoI were added to the N- and C-terminus, respectively, for sub-cloning into the pCAGGS expression vector. Staphylococcal nuclease fused to the TM domain of glycophorin A (GpA) in the pET-11a expression vector was generously provided by Dr. Karen Fleming (The Johns Hopkins University) (94). Analytical ultracentrifugation constructs containing either the wild-type or mutant Hendra F TM domain were cloned into pET11a using Xmal and XhoI sites at the 5’ and 3’ ends, respectively. Genes for the Ebola GP TM (residues 651-672), SARS CoV S TM (residues 1193-1227), Rabies GP TM (residues 429-461), and Influenza HA TM (residues 511-536) were ordered from GenScript and cloned into pET11a, as described for Hendra F TM. Constructs were sequenced to confirm sequence integrity. Anti-peptide antibodies to residues 527-539 of the Hendra F cytoplasmic tail were used to pull down F. PIV5 F immunoprecipitation was performed with a rabbit anti-peptide antibody to the PIV5 F C-tail residues 516-529. For immunoprecipitation, mAb 5G7 was used to detect the HeV F protein and the pre-fusion conformation of HeV F was detected using mAb 5B3, both antibodies were generously provided by Dr. Christopher Broder (Uniformed Services University of the Health Sciences).
TRANSIENT TRANSFECTION
All mutant or wild-type Hendra F, Hendra G, PIV5 F, or PIV5 HN constructs were transiently expressed using the mammalian expression vector pCAGGS allowing for high levels of protein expression from a chicken β-actin promoter. Cell lines were transiently transfected with plasmid DNA using Lipofectamine PLUS (Invitrogen, Carlsbad, CA), according to manufacturer’s protocol, unless noted otherwise.

GEL ELECTROPHORESIS, COOMASSIE STAINING, AND WESTERN BLOTTING
Protein samples were analyzed via 15% SDS-PAGE, unless otherwise noted. For recombinant protein expression, protein samples were taken pre- and post-induction to visualize protein expression via Coomassie staining. For Western blot analysis, immunoprecipitated protein was transferred to polyvinylidene difluoride (PVDF) membrane (Fisher) at 50V for 80min. After blocking with Odyssey block buffer (LI-Cor), membranes were incubated with anti-Hendra F mouse monoclonal antibody 5G7 at a 1:3000 dilution in Tris-buffered saline with 0.05% Tween 20 (TBS-T). Membranes were washed with TBS-T and incubated with horseradish peroxidase-conjugated goat anti-mouse (light chain specific) secondary antibody (Jackson Immuno Research) diluted 1:10,000. Membranes were washed again with TBS-T, developed with SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher) and visualized with the Bio-Rad ChemiDoc system.

SYNCYTIA ASSAY
Subconfluent Vero cells in 6-well plates were transiently transfected with pCAGGS-Hendra F and pCAGGS-Hendra G at a ratio of 1:3 using Lipofectamine and Plus Reagent (Invitrogen) per manufacturer’s protocol. Syncytia formation was observed 24 to 48h post transfection. Images were taken using a Nikon digital camera mounted atop a Nikon TS100 microscope with 10X objective. The fusion index ($f$) was calculated as $f=1-(C/N)$, where $C$ is the number of cells in a field after fusion and $N$ is the number of nuclei.
SURFACE BIOTINYULATION

Vero cells (confluency 80-90%) in 60-mm dishes were transiently transfected using Lipofectamine and Plus reagent (4 µg of DNA wild-type or mutant pCAGGS-Hendra F) according to manufacturer’s protocol. Eighteen to twenty-four hours post-transfection, cells were washed with PBS and starved for 45 min in DMEM deficient in cysteine and methionine. Cells were then labeled for 3 h with DMEM deficient in cysteine and methionine, containing Tran\(^{35}\)S-label (100 µCi/mL; MP Biomedicals). Cells were then washed 3 times with 3 mL of ice-cold PBS, and surface proteins were biotinylated using 1mg/mL EZ-Link Sulfo-NSH-Biotin (Pierce) in PBS with rocking for 35min at 4°C followed by incubation at room temperature for 15 min. Cells were then washed 2 times with ice-cold PBS and lysed with 500 µL of RIPA lysis buffer (100mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholic acid, 1mM phenylmethylsulfonyl fluoride (Sigma), and 25mM iodoacetamide (Sigma)). Cellular lysates were centrifuged at 136,500x\(g\) for 15 min at 4°C. The supernatant was removed to a 1.5mL microcentrifuge tube, and 4µL of Hendra F C-tail peptide antibody was added and incubated for 3h at 4°C with rocking. Proteins were then immunoprecipitated by incubating with 30µL of Protein A-Sepharose beads (GE Healthcare) for 30 min. The beads were washed with 2X RIPA + 0.30 M NaCl, 2X with RIPA + 0.15 M NaCl, and 1X with SDS Wash II (150 mM NaCl, 50 mM Tris -HCl, pH 7.4, 2.5 mM EDTA). After the beads were washed, 60 µL of 10% SDS was added, and the samples were boiled for 10min, removed to a new tube, and repeated with 40 µL of 10% SDS for a total of 100µL. Ten microliters of the supernatant was removed to analyze the total protein population. To the remaining supernatant, 30µL of Streptavidin beads (Pierce) and 400µL of biotinylation dilution buffer (20 mM Tris (pH 8), 150mM NaCl, 5mM EDTA, 1% Triton X—100, 0.2% bovine serum albumin) were then added for 1 h at 4°C with rocking. Hendra F was analyzed by 15% SDS-PAGE and visualized using the Typhoon imaging system (GE Healthcare). Band densitometry using ImageQuant 5.2 was performed for each experiment to quantitate the amount of F expressed, which was reported as % expression, the sum of F\(_0\) and F\(_1\), normalized to WT (Fig 2.1).
TIME COURSE IMMUNOPRECIPITATION

Wild-type Hendra virus F or TM mutants were transiently expressed in subconfluent Vero cells using Lipofectamine Plus (Invitrogen) as previously described. The next day cells were washed with PBS and starved for 45min at 37°C in cysteine-methionine-deficient DMEM. Cells were then labeled for 30min with Trans[^35S] metabolic label (100µCi/mL; MP Biomedicals). At appropriate time points, cells were washed three times with PBS and lysed with RIPALysis buffer, as described. Immunoprecipitation and imaging were performed as described for surface biotinylation.

RECOMBINANT PROTEIN EXPRESSION AND PURIFICATION

SN-TM domain centrifugation constructs were expressed as C-terminal fusions with staphylococcal nuclease (Fig 2.2A). All constructs were transformed into Rosetta-Gami cells (EMD Chemicals, Gibbstown, NJ) and grown at 37°C in 2X yeast extract-tryptone (YT) medium under the selection of 0.015 mg/mL kanamycin, 0.0125 mg/mL tetracycline, 0.05 mg/mL streptomycin, 0.034 mg/mL chloramphenicol, and 0.1 mg/mL ampicillin. The cultures were grown to an optical density at 600nm (OD 600) of 0.6 to 0.8, induced for protein expression by addition of 1 mm isopropyl β-d-1-thiogalactopyranoside (Sigma), grown for 4 h, and harvested by centrifugation. Cells were resuspended in a 1:20 culture volume of lysis buffer (50 mm HEPES, 2 mm EDTA, 1 mm phenylmethylsulfonyl fluoride, pH 8.0), subjected to three freeze-thaw cycles, and incubated with 0.1 mg/mL hen egg white lysozyme for 30 min on ice. The cell lysate was then sonicated for three-20 s pulses, 5mM CaCl₂ was added, and the solution was incubated for 30 min on ice. Insoluble protein was pelleted at 12,000 xg for 10 min. The supernatant was then purified using the detergent Thesit-290 (Sigma) and a single salt (1M NH₄OAc) extraction as described previously, except using 50mM HEPES instead of 20mM Tris-HCl (95). The supernatant containing the recombinant protein was then dialyzed overnight at 4°C against lysis buffer containing 0.1M NH₄OAc and 0.2% v/v Thesit and clarified by centrifugation at 15,000 xg for 15 min. The recombinant protein was purified by FPLC cation-exchange chromatography using a 1mL HiTrap SP FF column (GE Healthcare) and then exchanged into a solution containing 200mM NaCl, 20 mM Na₂HPO₄/NaH₂PO₄ (pH 7), 29% D₂O, and Zwittergent detergent 3-(N,N-
dimethylmyristyl-ammonio)propanesulfonate (C14SB) (Sigma), as previously described. Protein was dialyzed using Slide-A-Lyzer MINI dialysis units (10,000 MWCO; Pierce) and concentrations were determined by spectrophotometry, using $\varepsilon_{280}=17,420 \text{ M}^{-1}\text{cm}^{-1}$.

**Analytical Ultracentrifugation**
Sedimentation equilibrium (Fig 2.2) measurements were obtained at three different rotor speeds using a Beckman XL-A analytical ultracentrifuge equipped with an An-60 Ti rotor operated at 25°C. Protein concentrations corresponding to 280nm absorbance between 0.4 and 0.8 were utilized for determination of the best fit models. Attainment of sedimentation equilibrium was established as previously described (95). The buffer density was matched to that of C14SB detergent ($\rho = 1.04 \text{ g/mL}$) using D$_2$O, as described previously (95). The partial specific volume of each protein was estimated using SEDNTERP, and data analysis was performed using KaleidaGraph (Synergy Software) and HeteroAnalysis. Molecular weight values were fixed for analysis.

**Thermal Triggering Assay**
Subconfluent Vero cells in 60-mm dishes were transiently transfected with 4 µg of either wild-type or mutant Hendra F in pCAGGS using Lipofectamine and Plus (Invitrogen) according to the manufacturer’s instructions. Eighteen to twenty-four hours after transfection, cells were washed and replenished with DMEM + FBS at 37°C, or at 50°C. Samples treated with warmed media were then incubated in a 50°C water bath for 20 min. After heat treatment, the samples were immediately placed on ice and the media was replaced with ice-cold DMEM + FBS. Following a 30 min incubation on ice, cells were washed 2X with ice cold PBS, and incubated with 5 µg/mL mAb 5B3 in PBS + 1% BSA for 3 hr at 4°C to detect pre-fusion Hendra F. Cells were then washed 2X with ice cold PBS, lysed with RIPA + 0.15M NaCl, and centrifuged for 15 min at 136,500 x g. The resultant supernatant was immunoprecipitated using protein G Sepharose beads, and protein was analyzed via 15% SDS-PAGE. Hendra F was detected via Western blot analysis using mAb 5G7. Results were reported as % triggered $=100 \left[ 1 - \frac{F \text{ expression at } 55^\circ \text{C}}{F \text{ expression at } 37^\circ \text{C}} \right]$. 

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CO-EXPRESSION OF F PROTEIN WITH TM CONSTRUCTS

Subconfluent Vero cells in 35-mm dishes were transiently transfected with a total of 2µg of DNA. The DNA was transfected at a ratio of 2:1 (F:TM) for HeV F or PIV5 F with the TM constructs. For the control without the TM construct, HeV F or PIV5 F were co-transfected with empty pCAGGS at a ratio of 2:1. Eighteen to twenty-four hours post-transfection, cells were washed with PBS and starved for 45 min in DMEM deficient in cysteine and methionine. Cells were then labeled for 3 h with DMEM deficient in cysteine and methionine, containing Tran\textsuperscript{35}S-label (100 µCi/mL; MP Biomedicals). Cells were washed 2x with PBS and lysed with RIPA + 0.15M NaCl, as described for the surface biotinylation. The HeV F co-expression samples were immunoprecipitated with an antibody to the C-tail of HeV F, and the PIV5 F samples were immunoprecipitated with an antibody to the C-tail of PIV5 F. Samples were treated as described for surface biotinylation, run on a 15% gel, exposed to a phosphor-screen and imaged on the Typhoon imaging system (GE Healthcare). Band densitometry was performed with the ImageQuant software.

PEPTIDE INHIBITION ASSAY

Virus, either rgHMPV or PIV5-GFP, was pretreated for 30 min at room temperature with TM peptide corresponding to the TM domain sequence of the PIV5 F protein. The peptide was synthesized by LifeTein with the following sequence: VLSIIAICLGCLGLILILLSVVVWKLL. The peptide was solubilized in sterile DMSO. Virus was diluted in OPTI-MEM for an infection with an MOI=1. Vero cells (70-90% confluency) were washed twice with PBS and incubated with 500µL of peptide treated virus for 4h at 37°C. After the incubation, the infection media was removed, cells were washed 2X with PBS, and cells were left overnight with DMEM+FBS. The following day, cells were imaged for GFP-positive cells with a Nikon Axiocvert-100 microscope. GFP-positive cells were counted and normalized to determine peptide inhibition.

IMMUNOFLUORESCENCE

Cells grown in 6-well plates containing coverslips were transfected with HeV F. After 24hr, cells were washed in phosphate buffer saline (PBS) and fixed in 4%
paraformaldehyde for 15 min at room temperature. Cells were then permeabilized in 1% Triton X-100 for 15 min at 4°C followed by blocking in 1% normal goat serum and incubation with the corresponding primary antibody overnight at 4°C. The following day, cells were washed with 0.05% tween-PBS, secondary antibodies were added, and cells were incubated at 4°C for one hour. Coverslips were then mounted on glass slides using Vectashield mounting media (Vectorlabs, Burlingame, CA). Pictures were taken using a Nikon 1A confocal microscope and analyzed with the NIS-Elements software. All images were processed in Adobe Photoshop, with equivalent adjustments made to all panels.

**SEQUENCE ANALYSIS**

The sequences of 140 paramyxovirus F proteins were aligned and analyzed. First, the TM domains were predicted with the TMHMM Server v 2.0. Next, the sequences were aligned using the MUSCLE aligner based on similarity that is built into the Virus Pathogen Database and Analysis Resource (VIPRBRC) and cross-validated by the predicted TM domain regions. Finally, the frequency of β-branched amino acids was calculated at each position in the aligned TM domain region and graphed.

**STATISTICAL ANALYSIS**

Statistical significance for the triggering analysis quantitative data obtained was analyzed in GraphPad using Student's t test (**, p < 0.05).
Figure 2.1. Surface biotinylation. Vero cells were transfected with the construct of interest. 24h later cells were starved and radiolabeled with $^{35}$S. After the label, cell surface proteins were biotinylated. Lysed cells were first immunoprecipitated with the HeV F C-tail antibody to pull down the total fusion protein population. A sample of the total population was aliquotted, and the remaining sample was immunoprecipitated with streptavidin to pull down the surface population.
Figure 2.2. Sedimentation equilibrium analytical ultracentrifugation. A. Chimeric proteins were designed with the protein staphylococcal nuclease (SN) and the TM domain of interest. B. Upon centrifugation, the samples were brought to equilibrium and absorbance values were recorded. C. The data obtained was then fit to the equation shown to determine best fit, where $\alpha$ is the absorbance, $r$ is the radial position, $r_o$ is the reference position, $M$ is the molecular weight, $\bar{\nu}$ is the partial specific volume, $\rho$ is the solution density, $\omega$ is the rotor speed, and $\zeta$ is the offset coefficient. Figure modified from E.C. Smith.
CHAPTER 3: HENDRA VIRUS FUSION PROTEIN TRANSMEMBRANE DOMAIN CONTRIBUTES TO PRE-FUSION PROTEIN STABILITY

INTRODUCTION

Enveloped viruses, including members of the paramyxovirus family, such as measles virus, mumps virus, Sendai virus, and the zoonotic Hendra virus (HeV), utilize surface membrane proteins to promote the vital steps of attachment and membrane fusion. Membrane fusion of the viral envelope and target cell membrane, a critical early step in infection, is driven by large conformational changes in surface viral fusion (F) proteins. The paramyxovirus F protein is a prototypic class I fusion protein that is initially synthesized as a homo-trimer in a metastable pre-fusion conformation (96). The Hendra and Nipah virus F protein is synthesized in the secretory pathway, trafficked to the plasma membrane, endocytosed for cleavage and trafficked back to the cell surface in the fusogenically active conformation, $F_1+F_2$ (53). Through this entire process, the F protein must maintain the pre-fusion conformation. Upon triggering, the F protein irreversibly folds into a post-fusion conformation, a change that includes dramatic rearrangement of the ectodomain. Stabilization of the pre-fusion conformation is critical for viral stability and function, as premature triggering would result in a fusion dead viral particle. Understanding factors that control the stability of the pre-fusion conformation therefore would provide an avenue for disrupting viral membrane fusion.

To obtain the pre-fusion structure of the paramyxovirus parainfluenza virus 5 (PIV5) F protein, a trimeric coiled coil was engineered and added to the soluble portion of the fusion protein. Without the coiled coil, the PIV5 F protein could only be crystallized in the post-fusion conformation. This suggests that the F protein requires a domain to pin the protein in its pre-fusion conformation, implicating the TM domain in

pre-fusion protein stability (50). A recent study found that soluble HIV gp41 trimers, another class I fusion protein, could only be produced when the trimerization tag, foldon, was added to the protein (97). TM domains of viral F proteins have historically been characterized solely as membrane anchors. However, recent studies have shown that changes in length or single point mutations in the TM domain can result in modulation of class I, II, and III viral fusion protein expression and activity (86, 98-102). Despite these studies, the mechanism by which these mutations alter F protein activity and function remains unclear. Although the TM domain of these proteins appears to be important, few studies have directly analyzed TM-TM interactions, which is likely a result of the difficulty of working with such hydrophobic domains. One study utilized NMR to demonstrate that the HIV gp41 TM domain forms a trimer in bicelles, further implicating the importance of TM-TM interactions (103). Additionally, TM-TM interactions have been shown to be important in several biological systems, including signaling processes mediated by receptor tyrosine kinases, so understanding factors that affect TM-TM interactions could be implicated beyond viral membrane proteins (104-106).

A critical role for the TM domain in stability of the pre-fusion form was recently proposed for the herpes simplex virus gB (107), and several studies support the concept that viral fusion protein TM domains can self-associate, though the TM domain oligomeric form could not be determined with the assays utilized (108-110). Previously, our group utilized sedimentation equilibrium analytical ultracentrifugation (SE-AUC) to directly assess isolated TM-TM interactions. We were the first to demonstrate that paramyxovirus F protein TM domains self-associate in monomer-trimer equilibrium in the absence of the rest of the protein (95). Molecular dynamic simulations of influenza virus HA and HIV envelope glycoprotein TM domains support trimeric interactions, suggesting that stabilizing trimeric TM domain interactions may be characteristic of many viral fusion proteins (108, 111). Here, we analyzed the sequences of 140 TM domains from 19 paramyxovirus species and identified the presence of a β-branched residue heptad repeat. To understand what drives TM interactions in the Hendra virus F protein, the TM domain sequence was analyzed for association motifs; a heptad repeat leucine-isoleucine zipper (LIZ) was found in frame with the upstream leucine zipper in the heptad repeat B (HRB) domain. Studies have previously shown that a LIZ motif can
promote protein oligomerization in soluble proteins, and more recently in hydrophobic environments (112, 113). Mutagenesis indicated that the HeV F LIZ is important for TM-TM interactions, as well as overall protein expression and stability. More specifically, mutation of the F protein LIZ motif resulted in reduced stability of the pre-fusion conformation of HeV F, suggesting TM-TM interactions are important contributors to pre-fusion F protein stability. Together, our results suggest that disruption of HeV F TM-TM interactions affects the pre-fusion conformation of F, and contributes to the F protein triggering process that is required to drive membrane fusion.

The results presented here represent an effort on the part of several people. Andreea Popa identified the leucine/isoleucine zipper in the HeV F TM domain and performed initial transient transfection experiments of the mutants. She produced the HeV F TM mutations in the pCAGGS vector, which I subcloned into the pET11a vector for SE-AUC analysis. The sequence alignment was performed by Tamas Nagy, an undergraduate that worked in collaboration with our group and Dr. Hunter Moseley’s research group.

**RESULTS**

*Identification and oligomeric analysis of a L/I zipper in the HeV F TM domain*

In order to further define elements which drive TM-TM interactions in Hendra F, we analyzed the TM domain sequence for motifs involved in protein-protein association and identified a heptad repeat leucine-isoleucine zipper that continued through the TM domain in frame with the leucine zipper of the upstream HRB domain (Fig 3.1A). Previous studies have demonstrated that L/I zippers can mediate protein-protein interactions in soluble proteins via hydrophobic collapse (112, 114-116). This motif was also found to contribute to protein interactions in a membrane environment via a knob-in-hole packing mechanism (113, 117). To determine whether this motif could be involved in TM-TM association in other viruses, the sequences of 140 paramyxovirus F proteins were analyzed for amino acid frequency in the predicted TM domain (Fig 3.2). This analysis was composed of 19 unique viruses with multiple strains of each, representing each paramyxovirus genus except respirovirus (Table 3.2). The predicted TM domains
were aligned to look for a specific pattern related to a L/I zipper. Upon examination, a heptad repeat of β-branched residues (isoleucine, valine, threonine), which also included leucine, was identified (Fig 3.1B and table 3.2). This suggests that a heptad repeat, such as a L/I zipper, may be important for the TM domain across the viral family. To determine whether the predicted L/I zipper in the Hendra F TM domain contributed to TM-TM association, site-directed mutagenesis was used to replace the four L/I residues (L488+I495+I502+L509) with alanine resulting in a four point mutant, LIZ 4A. To directly analyze TM-TM interactions, we utilized chimeric proteins containing staphylococcal nuclease (SN) protein linked to the TM domain of interest and analytical ultracentrifugation, as previously described (95). The addition of SN aids in solubilization of the highly hydrophobic TM protein and also increases the extinction coefficient of the protein, which is important for absorbance based assays. Samples of the wild-type SN-TM and LIZ 4A SN-TM were brought to sedimentation equilibrium in a Beckman XL-A analytical ultracentrifuge, and radial absorbance data were obtained at 20,000, 25,000, and 30,000 rpm. The data were subjected to non-linear least squares fitting with equations modeling monomer and monomer-trimer sedimentation equilibria, as well as residual plotting with KaleidaGraph. Consistent with previous results, the data for the chimeric WT protein fit with a monomer-trimer equilibrium (blue, Fig 3.3A), as determined by residual plotting (95, 118). Additional curve fits were analyzed, such as a single species monomer (red line Fig 3.3A), but were a poor fit to the absorbance points. The chimeric LIZ 4A protein also demonstrated a best fit to a shallow monomer-trimer equilibrium curve (Fig 3.3B). When fit to a multi-species system (monomer-Nmer), the oligomeric state of the second species for both WT and LIZ 4A was found to be trimeric. The data points for LIZ 4A exhibited a shift in absorbance toward a smaller radial position suggesting a shift in equilibrium toward a much larger population of monomeric protein when compared to WT. To further confirm this shift in equilibrium for the LIZ 4A chimeric protein, an absorbance based apparent dissociation constant was calculated for WT and LIZ 4A. When normalized to WT at each spin speed, the LIZ 4A mutant displayed an approximate thousand fold decrease in association constant, suggesting weaker TM-TM interactions (Table 3.1). These results implicate the predicted L/I zipper motif in TM-TM association for the HeV F TM domain.
Mutation of the TM domain L/I zipper alters overall protein expression and fusion activity

The analytical ultracentrifugation data suggested a shift in equilibrium upon mutation of the L/I zipper motif in the isolated TM domain, consistent with reduction in TM-TM association. To determine how these mutations affected expression, intracellular transport, and function, the LIZ 4A mutant protein was analyzed for total and surface expression by cell surface biotinylation. The trafficking and cleavage pathway of the F protein ultimately results in a homo-trimer that is composed of the F1 and F2 fragments. The F2 fragment is small in size, so F1 is used to detect the cleaved, active form of F. When compared to the WT F protein, the LIZ 4A F protein exhibited a dramatic reduction in total protein expression, as indicated by the reduction in F1 detectable (Fig 3.4A). A reduction in protein expression of LIZ 4A F at the cell surface was also observed (Fig 3.4B), which could potentially affect membrane fusion. The presence of cleaved F protein on the cell surface was used as a measure of whether the F protein was properly trafficked. In order to test fusion activity, a syncytia assay was performed in which F and the attachment protein (G) were transiently transfected into cells, and the cells were visualized for the presence of syncytia (indicated by white arrows in fig 3.5). The LIZ 4A F protein exhibited a striking reduction in fusion index, a measure used to quantify fusion activity, with levels comparable to the mock control (Fig 3.5A). The complete loss of fusion activity exhibited by the LIZ 4A F protein indicated that the L/I zipper may contribute to overall protein stability or alter pre-fusion conformation stability. In addition, the single point mutants, L488A, I495A, I502A and L509A, were examined to determine the effect each had on the F protein. The single point mutants, L488A, I495A and I502A, exhibited a moderate reduction in total protein expression compared to the WT F protein (Fig 3.3A). To determine the effect these mutations had on membrane fusion, syncytia formation assays were performed. Each of the single point mutants displayed a moderate to WT level fusion index (Fig 3.5A&B). The single point mutant, L509A presented with a large reduction in total and cell surface protein expression levels, similar to that of LIZ 4A (Fig 3.4B). Interestingly, although LIZ 4A F and L509A F had comparable reduction in total and surface expression, LIZ 4A exhibited a fusion index at background levels, whereas the L509A fusion index was only
moderately reduced (Fig 3.4B). Although there was a reduction in expression levels, L509A had more fusogenically active F$_1$ than the LIZ 4A mutant, which would enable L509A to drive fusion, unlike LIZ 4A (Fig 3.4D). A small shift in molecular weight for the LIZ 4A and L509A constructs was observed. This may be the result of altered glycosylation, as it has been shown previously that there is a glycosylation site at residue N464. The change in TM-TM interactions may affect the glycosylation state of this upstream residue (36). Previously, it was shown that there is a correlation between cell surface expression and fusogenicity (73). The surface expression of LIZ 4A was around 50% of WT, however, the level of fusion activity was reduced to near background levels. This deviation suggested that LIZ 4A may be affecting overall protein stability. These results suggest that even when individual mutations have an impact on protein expression, it was the mutation of the four L/I zipper residues which caused a complete loss of F protein fusogenic activity.

**Mutation of the TM domain L/I zipper affects stability of the full length F protein**

The HeV F protein is trafficked through the secretory pathway and must then undergo a unique trafficking pathway through recycling endosomes for processing to the fusogenically active form of F by cathepsin L (53). The F protein is synthesized in the endoplasmic reticulum as an inactive trimer (F$_0$), trafficked to the plasma membrane, endocytosed and cleaved to the fusogenically active form of F (F$_1$+F$_2$). After cleavage, the active form of F must then be trafficked back to the plasma membrane (4). With this complicated trafficking pathway, the F protein must be stable over time so that it can ultimately arrive at the plasma membrane in its active, cleaved form. To test whether the LIZ 4A TM mutation affected the stability of the F protein over time, a pulse-chase experiment was performed with various time points analyzed up to 24 hours post-transfection (Fig 3.6). The WT HeV F protein displayed stability over a 24 hour time period. At early time points (0-1hr), the F protein was detected as the inactive uncleaved form, F$_0$. Expression levels increased from the 0hr time point. This is likely because the C-tail antibody does not recognize partially synthesized protein at the 0h time point. Between the two and four hour time points, the F protein was detected in both the uncleaved and cleaved forms, suggesting proper trafficking and cleavage of the F protein.
Ultimately at the final time points, only the cleaved, fusogenically active form of WT F was present (Fig 3.6A). Interestingly, the initial level of LIZ 4A F protein expression at the early time points (0 and 0.5hr) were comparable to that of WT, but quickly protein levels began to diminish over the later time points. The remaining LIZ 4A HeV F protein was processed as expected, but by 1hr post transfection, a decrease in expression was evident compared to the WT F protein. At 24 hours post-transfection, there was almost no detectable amount of LIZ 4A F present. Band density quantification showed the progressive loss of LIZ 4A F over time (Fig 3.6B). The reduction in LIZ 4A F protein did not appear to be a result of reduced protein synthesis, but could be attributed to overall misfolding of the protein, resulting in targeting for degradation or due to premature triggering of F to its post-fusion conformation. It has been suggested previously that prematurely triggered F protein is more susceptible to degradation, which could explain the reduction in LIZ 4A F detection (119).

Pre-fusion F protein stability is reduced with LIZ 4A mutation

In order to obtain crystal structures of the pre-fusion conformation of several viral F proteins, including PIV5 F, HeV F, and RSV F, trimeric tags were engineered onto the protein to prevent triggering to the post-fusion conformation (47, 50, 97, 120). This suggested that the F protein may require TM-TM association to maintain the pre-fusion conformation, until an appropriate event occurs to promote triggering to the post-fusion conformation. The LIZ 4A F protein was utilized to determine whether reduction in TM-TM association affected F protein triggering. Other groups have previously shown that changes in thermal conditions can promote triggering of the F protein (121-123). Based on the thermal triggering property of F, a novel assay was developed to test pre-fusion F protein stability. After cells were transfected to express the F protein, they were either maintained at 37°C or exposed to an elevated temperature of 55°C for a brief period. After heat exposure, the cells expressing the WT F protein or LIZ 4A mutant were then quickly cooled to prevent further conformational changes and incubated with an antibody, mAb 5B3, that was specific to the pre-fusion conformation of HeV F. This allowed for immunocapture of the population of F that remained in the pre-fusion conformation on the surface of the cell, thus representing the population that would drive
membrane fusion. The cells were then lysed and processed to determine the amount of F protein in the pre-fusion conformation. The WT HeV F protein was only minimally susceptible to thermal triggering at 55°C, as only approximately 15% of the WT protein triggered at this temperature (Fig 3.7A). In contrast, the LIZ 4A F protein exhibited a dramatic reduction in pre-fusion F detectable, corresponding to approximately 60% of the expressed protein triggered (Fig 3.7B). Additional experiments were performed with the WT HeV F protein at higher temperatures of 60°C, at which the majority of the WT HeV F protein surface population triggered to the post-fusion conformation (Fig 3.8). The susceptibility of LIZ 4A F to trigger at temperatures lower than the WT HeV F suggested that TM-TM association may contribute to pre-fusion stability of the F protein, with a shift in TM-TM association equilibrium resulting in a dramatic effect on the stability of the pre-fusion conformation.

**DISCUSSION**

While paramyxovirus F proteins have been studied extensively, the factors driving pre-fusion stability remain unclear. Upon triggering, the F protein undergoes a dramatic conformational change, refolding to the post-fusion conformation (Fig 3.9). This large, irreversible change in conformation drives membrane fusion between the viral and cellular membranes, a critical event in the viral life cycle. It has been shown that peptides that prevent conformational changes can be used as a therapeutic treatment, as in the case of Enfuvirtide for targeting of HIV gp41 (124). Maintenance of the pre-fusion state of the F protein is necessary until the F protein is in the appropriate location to drive membrane fusion. Stabilization of the pre-fusion conformation is therefore critical for viral stability and function.

Although the pre-fusion structure for several viral fusion proteins has been obtained, including Nipah virus F, HeV F, HIV env, and the human coronavirus HKU1 spike protein, the structure of each required modification with a trimeric tag, such as foldon or GCNt (47, 97, 125, 126). TM domains of viral F proteins have historically been considered to act primarily as membrane anchors, but these structural studies suggest that the TM domain may be important for pre-fusion F stability, as the soluble
trimeric tags mimic the proposed function of the TM domain. Previously, we have shown that several paramyxovirus F protein TM domains associate in a monomer-trimer equilibrium in the absence of the rest of the protein (95). In addition, replacement of F protein TM domains with foreign TM sequences, including a poly-leucine stretch, can result in folding defects (90, 127, 128). The TM domain of the measles virus F protein was found to modulate fusion activity by altering F protein interaction with its receptor binding protein, hemagglutinin (102). These studies suggest that the TM domain sequence and TM-TM interactions are specific and important for the stability of the F protein. Results from the data presented here further support the importance of the TM domain in F protein function and suggest that TM-TM interactions are important for HeV F protein stability and function. More importantly, these results demonstrate the significance of TM-TM association in the stability of the pre-fusion conformation of F, a critical point of control for viral membrane fusion.

A L/I zipper was identified in the TM domain of HeV F in frame with the upstream heptad repeat B domain. Here, sedimentation equilibrium analytical ultracentrifugation demonstrated that mutation of the L/I zipper motif resulted in reduced TM-TM association when the TM domain was studied in isolation. Previously, motifs responsible for TM-TM association in cellular transmembrane proteins include the GxxxG motif, polar amino acids, Ser/Thr clusters, and QxxS motifs. The GxxxG motif has been suggested to promote TM-TM interactions in the HIV env protein (129-131). Identification of an L/I zipper motif is a novel association motif for viral F protein TM domains (114). The sequence analysis of 140 paramyxoviruses revealed a heptad pattern of β-branched residues, suggesting that TM association motifs may be more flexible than strictly a leucine/isoleucine zipper. Mutation of the L/I zipper also resulted in alteration of overall protein stability and fusion activity, suggesting that TM-TM interactions are important for proper F protein function. The mutation of the entire L/I zipper dramatically reduced transient protein expression levels, abolished fusion activity and overall protein stability as demonstrated via pulse chase experiments. The L/I zipper and similar motifs could also be involved in TM-TM association of other class I viral fusion proteins, but there may be multiple motifs within a single TM domain that maintain TM-TM association.
Considering the requirement of a trimerization tag for crystallization of several paramyxovirus F proteins in the pre-fusion conformation, it is reasonable to predict that TM-TM interactions are involved in maintaining pre-fusion F protein stability. Various groups have previously shown that thermal treatment can be utilized to trigger the fusion protein to its post-fusion conformation. This characteristic of the F protein was used to develop an assay that demonstrated that mutation of the L/I zipper in the HeV F TM resulted in a protein that was dramatically less stable in the pre-fusion conformation. It is important to remember that the F protein must ultimately trigger and drive membrane fusion, so equilibrium must be maintained with TM-TM interactions not being too strong or too weak. The results here suggested that shifting the TM-TM interactions toward a monomeric state dramatically destabilized the pre-fusion conformation of the F protein. The proposed model suggests that TM-TM interactions are important for stability of the F protein in its pre-fusion conformation (Fig. 3.9). We hypothesize that, on the other end of the spectrum, if TM-TM interactions are too strong, the F protein will not be able to trigger to the post-fusion conformation. Here we propose that TM-TM interactions could also be important in vivo, and that modulation of those interactions may result in a fusion dead particle, as a result of premature triggering of the F protein. Together, these results suggest that TM-TM interactions serve to pin the F protein in its pre-fusion conformation, though a dynamic equilibrium ultimately allows triggering.

Beyond viral F proteins, TM-TM interactions have been shown to be important in several cellular processes. A number of studies have shown that TM-TM interactions are necessary for proper signaling of receptor tyrosine kinases, such as ErbB/Neu receptors and EGFR. A recent report found that targeting the ErbB TM domain with TM domain derived peptides could delay tumor growth (105, 132). Additionally, the β-amyloid peptide, which helps mediate plaques typical of Alzheimer’s disease lesions, was found to contain critical TM domain residues that contribute to β-amyloid peptide oligomerization (133). The identification of a L/I zipper in the TM domain of the Hendra viral F protein may provide insight into motifs responsible for TM-TM association in cellular proteins. These studies implicate the TM domain as not just an anchor in the membrane, but more importantly as a domain essential for the function of the full length viral F protein. When considering methods for targeting the viral F protein for therapeutic
purposes, it may be reasonable to consider targeting the TM domain, as it has been shown here to contribute to pre-fusion stability and overall F protein function.
Figure 3.1. LIZ motif in HeV F TM domain. A. Diagram of the fusogenically active, disulfide linked heterodimer F protein with the HeV F TM sequence below. Domain structure of F includes the fusion peptide (FP), heptad repeat A and B (HRA, HRB), transmembrane domain (TM), and the cytoplasmic tail (CT). Mutations for the LIZ 4A HeV F construct are indicated in red in the sequence below WT F. Andreea Popa identified the LIZ and designed the mutations in the pCAGGS expression vector. B. The frequency of β-branched residues in the predicted TM domains of 140 viruses in the paramyxovirus family is shown graphically and appears in a heptad repeat pattern (positions 480-510). Tamas Nagy performed the sequence alignment and determined the frequency of β-branched residues.
Figure 3.2. Amino acid frequency in the TM domain of 140 paramyxoviruses. The intensity of each square represents the frequency of the residue.
Figure 3.3. Sedimentation equilibrium analysis revealed a shift in monomer-trimer equilibrium for LIZ 4A SN-TM. Samples were prepared in C14SB detergent micelles, and absorbance data were collected at 20,000 rpm in a Beckman XL-A analytical ultracentrifuge. WT SN-TM (A) and LIZ 4A (B) data points fit to a monomer (red line) and monomer-trimer (blue line) model. Residual fitting is shown above for both curve fits, with monomer residuals in red and monomer-trimer residuals in blue.
Table 3.1. Best fit model and relative apparent association constant ($K_a^*$) for purified SN-TM constructs. Analysis was performed at three different speeds. Data are shown for monomer and monomer-trimer curve fits. Based on $\chi^2$ and residual fitting, the monomer-trimer curve fit was most appropriate for WT and LIZ 4A. The relative apparent association constant ($K_a$) is normalized to the WT SN-TM construct for each spin.

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Figure 3.4. Total and surface expression levels for LIZ 4A F. Total protein (A) and cell surface (B) expression levels of transiently transfected F constructs after a 3h metabolic label, surface biotinylation and immunoprecipitation. C. Total (black bars) and surface (white bars) expression levels of F₀+F₁ were determined by densitometry and normalized to WT levels. D. The amount of cleaved F was determined by densitometry as F₁/(F₀+F₁). The average represents three independent experiments. Each mutant was compared to the WT F protein using the Student’s t test. *, p<0.05; **, p<0.005; ***, p<0.0005
Figure 3.5. The LIZ 4A mutation completely abolished F-mediated fusion activity. A. Vero cells were transfected with the HeV G attachment protein and WT F, or each of the L/I zipper mutants. Syncytia formation was analyzed 24 h post transfection; images were taken with a Nikon TS100 microscope. White arrows indicate syncytia. Images are representative and quantification (B) represents three independent experiments. **, $p<0.005$
Figure 3.6. LIZ 4A F was less stable over time than the WT F. A. A pulse chase experiment was performed to monitor WT F or LIZ 4A F protein expression and processing. After a 30 min metabolic label, samples were taken at various time points, immunoprecipitated, analyzed on a 15% SDS PAGE, and exposed to a Phosphor screen for imaging. B. Quantification of F₀+F₁ was determined by densitometry and normalized to the zero time point for each construct. The average represents three independent experiments ± standard deviation. The Student’s t test was used to determine significance between WT F and LIZ 4A time points. *, p<0.05; **, p<0.005; ***, p<0.0005
Figure 3.7. LIZ 4A F triggered more readily than WT F at 55°C. A. Vero cells were transfected with WT or LIZ 4A F, thermally treated, and then antibody specific to the pre-fusion conformation of F (mAb 5B3) was directly added to cells. Immunoprecipitation was performed followed by Western blot analysis, probing for the F protein with mAb 5G7. Thermal triggering was performed at 55°C for both constructs. B. Quantification was determined via band densitometry, normalized to 37°C for each construct, and reported as % triggered \[100 \times (1 - \frac{F \text{ expression at } 55°C}{F \text{ expression at } 37°C})\]. Results represent three independent experiments. **, p < 0.005
**Figure 3.8. F protein triggering at 60°C.** WT HeV F and LIZ4A HeV F were thermally treated and immunoprecipitated to detect the pre-fusion conformation of F, as described. The WT F and LIZ 4A F were not detectable in the pre-fusion conformation after the 60°C treatment.
Table 3.2. Virus and associated accession # for the sequences analyzed in Fig 1B.

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Table 3.3. **TM domain heptad repeat alignment.** F protein TM domain sequence three different paramyxovirus genera, highlighting the heptad repeat of β-branched and leucine residues. The TM domain was predicted with the TMHMM server v 2.0.

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Figure 3.9. Proposed model: TM-TM association affects triggering. Viral fusion proteins undergo a large conformational change upon triggering. TM-TM interactions are important for maintenance of the pre-fusion conformation. Reduced TM-TM association results in prematurely triggered F protein, while TM-TM association that is too strong may prevent triggering to the post-fusion conformation. A dynamic equilibrium must therefore be maintained in order for the F protein to properly trigger for membrane fusion.
CHAPTER 4: TARGETING THE F PROTEIN TM DOMAIN TO INHIBIT MEMBRANE FUSION

INTRODUCTION

Membrane proteins represent approximately 30% of all proteins encoded by the human genome and are important for many aspects of cell biology and physiology. Not surprisingly, it is estimated that about 50% of current drug targets are membrane proteins. Despite the clear importance of membrane proteins in cellular biology, their composition of both hydrophobic transmembrane regions and hydrophilic soluble domains have made it difficult to obtain structural data, with only 3,084 of 126,722 protein data bank (PDB) entries representing membrane proteins (PDBTM). Both cellular and viral membrane proteins are typically anchored to the membrane through a transmembrane domain, which has been shown to serve as more than an anchor and contributes to the function of the protein. The difficulty of working with highly hydrophobic transmembrane domains has previously excluded these domains from being targeted for therapeutic development. However, advancements in understanding the TM domain of these proteins have recently been aided by improvements in cryo-electron microscopy and NMR. The TM domain of HIV gp41 was revealed as two helices linked by a flexible region, based on high resolution NMR data (134). Previously, a GxxxG motif was identified within the TM domain of gp41 and found to be important for self-assembly of the TM domains in a membrane environment. Virus-cell fusion was also found to be inhibited when cells treated with homologous gp41 TM peptide were infected with virus (131). Based on the structure of the TM domain, rational drug design may now be used to more accurately target the gp41 TM domain and potentially modulate fusion.

The life cycle of enveloped viruses requires the fusion of the viral envelope with a target cell membrane. The fusion protein is essential for viral entry and, therefore, has been studied to aid in development of antivirals. There are several points in the early stages of the fusion process that can be targeted for disruption: the binding step mediated by the attachment protein, the interaction between the attachment protein and fusion protein that mediates triggering, and the overall refolding of the fusion protein. While there is little sequence homology among class I fusion proteins, the steps critical for
membrane fusion appear to be conserved. Although the role the ectodomain plays in fusion has been studied for a long time, only recently has the role of the TM and intraviral domain been studied. Class I fusion proteins, including those of the paramyxovirus family, are ultimately folded as trimers and it has been shown that the TM domain is important for proper folding and function of the F protein. Replacement of the TM domain with other F protein TM domains or lipid anchors alters F protein function. Additionally, mutation of motifs known to promote protein oligomerization, such as GxxxG motifs, resulted in incomplete fusion (129, 135). The importance of TM oligomerization can also be seen with cellular proteins. For example, the protein ultimately responsible for plaque formation and development of Alzheimer disease, amyloid precursor protein (APP), must dimerize for the generation of amyloid-β (Abad, 2009). Neuropilin-1 (NRP1), a coreceptor for vascular endothelial growth factor receptor 2 (VEGFR2), represents another example in which the TM domain is important for function and the oligomeric status of the protein is important. The NRP1 TM domain contains a GxxxG motif that is required for dimerization and ultimately downstream signaling. It has been shown that targeting the NRP1 TM domain with synthetic peptides could inhibit glioma tumor growth in vivo (136). Together, these studies exemplify the potential of targeting the transmembrane domain of proteins for therapeutic drug design.

Previously, we have shown that the TM domain of the HeV F protein associates in isolation and contributes to overall protein stability. To further understand the role of the TM domain in protein folding, stability or membrane fusion, the TM domain was targeted as a potential modulator of membrane fusion. Since the TM domain has been shown to be important for F protein function and self-associates, it was hypothesized that introduction of exogenous TM protein would disrupt the TM-TM interactions in the native F protein, resulting in premature triggering or protein misfolding. To test this, exogenous Hendra F TM constructs were co-expressed with the wt F and overall protein expression was analyzed. Co-expression of the exogenous Hendra F TM constructs dramatically reduced the expression of Hendra F. However, the co-expression of exogenous Hendra F TM constructs with PIV5 F did not have a dramatic effect on PIV5 F expression levels, suggesting that the interaction of the exogenous TM constructs is sequence specific. Additionally, a TM peptide homologous to the PIV5 F TM domain
was used to inhibit viral infection in cells. Together, these results further emphasize the importance of the TM domain in the F protein function and also identify the TM domain as a potential antiviral target.

RESULTS

Exogenous TM constructs reduced HeV F expression

To test whether the TM domain of the fusion protein could be targeted, exogenous TM constructs were designed based on the HeV F protein sequence. Three constructs were designed containing a signal peptide, a variable linker, the full length TM domain and C-tail linked to an HA-tag (Fig 4.1B). A signal peptide was included to target the protein to the endoplasmic reticulum because during translation the HeV F protein is cotranslationally inserted into the endoplasmic reticulum. The variable linker represented varying lengths of the HRB domain upstream of the TM domain and denoted as short linker, long linker or HRB TM (Fig 4.1C). These constructs were then co-expressed with the full length HeV F protein in Vero cells at a DNA transfection ratio of 2:1 (F:TM). A radiolabel surface biotinylation was used to determine the expression levels of HeV F, as well as whether the F protein was trafficked to the cell surface. When HeV F was co-expressed with an empty vector as a control, the F protein was as two bands, F0 (uncleaved) and F1 (cleaved) in both total overall expression and the surface population (Fig 4.2 A&B). Upon co-expression of each of the TM constructs, total and surface expression of HeV F was reduced to 20-30% when compared to the HeV F expressed with empty vector (Fig 4.2C).

In addition to expression levels, the proteolytic processing of F was analyzed by calculating cleavage \((F_1/(F_0+F_1))\). HeV F co-expressed with the empty vector resulted in approximately 40% cleavage in the total and surface populations (Fig 4.2D). Co-expression of the short and long linker TM constructs with HeV F significantly reduced cleavage in the total population to approximately 26% and 27%, respectively. Interestingly, the amount of cleaved protein detected on the surface was only significantly reduced when HeV F was co-expressed with the long linker TM. To determine whether the effect was concentration dependent, HeV F and the short linker
TM was co-expressed at additional ratios of 10:1 and 20:1. As the amount of HeV F DNA transfected was increased the levels of HeV F detected also increased, as expected (Fig 4.3A). However, the levels of TM detected did not change, which could be a result of quick turnover of the small TM protein. A time course analysis was performed to determine whether the initial synthesis of HeV F was reduced upon co-expression of the TM constructs, or if the presence of the TM proteins reduced the stability of the F protein over time. At the zero time point, the levels of HeV F detected when co-expressed with the short linker TM is reduced when compared to the co-expression with empty vector (Fig 4.3C). It is important to note that the F protein cannot be detected until it is fully synthesized because the antibody used for immunoprecipitation detects the C-tail. The protein that is synthesized undergoes cleavage, producing fusogenically active F1, as seen in the 2 hr and later time points. Quantification of these preliminary results suggests that the co-expression of the short linker TM correlates with an increased rate of protein degradation. The F0+F1 expression levels immediately start to decrease at the 1hr time point (Fig 4.3D). Overall, the addition of the homologous TM constructs resulted in a dramatic reduction in HeV F protein expression.

To further characterize the co-expression, immunofluorescence was used to determine whether the exogenous TM constructs were localized in the same compartments of the cell as the HeV F protein. When HeV F was co-expressed with empty vector in Vero cells, the F protein was primarily detected near the nucleus by a monoclonal antibody to the F protein (7F7) in what is likely the endoplasmic reticulum. For co-expression with the TM constructs, the TM protein was detected with an anti-HA antibody (Roche). All three of the TM constructs appeared to express throughout the cell and were mostly detected near the nucleus. The overlaid images show that the HeV F protein and each of the TM constructs are expressed in similar regions of the cell (Fig 4.4). This is to be expected, as the TM proteins were tagged with a signal peptide to target the protein to the endoplasmic reticulum. Co-immunoprecipitation of HeV F with the TM constructs was attempted, however the assay was not successful. This may be the result of the dramatic reduction in overall F protein levels. Though a direct interaction could not be explicitly determined, the immunofluorescence data suggests that the F and TM proteins are expressed in similar regions of the cell.
Fusion activity is reduced upon exogenous TM expression

Though the presence of the exogenous TM constructs dramatically reduced the levels of HeV F detected in cells, there was still a small population of F detected. A syncytia fusion assay was used to determine the effect the presence of the TM proteins had on F protein function. The F protein and its prototypic attachment protein, G, were co-expressed in Vero cells with each of the TM constructs. After 24hr, the cells were imaged to visualize syncytia formation. HeV F and G co-expressed with empty vector resulted in syncytia formation, as indicated by the white arrows in figure 4. When the TM constructs were introduced into the transfection, syncytia formation was ablated (Fig 4.5). The reduced overall expression levels of HeV F would explain a reduction in syncytia formation. Additionally, co-expression of HeV F with the short linker and long linker TM proteins resulted in reduced protein cleavage, further (Fig 4.2D) reducing the presence of fusogenically active F protein. The co-expression of the TM constructs resulted in a dramatic reduction in membrane fusion activity. Previously, Clint Smith determined the correlation between wild-type surface expression levels and the resulting fusion activity. Based on his findings, a mutant that expresses at 20-30% when normalized to wild-type would be expected to exhibit fusion levels at 30-40% of wild-type. Interestingly, the co-expression of the TM constructs reduced HeV F expression to these levels, but fusion activity was ablated. The lack of fusion seen may be the result of pre-mature triggering of the fusion protein, or the presence of the TM proteins could somehow prevent the fusion protein from being able to trigger.

The effects of the exogenous TM constructs are specific

The previous results demonstrated that the HeV F protein is deleteriously affected upon expression of homologous TM constructs. To determine whether the TM proteins were specifically targeting the HeV F protein, analogous experiments were performed with another class I fusion protein of the paramyxovirus family, PIV5 F. The same DNA transfection ratio of 2:1 was used for transfection of PIV5 F with the TM constructs. Co-expression of the TM constructs with PIV5 F in Vero cells demonstrated no change in total F protein expression levels (Fig 4.6A). The PIV5 F protein was able to drive
membrane fusion and promote syncytia formation in the presence of the exogenous TM constructs, as indicated by white arrows (Fig 4.6B). Together, these data indicate that the TM constructs designed based on the HeV F TM domain do not produce the same effect when expressed with PIV5 F as co-expression with HeV F, suggesting that the effect may be specific.

**TM peptide treatment reduces viral infection**

The results presented thus far have utilized transient transfection to demonstrate that the TM domain can be targeted. In order to determine if the F protein TM domain could be targeted in a more physiologically relevant system, an infection assay was performed. The Hendra virus is a BSL-4 pathogen, so the infection assay was executed with two other paramyxoviruses, PIV5 and human metapneumovirus (HMPV), which both utilize class I fusion proteins to mediate membrane fusion. The recombinant viruses used contained GFP, which allowed for visualization of infection. A peptide was designed based on the sequence of the PIV5 F TM domain and did not include the C-tail or HRB domain. The highly hydrophobic peptide was resuspended in DMSO. rgHMPV-GFP or rgPIV5-GFP were treated with varying concentrations of peptide for 30min and then added to Vero cells to allow for infection (MOI=1). After 24hr, the cells were imaged to count GFP+ cells. When the viruses were treated with DMSO (0μM peptide), the infection for both appears to be widespread. Treatment with TM peptide reduced the number of GFP+ cells for PIV5 (Fig 4.7). Addition of 5μM of peptide resulted in approximately 50% reduction in PIV5 infection. The HMPV samples treated with peptide did not exhibit a reduction in GFP+ cells, suggesting that the TM peptide utilized was specific to PIV5. Together, these results suggest that the TM domain can be targeted to disrupt F protein function and viral infection.
DISCUSSION

The fusion protein drives the fusion of the viral and cellular membranes, a key early step in the entry of enveloped viruses. Previously, various methods have been used to disrupt the fusion process: premature triggering of the fusion protein, disruption of fusion and attachment protein cooperation, and prevention of receptor binding. Targeting the conformational change the fusion protein undergoes to drive fusion has primarily focused on the soluble ectodomain of the protein. The HRB domain of the Sendai virus fusion protein was used to develop an inhibitor of membrane fusion. The inhibitor did not prevent the virus from binding the target cell, but prevented membrane fusion (137). In addition, the heptad repeat domain of HIV gp41, respiratory syncytial virus, human parainfluenza virus type 3, and measles viruses were utilized to develop antiviral peptides that were able to block membrane fusion (138). The heptad repeat domain at the C-terminus of three paramyxoviruses was shown to be a viable target for peptide inhibition. Cholesterol conjugated peptides designed to represent the heptad repeat domain of human parainfluenza virus 3 (hPIV3), Hendra or Nipah virus fusion protein were found to inhibit fusion. The tagging of cholesterol to the peptides appeared to increase the inhibitory activity of the peptides when compared to previous studies, which is likely the result of specifically targeting the peptides to the plasma membrane (139, 140). An exciting in vivo study with measles virus found that fusion protein heptad repeat derived peptides could self-assemble and, upon insertion into cell membranes, increase fusion inhibition potency (141). This study demonstrated that intranasal delivery of these lipid-conjugated peptides could inhibit infection in cotton rats. An analogous study with hPIV3 fusion protein heptad repeat derived peptide found that addition of cholesterol to the peptide increased inhibition of cell-cell fusion. This study also introduced a linker region between the cholesterol tag and the heptad repeat sequence; varying the linker modulated the levels of cell-cell fusion. An in vivo experiment with these peptides showed a significant decrease in viral load when cotton rats were treated subcutaneously after viral infection (142). Other regions of the F protein that have been targeted to prevent membrane fusion include the fusion peptide (143).
We have previously shown that the TM domains of several paramyxovirus F proteins associate in isolation, and, as discussed in a previous chapter, disruption of TM-TM association resulted in a HeV F protein that triggers prematurely. Based on these previous studies, we hypothesized that exposing the native F protein to exogenous TM protein would disrupt the overall structure of the F protein: the exogenous TM protein would interact with the TM domain of the native F protein, ultimately disrupting the trimeric interaction that the F protein requires for proper folding. The structural disruption would lead to either pre-mature triggering or overall misfolding.

Studies with influenza virus, Sendai virus and measles virus have previously shown that the TM domain plays a role in the function of the F protein and is sequence dependent. Based on this premise, the TM constructs designed were homologues of the TM domain of HeV F. The co-expression of the TM constructs dramatically reduced F protein levels overall and may have reduced the initial synthesis of F (Fig 1 and 3). To determine whether initial synthesis was reduced, a pulse-chase with much shorter time points (5, 10, 15, 30 min) would need to be performed. The exogenous TM protein may have interacted with the F protein early in synthesis, causing a misfolding event and eventually degradation. On the other hand, the interaction of the exogenous TM construct may have caused premature triggering, which would also result in the protein being targeted for degradation. In the time course co-expression experiment, the F protein that was successfully folded in the presence of the short linker TM appeared to be properly cleaved. The successful cleavage event and presence on the cell surface (Fig 2 and 3) suggests that the exogenous TM proteins were not altering proper trafficking of the portion of the F protein that successfully exited the ER. From the data presented here, it is unclear whether the F protein was misfolded or prematurely triggered. To distinguish the fate of the F protein in the presence of exogenous TM protein, immuno-capture could be performed to determine whether the F protein is still detectable in the pre-fusion conformation. If the protein is misfolded, the protein may not be detected by a pre-fusion antibody. To determine if misfolding is occurring, co-localization studies with various cellular degradation markers (Rab 7, late endosomal marker; Rab11, autophagosome; Lamp1, lysosome; PSMA1, proteasome target) may be necessary.
The specificity of the effect of exogenous TM protein was determined by co-expressing the HeV F TM protein with PIV5 F. The co-expression of the exogenous TM protein did not affect PIV5 F in terms of expression or function (Fig 6), which supports the premise that the TM-TM interactions are specific and not simply the result of proximity or hydrophobicity. When considering the TM proteins for the development of viral therapeutics, specificity is important so that off-target drug effects can be minimized. The infection assay served as a proof of concept tool, demonstrating that the TM peptide could specifically affect viral infection. We hypothesize that the peptide treatment of the virus samples allowed the TM peptide to insert into the viral membrane and trigger or misfold the F protein. In either case, the virus is left with F protein that is not functional for membrane fusion. When considering the use of the TM domain as a potential viral inhibitor, the solubility of the peptide needs to be overcome. The experiments performed within utilized peptide solubilized in DMSO. In the infection experiment, it was apparent that the peptide tended to aggregate and may not have fully incorporated into the media. A recent publication demonstrated that HRB peptides conjugated with cholesterol were able to inhibit membrane fusion and acted as a broad spectrum antiviral for two paramyxoviruses (142). To maintain specificity, the TM peptides could be redesigned based on their target F protein TM domain, but also include a portion of the upstream HRB domain. The inclusion of the HRB domain may help overcome the solubility issue and also provide a two-fold means of preventing fusion, as the HRB has previously been shown to be a region important for membrane fusion (as discussed previously).

Beyond viral proteins, the concept of disrupting TM-TM interactions may prove to be a viable therapeutic option, too. The tyrosine kinase receptor, ErbB2, was found to be overexpressed in high-grade inflammatory breast cancer. ErbB2 requires dimerization in order to trigger downstream signaling cascades that include the MAPK pathway. TM peptides were designed to disrupt ErbB2 TM dimerization and were found to reduce tumor cell growth and metastasis (92). The TM domain may serve as a viable domain for drug design for other tyrosine kinase receptors, as well. Another study with NRP1 exploited the heterodimerization of NRP1 with Plexin-A1, a protein highly expressed in glioblastoma. A synthetic TM peptide was used to disrupt TM interactions between
NRPI and Plexin-A1, resulting in reduced glioblastoma cell proliferation. The TM peptide was designed based on the NRPI TM domain and diversifies the potential of disrupting TM-TM interactions beyond homo-oligomerization, to hetero-oligomerization (144). Overall, these studies demonstrate that the TM domain may be a viable target for antiviral therapeutic development.
Figure 4.1. Linker TM construct design. Domain structure of the di-sulfide linker heterodimer F protein. FP, fusion peptide; HRA and HRB, heptad repeat A/B; TM, transmembrane domain; CT, C-terminal tail. Below the F protein is the design for exogenous TM constructs, containing a signal peptide, a linker composed of varying lengths of the HeV F HRB domain, the HeV F TM domain and CT, and an HA tag. Residues of the HRB domain used for the variable linker are indicated.

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<td></td>
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<tr>
<td>long linker: 478-487</td>
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<tr>
<td>HRB: 459-487</td>
<td></td>
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Figure 4.2. Co-expression of HeV F with TM proteins reduces total and surface expression. Surface biotinylation was used to analyze total (A) and surface (B) expression of HeV F when co-expressed with empty vector or the TM constructs. Expression (C) and F protein cleavage (D) are quantified using band densitometry (ImageQuant). *, p<0.05 student’s t-test
Figure 4.3. Presence of exogenous TM proteins reduces HeV F initial synthesis. A. HeV F was co-expressed with the short linker TM at varying ratios. As the ratio increases, the levels of F detected increase, as quantified in B. C. A radiolabel time course assay was performed when HeV F was co-expressed with the short linker TM. D. Band densitometry for time course.
Figure 4.4. Immunofluorescence shows co-localization of HeV F with the TM constructs. Cells were transfected with HeV F and the TM proteins and immune-labeled with an antibody against HeV F, 7F7, and an antibody to detect the TM proteins, anti-HA.
Figure 4.5. Syncytia formation is ablated upon co-expression of the TM proteins with HeV F. Cells were transfected with HeV F, the attachment protein (G), and the TM constructs. After 24h, cells were imaged for syncytia formation, indicated by white arrows.
**Figure 4.6. PIV5 F expression and fusion activity are not affected by HeV F TM proteins.** A. PIV5 F expression levels were determined when co-expressed with the TM proteins by radiolabeled immunoprecipitation. Expression levels were determined by band density (C). B. Syncytia formation assay revealed that co-expression of TM constructs did not inhibit PIV5 F fusion activity. Syncytia indicated by white arrows.
Figure 4.7. **TM peptide inhibits viral infection.** A. GFP viral samples, PIV5 or HMPV, were treated with TM peptide designed to represent the PIV5 F TM domain, then cells were infected with treated virus. GFP+ cells were imaged 24hr later and counted for quantification (B).
CHAPTER 5: VIRAL FUSION PROTEIN TRANSMEMBRANE DOMAINS EXHIBIT TRIMERIC ASSOCIATION ACROSS SEVERAL VIRAL FAMILIES

INTRODUCTION

Membrane fusion is a critical event in the life cycle of an enveloped virus. Fusion of the viral envelope with the target cell membrane is mediated by one of the surface glycoproteins, the fusion protein, which studs the surface of the virus. The fusion protein undergoes a dramatic conformational change from its metastable pre-fusion conformation to the post-fusion conformation when triggered by an event such as receptor binding or a pH change in the surrounding environment. The change in structure is an essentially irreversible process, so it is important for the virus to maintain the pre-fusion structure until triggering is appropriate. Factors that contribute to stability of the pre-fusion state of the fusion protein have been identified in the ectodomain of several viral fusion proteins, including residues within the stalk domain. Though it is often thought to simply act as an anchor, the TM domain of viral fusion proteins has been implicated in protein stability and function as well. The influenza virus fusion protein (HA) was engineered to replace the TM domain with a lipid anchor, glycosylphosphatidylinositol (GPI), to elucidate the role of the TM domain in the fusion protein. The lipid-anchored HA protein was only able to promote hemi-fusion, implicating the TM domain in fusion protein function (87). Additional studies with Influenza virus HA have demonstrated that specific residues within the TM domain are important for the fusion protein oligomeric state and function (86, 98, 145). Trimerization of the HIV gp41 TM domain was found to enhance neutralizing antibody binding to the membrane proximal external region when compared to monomeric TM (146). Together these studies make it evident that the TM domain is of importance for the fusion protein, though the details require further study.

Previously, we have shown that the HeV F TM domain self-associates in a monomer-trimer equilibrium, based on analysis of the SN-TM chimera in isolation via sedimentation equilibrium analytical ultracentrifugation (95). Since the fusion proteins of class I and III viral fusion proteins need to form a trimer to properly function, we hypothesized that it is likely that the TM domain of these fusion proteins also associates
in a monomer-trimer equilibrium. Additionally, essentially all of the pre-fusion crystal structures that have been obtained to date required the addition of a trimeric tag, regardless of viral family or fusion protein class, further suggesting that the TM domain of these proteins likely self-associates. The structure of the respiratory syncytial virus (RSV) fusion protein in the pre-fusion conformation was determined upon addition of a C-terminal fibritin trimerization domain (120). Other pre-fusion crystal structures include that of PIV5 F, HIV gp41, Hendra virus F, and the coronavirus spike protein, all of which required the addition of a coiled-coil for stabilization in the pre-fusion conformation. This requirement across various fusion proteins suggests that TM-TM interactions may be broadly relevant.

Viral fusion proteins from four single stranded-RNA viral families were chosen to analyze TM-TM interactions beyond the paramyxovirus family. The viruses represent several major human pathogens: Ebola, influenza, severe acute respiratory syndrome (SARS), and rabies. The Ebola fusion protein (GP), influenza virus HA, and SARS CoV spike protein (S) are class I viral fusion proteins, like fusion proteins of the paramyxovirus family. The fusion proteins in this class undergo a dramatic structural rearrangement that is irreversible, so it is important to have mechanisms in place to maintain the pre-fusion conformation. The pre-fusion structure of the Ebola virus glycoprotein (GP) was determined upon binding of a human antibody, which essentially clamped the GP1 and GP2 subunits together, preventing triggering. The Ebola virus GP has two subunits that mediate attachment (GP1) and fusion (GP2) (147). Influenza virus HA was the first fusion protein crystallized in its pre-fusion conformation (148). Influenza HA is cleaved into a disulfide linked hetero-dimer for activation (HA1, attachment; HA2, fusion). The pre-fusion HA structure revealed that the fusion protein exists as a trimer with the fusion peptide buried within the trimer interface (69). SARS CoV S, like Ebola and influenza fusion proteins, requires a cleavage event for activation, resulting in two fragments, S1 (attachment) and S2 (fusion). A recent cryo-electron microscopy study described several pre-fusion intermediates in which the S1 subunit was found on top of the S2 subunit in different “up” (active, with exposed receptor binding sites) or “down” (receptor-binding inactive) positions (149). The pre-fusion structure of a closely related coronavirus spike protein, HKU1 S, was reported recently. Similar to the
pre-fusion intermediates described for SARS CoV S, the attachment subunit, S1, was found on top of the fusion subunit, S2. This arrangement is predicted to prevent the fusion protein triggering until receptor binding occurs (126). Rabies GP, a class III viral fusion protein, is a member of the *rhabdoviridae* family, which includes the well-studied vesicular stomatitis virus (VSV). There is limited structural information about the rabies GP. Unlike class I viral fusion proteins, some class III viral fusion proteins are able to reverse the refolding process and return to the pre-fusion conformation. The selected fusion proteins are all trimeric and require a large conformational change to drive membrane fusion. Mutagenesis studies with SARS CoV S, Ebola GP, and influenza HA have all implicated the TM domain as important for proper folding and/or function of the fusion protein, however none of the crystal structures described to date include the TM domain (86, 87, 150, 151). With the knowledge that the TM domain of several paramyxovirus fusion protein TM domains oligomerize and appear to contribute to fusion protein function, I wanted to determine whether this property was applicable beyond paramyxoviruses. Utilizing the sedimentation equilibrium AUC system that has been established, chimeric proteins containing the fusion protein TM domain of interest fused with the protein staphylococcal nuclease were analyzed for oligomerization, as described previously.

**RESULTS**

The TM domain of each fusion protein was predicted using the TMHMM server v2.0 (Table 5.1). The SN-TM chimeric proteins were expressed in E.coli and purified into detergent micelles at pH 7. The TM residues used for each construct were: Ebola GP, 651-672; influenza HA, 511-536; SARS CoV S, 1193-1227; Rabies G, 429-461 (Table 5.1). For centrifugation, the protein samples prepared in detergent micelles were density matched using deuterated water, to negate any contribution to sedimentation by the micelle itself. As a result of this preparation, any changes in sedimentation, as measured by absorbance, is the result of protein oligomerization.
**TM-TM association at pH 7**

To determine the best fit for each construct, the data points were fit to multiple models varying from monomer only to more complicated fits, such as monomer-trimer-Nmer. Residual plotting, $\chi^2$ and R values were used to choose the best fit, and, when more than one model was applicable, the simplest model was chosen. Analysis of the Ebola GP and Rabies GP SN-TM constructs resulted in a monomer-Nmer fit of approximately 1:3, suggesting a best fit model of monomer-trimer for either. The curve fit for each at 20,000rpm is shown with the residuals plotted above (Fig 5.1). Each analysis was performed at three speeds, 20, 25, and 30krpm. Although these are fusion proteins of different class (I and III, respectively), both exhibit the monomer-trimer equilibrium, supporting the overall trimeric structure of the full length fusion protein. The SN-TM constructs for influenza HA and SARS CoV S were determined to best fit a monomer-trimer-hexamer equilibrium. Again, the residual plotting and curve fitting parameters are shown (Fig 5.1, table 5.2). This fit adds another oligomeric species when compared to Ebola GP and rabies GP. The slight variation, when compared to the other class I fusion proteins, may be the result of association between trimers of the SN-TM constructs.

**TM-TM association at pH 5**

Low pH has been described as a trigger for many viral fusion proteins. The influenza HA protein requires exposure to low pH to initiate the fusion protein conformational change that drives membrane fusion. The rabies GP was found to require exposure to pH below 6.2 in order to drive membrane fusion. At pH 6.7, equilibrium occurs in which approximately half of the GP on the rabies virion are in an inactivated state, but fusion does not occur. These results suggest that there may be some critical concentration of fusion active GP that is required for membrane fusion (152, 153). It has been shown that the Ebola GP requires low pH; however, the low pH seems to be important for the activity of the cathepsins that cleave GP into its active conformation, not the protein conformational change (154). The SARS CoV S protein, like Ebola GP, requires low pH exposure for the cathepsin cleavage activity, but the acidic pH is not necessarily responsible for the conformational change the fusion protein undergoes (155,
To determine whether TM-TM association was affected by exposure to low pH, the SN-TM constructs were prepared at pH 5 and analyzed via SE-AUC. Again, the data points were first fit to a monomer-Nmer curve (Table 5.2), and then fit to multiple species curves. The SN-TM constructs for the Ebola GP, influenza HA, and SARS CoV S best fit to a monomer-trimer-hexamer equilibrium, as determined by residual plotting, $\chi^2$, and R values (Fig 5.2). Interestingly, the influenza HA SN-TM protein exhibited a monomer-trimer equilibrium at pH 7. The rabies GP SN-TM protein, on the other hand, continued to exhibit a monomer-trimer equilibrium. From these results, it is apparent that the fusion protein TM domain various viral families self-associate, most typically as a trimer.

**DISCUSSION**

Direct analysis of TM domains has been limited, largely as a result of the inherent difficulty of working with such hydrophobic domains. Sedimentation equilibrium AUC has provided a powerful tool for directly studying the TM domain of viral fusion proteins, providing further insights into the importance of this elusive domain. Other systems have been used to study TM domain dimerization, such as the TOXCAT system; however these systems are unable to characterize higher order oligomeric species. The data here demonstrate that trimeric TM-TM interactions occur for class I and III viral fusion proteins of different viral families. Class I and III viral fusion proteins are known to be synthesized as trimers, so it is not surprising to find that the TM domain of these proteins oligomerizes as a trimer. More importantly, these studies provide a tool to elucidate the residues that are critical for TM-TM association, and, therefore, potentially critical for the proper folding and function of the full length fusion protein.

The best fit for SARS CoV S and Ebola GP SN-TM constructs at pH 7 included an additional higher order species, a hexameric species. This hexameric species is likely the result of two sets of trimeric SN-TM interacting with one another. The Nipah virus F protein was recently found to oligomerize, forming higher order species up to a hexamer of trimers (125). The pre-fusion crystal structure of Nipah virus F protein found that six of the fusion protein trimers interacted in a ring structure that may contribute to pre-fusion protein stability. The close proximity of the fusion protein in this tertiary structure
may provide a platform for the interaction of the trimeric TM domains. Additionally, an earlier study with influenza virus HA demonstrated that HA proteins located outside of the site of contact were also important for membrane fusion. By interfering with the HA outsiders, membrane fusion was inhibited, suggesting a potential role for higher order fusion protein oligomerization in membrane fusion (157). Interestingly, the influenza HA SN-TM construct fit a monomer-trimer equilibrium at pH 7 and then best fit a monomer-trimer-hexamer equilibrium at pH 5. The addition of a higher order species at the lower pH may support the idea that higher order oligomerization could be important for membrane fusion, and that the TM-TM interactions may contribute to the oligomerization. In combination with the functional biochemical assays, the data obtained from sedimentation equilibrium AUC provides an instrument to study the contributions of the TM domain to fusion protein function.
Table 5.1. F protein TM sequence of viral F proteins from four different viral families. TM domain amino acid sequence of Ebola GP, Influenza HA, SARS Co-V S, and Rabies GP used to produce SN-TM constructs for SE-AUC.

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Virus family</th>
<th>Class</th>
<th>TM sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ebola GP</td>
<td>Filoviridae</td>
<td>I</td>
<td>WIPAGIGVTGVIIAVIALFCIC</td>
</tr>
<tr>
<td>Influenza HA</td>
<td>Orthomyxoviridae</td>
<td>I</td>
<td>NEIKGVKLSSMGVYQILAIYATVAGS</td>
</tr>
<tr>
<td>SARS Co-V S</td>
<td>Coronaviridae</td>
<td>I</td>
<td>KWPWYVWLGFIAGLIAVMVTLCCMTCCSCLK</td>
</tr>
<tr>
<td>Rabies GP</td>
<td>Rhabdoviridae</td>
<td>III</td>
<td>HERISGVDLGLPNWGKYVLLSAGALTALMLIIF</td>
</tr>
</tbody>
</table>
Figure 5.1. Best fit curve fitting for each SN-TM construct at pH 7. Absorbance data and residuals were plotted for each of the four SN-TM constructs: Ebola GP, Influenza HA, SARS CoV S, and Rabies GP. Data shown are from the 20,000rpm spin and are representative of two sample preparations.
Figure 5.2. Best fit curve fitting for each SN-TM construct at pH 5. Absorbance data and residuals were plotted for each of the four SN-TM constructs: Ebola GP, Influenza HA, SARS CoV S, and Rabies GP. Data shown are from the 20,000rpm spin and are representative.
Table 5.2. Best fit model for each SN-TM construct. Analysis was performed at three different speeds and pH 5 or 7. Data are shown for the monomer-Nmer curve fit from the 20,000rpm spin. The best fit was determined by residual plotting, $\chi^2$, and R values.

<table>
<thead>
<tr>
<th>F protein</th>
<th>pH</th>
<th>Monomer Nmer</th>
<th>Best fit</th>
<th>$\chi^2$</th>
<th>R</th>
</tr>
</thead>
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<tr>
<td>Ebola GP</td>
<td>7</td>
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<td>0.00304</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1 : 5.12±0.21</td>
<td>1:3:6</td>
<td>0.00142</td>
<td>0.998</td>
</tr>
<tr>
<td>Influenza HA</td>
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<td>1 : 3.03±0.23</td>
<td>1:3</td>
<td>0.00449</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
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<td>0.00198</td>
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<tr>
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<td>1:3:6</td>
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<tr>
<td></td>
<td>5</td>
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<td>0.00350</td>
<td>0.999</td>
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<tr>
<td>Rabies GP</td>
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<td>0.00480</td>
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<tr>
<td></td>
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Membrane fusion is a critical process important in cellular and viral physiology. In cellular biology, membrane fusion is necessary for the life cycle of many proteins, from trafficking a newly synthesized protein to its site of action to ultimately leading to the degradation of that protein upon fusion of late endosomes with the lysosome. Membrane fusion is also important for fertilization (sperm-oocyte fusion), formation of muscle fibers, which requires the fusion of myoblasts to create large, multinucleated cells, and the formation of osteoclasts (macrophage-macrophage fusion), which is important for bone remodeling (158-160). Likewise, enveloped viruses rely on membrane fusion to initiate an infection event. Without the proper protein machinery, an enveloped viral particle would not be able to infect a cell and eventually propagate. With such a critical function, it is to be expected that the fusion machinery is finely tuned and regulated. After the virus has located and bound to a target cell, the fusion protein is responsible for mediating an incredible task: merging two lipid bilayers together. Fusion of two membranes requires the input of energy to overcome the kinetic barrier that is produced by the repulsive hydration force as the two bilayers come together. To complete this task, the fusion protein, initially found in a metastable conformation, undergoes dramatic, typically irreversible, structural rearrangements that drive membrane fusion. Structural data has provided some insights into the mechanism of fusion. Since 2005, nearly 300 structures of viral fusion proteins have been submitted to the Protein Data Bank (PDB). Unfortunately, these structures largely represent a few viruses, such as HIV env and influenza HA. More importantly, these structures are static and provided limited information regarding the dynamic fusion process.

The triggering process has been an area of interest for many viruses, as it has been proposed that disrupting or prematurely triggering the fusion protein is a viable therapeutic option. Studies of class I viral fusion proteins have largely focused on regions within the ectodomain as regulators of triggering. Examples include targeting the FP domain or the heptad repeat stalk domain of viruses, such as HIV, measles, and influenza.
Due to the difficulties of working with hydrophobic domains, few studies focus on the fusion protein membrane anchor, the TM domain. Additionally, the structural data for most fusion proteins does not include the TM domain, because of the complexity in crystallizing hydrophobic proteins. Despite this, all the class I and III pre-fusion crystal structures published to date required a trimeric tag at the C-terminus of the protein, implicating the TM domain in pre-fusion protein stability. Mutagenic studies of the fusion protein TM domain suggest a functional role for the TM domain beyond anchoring (influenza HA, measles virus F, and Sendai virus F) (90, 98, 102, 128, 137, 164). For example, replacement of the influenza HA TM domain with a lipid anchor, glycosylphosphatidylinositol (GPI), resulted in a fusion protein that could only achieve hemi-fusion. Interestingly, the GPI anchor only permeates one leaflet of the lipid bilayer, whereas the TM domain of influenza HA crosses both layers, suggesting an importance for TM length in membrane fusion activity (86, 87). Additionally, it was shown that there are length requirements for the TM domain of influenza HA. Truncations that shortened the TM domain from 27 amino acids to less than 17 amino acids resulted in a fusion protein that was only able to mediate hemi-fusion. To promote full fusion and lipid mixing, the TM domain may need to span the entire lipid bilayer and, for influenza HA, the truncation of less than 17 amino acids may not result in a TM domain that can span the bilayer (101). The class III viral fusion protein, VSV G, also demonstrated a length requirement for its TM domain. Truncation of the VSV G TM domain from 20 amino acids to 14 amino acids resulted in a fusion protein that could not promote syncytia formation. Interestingly, the TM domain truncation removed a glycine residue; introduction of a glycine residue in the 14 residue TM domain increased fusion activity to approximately 80% (165). These results suggest an important interplay between TM domain length and specific amino acid sequence for fusion protein function. Here, we demonstrate the importance of the Hendra virus F protein TM domain in triggering and the potential for targeting the F protein TM domain to prevent viral entry.
The paramyxovirus family contains many important human pathogens, including measles, mumps, and the zoonotic Hendra and Nipah viruses. These viruses utilize class I fusion proteins to fuse their lipid envelope with a target cell membrane. Therefore it is absolutely critical that the fusion protein only triggers when it can promote a productive membrane merger event. Previous work by E.C. Smith and Andreea Popa demonstrated that manipulation of the HeV F TM domain could alter protein trafficking and function (95, 99, 100). In her studies, Andreea Popa identified a leucine/isoleucine zipper (LIZ) in the HeV F TM domain. To build upon our understanding of TM-TM interactions, the LIZ, a motif known to promote protein oligomerization, was mutated for functional studies. By replacing the LIZ with alanine residues, the data presented here demonstrated that TM-TM association was dramatically reduced when analyzed in isolation by SE-AUC. This LIZ mutant provided a great tool to study the importance of TM-TM association in F protein folding and function. In transient transfection experiments, the LIZ mutation dramatically reduced HeV F protein expression and fusion activity, as determined by surface biotinylation and syncytia formation assay, respectively. The protein that was successfully synthesized was found to trigger more readily than the wild type F protein when analyzed by a thermal triggering assay. These results demonstrate that the HeV F TM domain is not only sequence specific but contributes to pre-fusion protein stability. Furthermore, sequence alignment of the fusion protein TM domain of approximately 140 additional paramyxoviruses revealed the presence of a heptad repeat of β-branched residues (with the addition of Leu). This heptad repeat may be important for TM-TM interactions, and even pre-fusion stability, beyond the Hendra virus.

As stated before, triggering must be spatially and temporally regulated, so pre-fusion maintenance is critical. Because of this, it is likely that multiple motifs within the TM domain contribute to pre-fusion protein stability. The AxxxG motif was shown to be important for TM-TM interactions of HeV F via SE-AUC, though the reduction in $K_a$ was approximately 5-fold for the G508A mutant, which is considerably less than the approximately thousand fold decrease in $K_a$ with the LIZ mutations (95). To determine if these motifs have an additive effect, the AxxxG and LIZ motifs could be mutated.
simultaneously and analyzed for TM-TM association and functional changes. The major
hurdle to overcome would be the reduced protein expression caused by the LIZ mutation.
This may be addressed by synthesizing the protein in cells at a reduced temperature, to
hopefully permit more successful folding events. Due to the dramatic changes in TM-TM
association shown here for the LIZ mutation and previously for the AxxxG motif, it is
likely that mutating both motifs would further decrease TM-TM association. This further
reduction would probably deleteriously affect folding and fusion activity. In addition to
the leucine zipper and GxxxG motif, there have been other motifs shown to be important
for TM-TM oligomerization. For example, the presence of polar residues within the TM
domain can promote hydrogen-bond formation and aid in oligomerization. A study with
model transmembrane peptides demonstrated that oligomerization of the helical peptides
was stabilized by side chain packaging of asparagine residues (166). Aromatic residues
have been demonstrated to be important for TM-TM interactions in both model and
physiological systems. The TM domain of SARS CoV S has an aromatic motif at the N-
terminus of the TM. Mutation of these aromatic residues, specifically the tryptophan
residues, resulted in a fusion protein that was severely crippled in its ability to drive
membrane fusion (150). Other motifs may also be involved in TM-TM interactions that
have yet to be identified. Molecular dynamics studies in combination with mutagenesis
experiments could aid in identifying additional association motifs.

Another approach could instead enhance TM-TM interactions. When the fusion
protein transitions from the pre-fusion to the post-fusion conformation, the stalk domain
transitions from a coiled coil (trimer) to a six-helix bundle. From the structural data, the
post-fusion conformation appears to place the TM domains in close proximity with the
hydrophobic FP domains. Due to the dramatic structural rearrangements and consistence
with the previous SE-AUC experiments, it is suggested that the TM-TM interactions are
in equilibrium, continuously associating and dissociating with one another. If the TM-TM
interactions were stabilized, I hypothesize that the F protein would not trigger efficiently.
More specifically, a fusion protein with stronger TM-TM interactions would require a
much higher heat treatment for triggering in the thermal triggering assay used previously.
To test this, the TM domain could be mutated to include cysteine residues that could then
be utilized to promote disulfide bonds between the TM domains. The mutations would

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need to be made near either termini of the TM domain because disulfide bonds will not form when the cysteine residues are buried in the membrane. The syncytia fusion and thermal triggering assays could then be utilized to test the function of the disulfide linked mutant. By shifting the TM-TM equilibrium toward more tightly associating trimers, it is likely that the fusion protein would not be able to trigger and therefore could not promote membrane fusion. To further monitor the conformational change of the fusion protein, conformation specific monoclonal antibody or heptad repeat peptide binding could be monitored. To dissect the stage at which enhanced TM-TM association affects the fusion protein, a lipid mixing assay could also be used to determine fusion intermediates. Lipid mixing could be monitored by preparing the fusion protein in liposomes that contain fluorophore-conjugated lipids. The fluorescence resonance energy transfer (FRET) between 7-nitro-2-1,3-benzoxadizaol-4-yl (NBD) and rhodamine is utilized to monitor lipid mixing. Upon fusion, the local concentration of fluorophores is decreased, so rhodamine can no longer absorb the energy emitted from NBD, which can be detected as a change in fluorescence (167, 168). When this method is modified to monitor content mixing, fusion intermediates can be detected.

Class I viral fusion proteins are synthesized as homo-trimers, so it is logical to consider protein-protein interactions when attempting to dissect the mechanism of fusion protein triggering and membrane fusion. However, the fusion protein is also a type I integral membrane protein and, therefore, directly interacts with the lipid bilayer in which it is embedded. Since the lipid bilayer of a cell is a tightly packed, fluid entity, it is likely that the lipid content surrounding the TM domain would affect TM-TM association and, as a result, protein function. Upon maturation, viral particles ultimately bud from the plasma membrane and are enveloped in a lipid bilayer that is of similar composition to the plasma membrane. The plasma membrane is composed primarily of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) with other sphingolipids and cholesterol present at lower concentrations. Interestingly, cholesterol has been shown to be important across the viral life cycle, including viral fusion, assembly and budding. More specifically, lipid rafts, areas of membrane that are enriched in cholesterol and glycosphingolipids, are utilized by many viruses to complete fusion. The membrane envelope of many viruses actually contains more cholesterol than the typical plasma membrane.
membrane from which they are taken (151, 169-172). HIV has been shown to require cholesterol in both the viral membrane and the target cell membrane to mediate efficient membrane fusion (173). The lipid rafts can increase the local concentration of the viral fusion protein within the viral envelope, or the cellular receptor on the plasma membrane. Here, we have shown that a decrease in TM-TM association resulted in a fusion protein that was more readily triggered than the wild type. The lipid environment surrounding the TM domains may also contribute to TM-TM association, especially since it has been shown in many cases that cholesterol is found in the viral envelope. A caveat of the SE-AUC system used in our studies is that the SN-TM constructs are purified into lipid micelles, not bilayers. Though the centrifugation data provides insight into relative changes in TM-TM association in micelles, the TM domain naturally resides in a lipid bilayer, which may have additional effects on association. Based on the general requirement for cholesterol in the viral envelope, I hypothesize that the lipid environment affects TM-TM association and therefore affects fusion protein triggering. To test this hypothesis, the fusion protein would need to be expressed in a lipid bilayer system, such as Nanodiscs or solid supported lipid bilayers, and analyzed to determine fusion protein triggering. These in vitro experiments would allow for the manipulation of lipid content, such as decreasing cholesterol content or changing lipid distribution across the bilayer leaflets. For example, increasing the ratio of PE to PC in the lipid bilayer may result in an environment in which the fusion protein is more easily triggered. The cone shape of the PE lipid would enhance lipid disorder, which could result in a reduction in TM-TM association. Viral like particles could also be used to test the effect of varying lipid conditions on not only fusion protein triggering, but also particle budding. Depletion of cholesterol in the plasma membrane would likely result in reduced production of viral like particles, as it has been shown that budding typically occurs at lipid rafts.

Returning to the classic influenza HA TM example, replacement of the TM domain of HA with a GPI lipid anchor resulted in a fusion protein that was only able to mediate hemi-fusion. GPI resides in the outer layer of the lipid bilayer and only permitted lipid mixing, suggesting an importance for TM length in membrane fusion (86, 87). Interestingly, the addition of chlorpromazine was able to rescue full membrane fusion with the GPI anchored HA protein. Chlorpromazine typically resides in the inner bilayer
of the leaflet and has a destabilizing effect. On the other hand, methochlorpromazine, which partitions to the outer leaflet, could not rescue membrane fusion (98). These results suggest that the TM domain must span the lipid bilayer and destabilize the lipid bilayer for efficient membrane fusion. Further supporting the idea that the TM domain is important for membrane fusion, isolated peptides derived from different viral fusion protein TM domains have been shown to promote fusion in model systems alone (174). Though there are limited studies regarding the direct interactions between the TM domains of viral fusion proteins and lipids, these studies suggest that the combination of the TM domain and particular lipid environments results in destabilization of the bilayer, which permits fusion (175).

In the process of membrane fusion, a dimpling of the viral envelope, as well as the cell membrane, must occur to initiate the formation of the hemifusion stalk. Insertion of the FP into the target cell membrane is thought to initiate the dimpling of the cell membrane (176). The perturbation of the cellular membrane is probably enhanced by the presence of multiple fusion proteins inserting their FP into a localized area of the membrane. In the native virion, there has been no evidence of dimpling of the envelope prior to membrane fusion; however it is necessary for successful fusion. It is possible that the triggering event and insertion of the FP into the cell membrane results in a change in TM structure that aids in the dimpling of the viral membrane. Interestingly, almost all of the changes described here occur after particle binding, so the change in lipid environment or the TM domain specifically affects membrane fusion and not receptor binding.

**DISRUPTING TM-TM INTERACTIONS: POTENTIAL FOR A NOVEL DRUGGABLE TARGET**

In order to fully exploit the fusion protein TM domain, I proposed to target TM-TM association to prevent membrane fusion and potentially infection. Considering the propensity for TM-TM association and the contribution to pre-fusion stability described previously, it seemed plausible that TM protein derived from the fusion protein could be used to disrupt TM-TM association, thereby disrupting fusion protein function. I hypothesized that the exogenous TM protein would interact with one of the TM domains of the fusion protein trimer. This interaction would essentially displace a TM domain of
the native fusion protein and result in premature protein triggering or misfolding (Fig 6.1). The data presented here demonstrated that exogenous TM protein could be co-expressed with Hendra F and specifically reduced protein expression and membrane fusion. Furthermore, virus treated with TM peptide derived from the viral fusion protein exhibited reduced infectivity. Like the transient transfection experiments, the TM peptide seemed to be specific in its interaction: the PIV5 F TM peptide did not significantly reduce HMPV infection. These results shine light on an additional role for the fusion protein TM domain as a druggable antiviral target. The proof of concept experiments presented here provide the starting foundation for potential drug candidates, although there are several stipulations to be overcome.

Within the past decade, peptides have been increasingly studied in applications for biotechnology and therapeutic design. As of 2015, there were at least 60 US Food and Drug Administration (FDA) approved peptide medicines on the market. Peptide medicine has several strengths and weaknesses. Peptides can be highly selective and potent, however they may be physically unstable, prone to aggregation, and have a short half-life. Efforts have been made to address many of these weaknesses, such as expanding plasma half-life by identifying and removing enzymatic cleavage sites from the peptide. Regardless of some of the potential shortcomings, there have been several successful peptide medicines developed. The most well-known anti-viral peptide is the first FDA approved HIV entry inhibitor, T20 (enfuvirtide). Enfuvirtide, first approved in 2003, targets the fusion step of HIV infection and binds the transmembrane subunit, gp41 (177). More specifically, the peptide was derived from the amino acid sequence of the heptad repeat domain upstream of the TM domain. Ultimately, binding of enfuvirtide prevents the formation of the six helix bundle that is required to complete membrane fusion. Unfortunately, enfuvirtide requires twice daily injections because it is rapidly degraded and patients often suffer from injection site reactions (approximately 98% of subcutaneous injections) (178). Resistance to enfuvirtide has also been reported as a result of mutations in the heptad repeat motif of gp41, with only single or double point mutations resulting in high-level resistance (179). Despite these shortcomings, enfuvirtide and other peptide drugs represent an incredible step forward in drug design: rationally designed small molecules.
In recent years, these concepts have also been applied to hydrophobic targets. Despite the known difficulties of working with a highly hydrophobic TM domain, there are several examples where the TM domain of an integral membrane protein has been targeted to disrupt protein function. The TM domain of several receptor tyrosine kinases (RTK) have been studied and found to require oligomerization for downstream signaling. Neuropilin-1 (NRP1) and its signaling partner, Plexin-A1, dimerize to regulate cell migration and proliferation. Previously, Plexin-A1 was identified as a marker for glioblastoma. A study found that oligomerization of NRP1 and Plexin-A1 could be inhibited by introducing a TM peptide derived from the Plexin-A1, reducing glioblastoma cell proliferation (144). Not only does this study highlight the importance of the TM domain in receptor signaling, but it demonstrates the potential for the TM domain as a therapeutic target. A similar study with the tyrosine kinase receptor, ErbB, found that the TM oligomerization of ErbB-2 and ErbB-3 could be disrupted with a TM peptide homologous to the TM domain of ErB-2. These TM studies targeted integral membrane protein TM oligomerization, a concept that is applicable to many receptor signaling cascades, further broadening the potential applications of TM peptides as drug targets.

The data I presented here further demonstrates the potential of TM peptide drugs. When PIV5 was treated with TM peptide derived from PIV5 F, infection was reduced. I proposed that the TM peptide was able to insert into the viral membrane, wherein the TM peptide could then interact with the TM domain of the native fusion protein. The interaction of the TM peptide with the fusion protein may result in premature triggering or misfolding, therefore preventing membrane fusion and infection. As described earlier, peptides have their shortcomings in drug development, especially when there are hydrophobic residues present. In the infection experiments I performed, a large portion of the peptide aggregated and, as a result, was unlikely to integrate into the virions. The aggregation of peptide would need to be overcome for development of TM peptides as drugs. To aid in solubilization, the TM domain could be modified to include part of the upstream heptad repeat domain. This addition could provide a two-fold effect: the heptad repeat residues would increase solubility and it has been shown previously that the heptad repeat alone can be successfully targeted to prevent membrane fusion. Recent studies have started to build upon heptad repeat domain inhibitors by conjugating the peptide to
PEG or cholesterol. This conjugation with cholesterol targets the peptide to the membrane, further enabling the peptide to interact with the fusion protein. PEG, on the other hand, is used to aid in peptide solubility. Specifically, a group conjugated PEG to enfuvirtide and found that the conjugation increased half-life in a rat model when compared to the enfuvirtide alone (180). The heptad repeat domain upstream of the TM domain is a common target to prevent membrane fusion. Another study found that conjugating a heptad repeat peptide derived from HPIV3 with cholesterol enhanced antiviral potency (141). By instead adding a portion or the full length TM domain, the fusion protein could then be disrupted in two domains, further increasing the possibility of disrupting its function. Together, the data presented here further supports the potential of fusion protein TM-TM oligomerization as a target for preventing membrane fusion.
Figure 6.1. Model for TM peptide interaction with fusion protein.
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Max Steckler Award, UK ................................................................. April 2013
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PUBLICATIONS


**Presentations**


Oral Presentation, “Viral Fusion Protein Transmembrane Domains: From Basic Biophysics to Potential Therapeutics,” Indiana-Kentucky Border Section of the American Chemical Society Award Lecture, Kentucky Wesleyan College, April 2016.


Poster Presentation, “The fusion protein of Hendra virus contains a leucine-isoleucine zipper in the transmembrane domain that is important for association,” ASBMB, April 2013.
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