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**UNDERSTANDING THE MECHANISM OF PROTEIN-MEDIATED FUSION
THROUGH STRUCTURE/ FUNCTION ANALYSIS OF A NONENVELOPED
VIRUS-ENCODED 'MINIMALISTIC' FUSION PROTEIN**

by

Maya Shmulevitz

**Submitted in partial fulfillment of the requirements for the degree of doctor of
philosophy in microbiology.**

at

**Dalhousie University
Halifax, Nova Scotia
December 2001**

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- ❖ Chapter 1 is modified from results published in EMBO Journal (2000) 19:902-912.

- ❖ Parts of research completed for chapter 3 was done in collaboration with Dr. Richard Epand (Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5. epand@mcmaster.ca) including liposome fusion assays and CD spectrum analysis.

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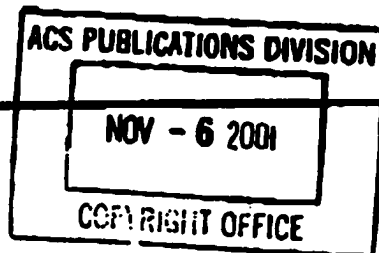
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ABSTRACT

Avian and Nelson Bay reoviruses are rare examples of nonenveloped viruses that encode a membrane fusion protein (p10). P10 is an integral membrane, type I, surface localized nonstructural protein. The life cycle of reoviruses and the unique characteristics of p10 suggest that unlike other biological membrane fusion proteins, p10-induced membrane fusion may not require regulation, specificity, rapidity or efficiency. Liposome fusion assays, structural studies, and mutational analysis show that p10 contains an internal fusion peptide with a beta-strand rich disulfide-bonded loop structure. Similar to the enveloped virus fusion proteins, the fusion peptide is proposed to promote fusion through destabilizing interactions with the target membrane. The p10 fusion peptide is directly responsible for rapid proteasome-dependent intracellular degradation of p10 suggesting that it is exposed upon p10 expression. The small size of the p10 ectodomain, the absence of heptad repeats shared by most enveloped virus- and intracellular vesicle fusion proteins, and the exposure of the p10 fusion peptide support that regulated conformational changes commonly associated with the activation of other biological fusion proteins are not necessary for p10. Mutational analysis shows that the glycine-rich transmembrane domain, intracellular palmitoylated cysteines and basic region are directly involved in the fusion process. That alterations to donor bilayer lipid packing favor membrane merger is a newly emerging concept. P10 also appears to exist as a monomer unlike all other oligomeric biological membrane fusion proteins. To explain the unique characteristics of p10, we propose that p10 is expressed in the fusion competent conformation. Our analysis of p10 may allow the minimal determinants for membrane fusion to be deciphered from auxiliary regulatory components found in other biological membrane fusion proteins.

LIST OF ABBREVIATIONS USED

2HAC	p10 with 2 HA epitopes at the C-terminus
2HAN	p10 with 2 HA epitopes at the N-terminus
2HANE	the ectodomain of p10 with 2 HA epitopes at the N-terminus
ARV	avian reovirus
ASLV	avian sarcoma/leukosis virus
ASLV	avian sarcoma-leukosis virus
BFA	brefeldin A
BRV	baboon reovirus
CD	circular dichroism
DIG	detergent insoluble glycolipid-rich membrane
EBV	Epstein Barr virus
ERAD	endoplasmic reticulum associated degradation
F	fusion
FAST	fusion associated small transmembrane proteins
GEM	glycolipid-enriched membranes
GP	glycoprotein
GPI	glycosylphosphatidylinositol anchor
HA	hemagglutinin
HBSS	Hanks balanced salt solution
HIV	human immunodeficiency
HN	hemagglutinin-neuraminidase
HTLV	human T-cell leukemia virus

LCMV	lymphocytic choriomeningitis virus
LPC	lysophosphatidylcholine
MRV	mammalian reovirus
NBV	Nelson Bay virus
NDV	Newcastle disease virus
OA	oleic acid
PEG	poly(ethylene glycol)
QM5	quail fibroblast cell line
RBC	red blood cells
RSV	respiratory syncytial virus
SFV	Semliki forest virus
SIN	Sindbis virus
SIV	simian immunodeficiency virus
SRV	snake reovirus
SV5	simian virus 5
TBE	tick-borne encephalitis virus
TM	transmembrane
VSV	vesicular stomatitis virus

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**INTRODUCTION: Characteristics of enveloped virus fusion proteins,
membrane fusion, and reovirus-encoded p10**

I. INTRODUCTION

My thesis describes the identification and structure/ function analysis of a novel membrane fusion-inducing proteins (p10) encoded by the nonenveloped avian reovirus and Nelson Bay reoviruse (ARV and NBV, respectively). Although research on membrane fusion proteins has proceeded for over 30 years, investigations have focused on the fusion proteins of enveloped viruses. P10 is the first example of a membrane fusion protein characterized from nonenveloped viruses. P10 is extremely small, bearing only 40 ectodomain, 20 transmembrane, and 40 intracellular residues. In comparison to the enveloped virus fusion proteins, p10 appears relatively simple in domain organization and potential for secondary structure. My thesis will attempt to demonstrate the unique characteristics of p10, and hopefully demonstrate how p10 will continue to contribute to the understanding of protein-mediated membrane fusion mechanisms.

To appreciate the uniqueness of p10, one first must be familiar with the present concepts of membrane fusion, and the complexities of enveloped virus fusion proteins. Thus, this introduction provides a comprehensive summary of the enveloped viruses, the characteristics of their fusion proteins, and the rationale for why these characteristics have evolved. By comparing the life cycle of enveloped versus nonenveloped viruses, it becomes evident that enveloped virus fusion proteins have more limitations / restrictions on the components necessary for membrane fusion function and its regulation as compared to ARV and NBV

p10. Many of the components of enveloped virus fusion proteins, which will be detailed in this introduction, are absent in p10 as detailed in the chapters that follow. The substantial knowledge obtained from studies on enveloped virus fusion proteins has permitted, by comparison, our investigations and ensuing understanding of p10. My thesis attempts to build on existing perceptions on the mechanisms of protein-induced membrane fusion. The introduction will summarize these existing suppositions and aid in understanding the implications of our findings related to p10.

I.A. OVERVIEW: MEMBRANE FUSION

The integrity of cellular membranes is pivotal to their function as compartmental barriers. The inherent structure of phospholipid bilayers and the physical properties of bilayer lipids impose a large energy barrier to the formation of fusion intermediates (Chernomordik and Zimmerberg, 1999, 1995; Siegel and Epanand, 2000, 1997; Siegel, 1999), and thus prevent spontaneous membrane fusion. Conversely, cellular fusion proteins facilitate membrane merger for essential processes including intracellular vesicle transport (Hanson, 1998; Jahn and Sudhof, 1999; Kondo et al., 1997), yeast mating (White and Rose, 2001), mitochondrial membrane fusion (Griparic and Blied, 2001) and the formation of zygotes, multinucleated myotubes and osteoclasts (Hernandez et al.; 1996 White, 1992). Furthermore, the entry of all enveloped viruses, including HIV, influenza, and other human pathogens involves membrane fusion induced by viral fusion proteins (Feldmann et al., 1999; Hernandez et al., 1996; Hoekstra

and Kok, 1989; Moore et al., 1993; Stegmann et al., 1989; White, 1990, 1992; Wiley and Skehel, 1987; Zimmerberg et al., 1993). Understanding the mechanisms of membrane fusion mediated by proteins from diverse sources has broad implications and is an important biological objective.

To date, the best-characterized fusion proteins are those involved in membrane fusion of vesicles and of enveloped viruses. Intracellular fusion is mediated by the SNARE (Soluble NSF Attachment protein REceptor) system. In addition to the integral membrane proteins found within the vesicle and target membrane (v-SNARE and t-SNARE, respectively), the SNARE system consists of many additional components thought to regulate the fusion process through protein-protein interactions and their corresponding conformational changes (Kondo et al., 1997; Rothman, 1994).

In comparison to intracellular fusion events, the fusion systems of enveloped viruses appear more simplified, usually consisting of a single integral membrane protein that becomes activated through conformational changes induced by specific environmental triggers such as low pH or receptor binding (Skehel and Wiley, 2000; Eckert and Kim, 2001). Membrane seeking amphipathic sequences called fusion peptides become exposed during fusion protein activation, and insert into target membranes. The lipid bilayer-destabilizing characteristics of fusion peptides favor the fusion of lipid bilayers (Colotto and Epand, 1997; Davies et al., 1998; Epand, 1998; Zimmerberg et al., 1991; Gray and Tamm,

1998; Han et al., 1999, Durrell et al., 1997; Tatulian and Tamm, 2000; Luneberg et al., 1995). In the simplest model for membrane fusion mediated by enveloped viruses, fusion peptide-membrane interactions are primarily responsible for membrane fusion.

The membrane fusion field is enormous and almost overwhelming, with opinions and evidence that suggest many alternative models (Example reviews; Dutch et al., 2000; Eckreck and Kim, 2001; Skehel and Wiley, 2000; Hernandez et al., 1996). The fusion proteins of enveloped viruses are extremely large (Figure 1.1), with the fusion peptide only representing approximately 20-40 residues. The remainder of the fusion proteins are involved in oligomeric interactions, conformational changes, and receptor binding roles. A clear picture of the minimal requirements for membrane fusion is blurred by the complexities of enveloped virus fusion proteins. To better appreciate this point, the next section attempts to describe the fusion proteins of enveloped viruses, their common characteristics, and the roles they must fulfill during the viral life cycle. A thorough understanding of the current models of protein-mediated membrane fusion will aid in appreciation of the unique characteristics of ARV and NBV-encoded p10. Chapters that follow this introduction will attempt to adapt the present models of enveloped virus fusion protein function to our novel findings on p10.

I.B. OVERVIEW: THE CHARACTERISTICS OF ENVELOPED VIRUS FUSION PROTEINS.

At best two steps are required for the fusion process. First, the opposing membranes must be brought into close contact. Despite the van der Waals attractive force between phospholipid bilayers, repulsive electrostatic interactions and hydration layers produce a substantial free energy barrier that prevents close membrane apposition (Wilschut and Hoekstra, 1984). Negatively charged phospholipids such as phosphatidylserine, found within biological membranes at 8-10% of the total lipid composition, produce electrostatic repulsion between membranes. Cations such as calcium can, therefore, function as fusogenic agents that promote membrane fusion by reducing ionic repulsion. The binding of water by all phospholipids creates a layer of water at the bilayer surface that must be eliminated for direct membrane contact. The energetic cost of dehydration can be compensated by reagents that assist in the dehydration step such as polyethylene glycol and dextran, or phospholipids which are less hydrating such as phosphatidylethanolamine.

Enveloped viruses contain receptor-binding domains either within their fusion protein or in separate proteins that permit initial interbilayer contact between the virus envelope and target cell. As will be discussed in detail, some fusion proteins also undergo conformational changes that decrease the distance between fusion peptide and transmembrane domains thereby bringing the opposing membranes into closer apposition.

The second requirement for fusion is some local packing defect in the bilayers to encourage intermembrane hydrophobic interactions. The greatest energy requirement for fusion is proposed to be the destabilization or disruption of apposed monolayers, which requires overcoming of the hydrophobic and bilayer curvature effects (Siegel, 1993a, b; Chernomordik et al., 1995). The hydrophobic effect refers to the thermodynamic drive for lipid conformations in which contact between the nonpolar portions of the lipids and water is minimized. Since nonpolar hydrocarbons cannot participate in hydrogen bonds with water, water molecules cannot favorably pack around lipid fatty acyl chains and are preferentially excluded. The hydrophobic effect is the major driving force stabilizing hydrated lipid aggregation. The curvature effect refers to the molecular shapes of lipids and critical packing that ensures optimal hydrophobic contact while maintaining correct cell shape. In simple terms, biological membranes require slight positive curvature, which is attained by incorporating (in addition to the cylindrical lipids) inverted conical shaped lipids into the outer leaflet, and conical lipids into the inner leaflet.

Hydrophobic, membrane-seeking sequences called fusion peptides common to all enveloped virus fusion proteins play an indispensable role in the fusion process. Fusion peptides insert into target cell membranes and cause local changes to lipid curvature and packing that destabilize the lipid bilayer and make membrane fusion more thermodynamically favorable.

In addition to the requirements for the fusion of lipid bilayers, the enveloped virus fusion proteins require methods of ensuring that the fusion process is specific and highly regulated. The fusion process must occur with the correct target membrane to ensure that the virus enters the correct cell. Furthermore, the fusion proteins must be regulated to ensure that fusion occurs at the right time. Regulation of timing ensures that fusion proteins do not prematurely function while expressed within infected cells, but only once incorporated into the envelope of viruses. Furthermore, regulated timing ensures that fusion occurs with the surface or endosomal membrane. In addition to specificity and regulation, the fusion proteins of enveloped viruses must function rapidly and efficiently. Viruses are in constant war with the immune system of their host, and cannot afford to remain bound to cells without fusion. Fusion must be efficient to ensure that the target cell is not damaged due to leakage. Enveloped viruses cannot tolerate a fusion process that requires superfluous time for accumulation and function of their fusion proteins. Thus, in addition to evolving the methods to juxtapose and destabilize membranes, enveloped virus fusion proteins have evolved elaborate mechanisms to confer specificity, regulation, rapidity, and efficiency.

I.B.1. Bilayer destabilization by fusion peptides

Using sequence and mutational analysis, fusion peptides of many enveloped virus fusion proteins have been identified. The location of the fusion peptide

differs amongst the fusion proteins of enveloped viruses. Fusion peptides located at the N-terminus of the transmembrane subunit (N-terminal) are found in most orthomyxoviruses, paramyxoviruses, and some retroviruses (White et al., 1983; Blumberg et al., 1985; Gething et al., 1986; Gallaher, 1987; Bosch et al., 1989). Internal fusion peptides have been found in the membrane-anchored subunits of Rous sarcoma virus (Hunter et al., 1983), vesicular stomatitis virus (Whitt et al., 1990), Ebola virus (Gallaher, 1996), and murine coronavirus (Luo and Weiss, 1998). Other viruses with N-terminal or internal fusion peptides are discussed in the sections to follow.

The characteristics of fusion peptides are summarized in the table below. N-terminal fusion peptides are only produced when precursor fusion proteins are cleaved into two subunits directly adjacent to the fusion peptide sequence. Internal fusion peptides have been identified within fusion proteins that are, and are not, proteolytically processed.

Characteristic	N-terminal fusion peptides	Internal fusion peptides
Requires cleavage?	Yes	Yes or No
Length	20-36	16-20
Structure	Mostly helical	Mostly looped
Amino-acid composition	Mostly apolar, G, A, P	Mostly apolar, G, A, P

The most convincing confirmation of the fusion peptide location is achieved using hydrophobic photolabeling techniques, which involve radioisotope-containing hydrophobic molecules incorporated into the membrane that can photochemically convert into reactive species and covalently bind peptides that are inserted within

the bilayer. Photolabeling also permits determination of conformation and depth of membrane insertion (Harter et al., 1989; Brunner 1989). The photolabeling experiments have demonstrated that fusion peptide insertion into the target membrane is a distinct step that precedes membrane fusion (Stegmann et al., 1991; Pak et al., 1994).

The ability of synthetic peptides corresponding to predicted fusion peptide sequences to induce fusion of liposomes has also become a standard assay for locating fusion peptides. The effects of amino acid substitutions within the synthetic peptides on liposome fusion correspond to the effects found when identical changes are made to the whole proteins and tested on biological membranes. Furthermore, the synthetic peptides respond to changes in environmental conditions such as pH similar to the whole fusion proteins. These observations have increased the validity of the liposome assay for use to identify fusion peptides (Freed et al., 1992; Pereira et al., 1995; Martin et al., 1996; Kliger et al., 1997; Pritsker et al., 1999; Ghosh and Shai, 1999; Wharton et al., 1988; Pereira et al., 1997). The liposome fusion assay has become a popular functional assay to show that synthetic peptides modeled according to predicted fusion peptides will: 1. associate with membranes; 2. cause changes to the lipid packing; and 3. induce membrane fusion. The ability of fusion peptides alone to induce membrane merger of liposomes substantiates their essential involvement in the fusion process catalyzed by whole enveloped virus fusion proteins.

The function of fusion peptides is believed to be dependent on hydrophobicity, amino acid composition, secondary structure, and orientation of insertion into the target membrane (Delahunty et al., 1996). The hydrophobicity must be sufficient to confer membrane-seeking qualities that would favor the spontaneous insertion of fusion peptides into lipid bilayers. The amino acid composition of fusion peptides, often rich in glycines and alanines in addition to other apolar residues, influences secondary structure, protein-protein interactions, distribution of charge and bulkiness, and would clearly influence the specific interactions between the fusion peptide and membranes. Glycine residues are found at an approximate spacing of every four residues within many fusion peptides and may be important in producing a 'glycine face' that would alter peptide shape, protein-protein interactions, or protein-lipid interactions. The fusion proteins of tick-borne encephalitis virus (TBE) and influenza share a tetrapeptide sequence, GLFG, which was shown to be critical for membrane fusion by mutagenesis (Qiao et al., 1999; Steinhauer et al., 1995). A phenylalanine-glycine pair is also found in almost all fusion peptides of enveloped virus fusion proteins (Durrel et al., 1997; Pecheur et al., 1999; Rodriguez-Crespo et al., 1995). The N-terminal fusion peptides can be modeled as β -strands or α -helices with one very hydrophobic face, and the conformation appears to be important in their interactions with membranes (Nieva et al., 1994; Gray et al., 1996). Extensive studies have confirmed that the hydrophobicity, amino acid composition and structure of N-terminal fusion peptides are essential for destabilizing effects on lipid bilayers. A

thorough discussion on these characteristics of N-terminal fusion peptides is provided in reviews by Martin et al. (1999), Fujii (1999), and Durell et al. (1997).

Oblique orientation of fusion peptides with respect to the plane of the membrane has been predicted to contribute to the membrane destabilizing qualities of fusion peptides. Synthetic peptides made according to the fusion peptides of influenza (Tatulian et al., 1995), human immunodeficiency (HIV-1) (Pritsker et al., 1999), simian immunodeficiency virus (SIV) (Martin et al., 1993), Sendai virus (Rapaport and Shai, 1994), bovine leukemia virus (Voneche et al., 1992), Newcastle disease virus (Brasseur, 1991) and measles virus (Brasseur et al., 1990) insert into lipid bilayers in an oblique orientation. The oblique angle is predicted to expand the interior of the bilayer relative to the bilayer surface, to disturb the lipid packing order, and to promote increased negative curvature of the outer leaflet that is required for membrane merging (Epanand et al., 1994).

Another suggestion is that oligomerization of fusion peptides within penetrated membranes is important for their activity (Kliger et al., 1997). Oligomerization of membrane anchored fusion peptides was, however, shown to be dispensable for membrane fusion in separate investigations (Pecher et al., 1999). The fusion peptide of the influenza HA2 subunit also appears to exist as a monomer within the membrane (Macosko et al., 1997).

Like N-terminal fusion peptides, internal fusion peptides are short, hydrophobic and membrane seeking. Internal fusion peptides share similar characteristics such as composition and proposed activity on target membranes (White, 1992). Unlike the N-terminal fusion peptides, loop structures rather than α -helical or β -strand structures are common with internal fusion peptides. The internal fusion peptides within the fusion proteins of TBE and avian sarcoma-leukosis virus (ASLV) were found to have a disulfide-stabilized loop structure by crystallography and mutational analysis, respectively (Allison et al., 2001; Delos et al., 2000; Hernandez and White, 1998; Delos and White, 2000). Proline residue(s) located near the middle of internal fusion peptides appear essential for vesicular stomatitis virus (VSV) G (Fredericksen and Whitt, 1995; Zhang and Ghosh, 1994), semliki forest virus (SFV) E1 (Levy-Mintz and Kielian, 1991), Ebola virus GP (Ito et al., 1999), and avian leucosis and sarcoma virus TM subunit (Hernandez and White, 1998). Glycine residues have also been demonstrated as essential for the function of the fusion peptides of Ebola virus (Ito et al., 1999), VSV (Fredericksen and Whitt, 1995; Zhang and Ghosh, 1994), and SFV (Levi-Mintz and Kielian, 1991). The proline and glycine residues may function to promote loop formation, similar to the proposed 'shape-determining' role of glycine residues in N-terminal fusion peptides. In comparison to N-terminal fusion peptides, studies on internal fusion peptides are relatively premature. Future studies will hopefully combine the understanding of N-terminal and internal fusion peptides to decipher the mechanism by which fusion peptides induce bilayer disruptions.

I.B.2. Transport of enveloped virus fusion proteins to the cell surface

Enveloped viruses are faced with a unique predicament with respect to the efficient expression and trafficking of fusion proteins. The fusion proteins of enveloped viruses must remain non-functional until they are within the viral envelope and exposed to the correct conditions supporting entry and infection. Yet, both low pH and cellular receptors are encountered during export to the cell surface, triggers that could result in premature fusion activation. To avoid this, enveloped viruses have developed strategies to maintain their fusion proteins in fusion incompetent states during intracellular transport.

The influenza virus encodes a membrane channel producing protein (M2) that prevents acidification of the Golgi compartment (Sugrue et al., 1990). Furthermore, similar to the human immunodeficiency virus (HIV) fusion glycoprotein and other cleaved fusion proteins (Figure I.1), the influenza HA undergoes proteolytic cleavage late during intracellular transport. Only after the production of the two subunits (HA1 and HA2) is the fusion potential of HA acquired. The rhabdovirus G protein does not undergo cleavage and instead, has evolved a different strategy to prevent early stimulation. A reversible inactive state is conferred to the G protein during export. The togaviruses and flaviviruses export the fusion protein in association with another protein partner that prevents the homo-oligomerization required for fusion activity. In all of these

examples, since the fusion proteins are absolutely essential for the entry and propagation of enveloped viruses, premature inactivation is prevented. Thus, the life cycle of enveloped viruses has imposed additional requirements on protein composition during the evolution of enveloped virus fusion proteins.

I.B.3. Binding and fusion activating triggers: regulation and specificity of membrane fusion.

Biological membrane fusion must be rapid and highly selective. Efficient membrane fusion must also occur without leakage of contents from within the membrane compartments. For these restrictions to be met, biological fusion must be tightly regulated. For enveloped viruses, regulation of the fusion process is essential to ensure that fusion proteins do not undergo premature activation prior to virus assembly and budding, and to ensure that membrane fusion occurs only between the virus envelope and the correct target cell. Premature activation would irreversibly inactivate glycoproteins leading to the production of non-infectious virus particles. Furthermore, activation would expose hydrophobic domains such as the fusion peptide that would cause aggregation in the absence of target membranes. On the other hand, these fusion proteins must simultaneously rapidly respond to proper stimuli for entry into the correct host cells.

The glycoproteins present within the lipid envelope of viruses must fulfill at least two requirements to permit entry through membrane fusion, binding and

regulated fusion (Eckert and Kim, 2000 and references within). Glycoproteins undergo high affinity and specific binding to cells through numerous non-covalent interactions. Viruses vary in their use of receptors, which include protein molecules (such as CD4 for HIV, and the C3Dd complement receptor for Epstein Barr virus (EBV)), carbohydrates (such as gangliosides for Sendai virus, sialic acid for influenza, and heparin sulfate for herpes simplex virus) or glycolipids or phospholipids (such as phospholipids for rabies virus). The choice of molecule used for virus binding is important as it confers tissue and species tropism and internalization of virus through endocytosis. Binding is essential for ensuring specificity of the fusion process. Furthermore, for some viruses, binding provides the trigger for fusion activation.

For viruses that enter through the endocytic pathway, binding to receptors plays a role in determining specificity for the target membrane, while low pH functions to regulate the activity of the fusion proteins by inducing structural changes. When TBE, influenza, SFV, sindbis virus (SIN), and vesicular stomatitis viruses are artificially exposed to low pH, their fusion with target membranes does not require the presence of a protein or carbohydrate receptor binding (Stegmann et al., 1987, 1995; Bron et al., 1993; Nieva et al., 1994; Moesby et al., 1995; Corver et al., 1997, 2000; Smit et al., 1999; Moor et al., 1999). In biological systems however, encountering of the low-pH environment through endocytosis is contingent on previous binding to the target cells and ensures specificity of target membrane prior to activation of membrane fusion. In contrast, some viruses

such as paramyxoviruses, herpesviruses and retroviruses do not depend on acidic environments and are capable of entry through membrane fusion with the host cell membrane. For these viruses, attachment to the receptor ensures specificity for the target membrane but also regulates the activating structural changes in the fusion protein necessary to initiate the fusion event.

For regulated fusion, fusion proteins must be capable of assuming both inactive and activated states, depending on the presence of correct environmental triggers. To accomplish this, enveloped virus fusion proteins take on at least two states. Triggers for the fusion process such as low pH and cell attachment promote conformational changes that culminate with the formation of the fusion active state that induces membrane merger. In the kinetic model, the primary inactive state is metastable and the final state is significantly more stable. The final state or an intermediate state would be active in induction of lipid bilayer fusion. Upon triggering, the metastable state would naturally refold into more stable conformations through a 'spring-loaded' mechanism. The kinetic model can be broken up into the following steps and requirements:

1. Folded fusion proteins are often modified by either proteolysis or partner protein binding to trap the fusion proteins in a metastable inactive state that would optimally refold into a stable active state upon triggering. Denaturing reagents or heat can, therefore, mimic the effects of the biological fusion trigger by inducing refolding into the more stable state.

2. **Specificity permits fusion at the correct time and with the correct target membrane. Domains that bind host cell receptors help determine specificity, and are found either integrated into the fusion glycoprotein or as a separate glycoprotein.**

3. **Upon binding and, for some viruses, low pH following endocytosis, a conformational change takes place that results in the exposure of a fusion peptide. Fusion peptides participate in the fusion process by binding to, and destabilizing donor and/or target membranes.**

4. **Conformational changes culminate with the formation of the final stable state.**

The formation of more stable conformations or complexes is supported by the finding that elevated temperatures and other denaturants are capable of inducing conformational changes that create the final stable form associated with the activated fusion protein. This has been demonstrated for the influenza HA and for several members of the paramyxoviridae family (Carr et al., 1997; Baker and Agard, 1994; Wharton et al., 2000; Paterson et a., 2000).

In contrast, the thermodynamic partitioning model suggests that the primary state is more stable than other possible conformations. Upon changes to amino acid

residue charges induced by acidic conditions, however, the original state is no longer stable and new, fusion active states are promoted.

In addition to being necessary for the regulation of fusion proteins, conformational changes are believed to be thermodynamically coupled to the fusion reaction. The energy that is released during transition to more optimal states is thought to drive the unfavorable lipid bilayer changes necessary for membrane merger.

In conclusion, the enveloped viruses must encode fusion proteins that are capable of fulfilling multiple requirements. The fusion proteins must be expressed in inactive states to ensure that they are not exhausted prior to incorporation into budded particles. Not only must they be made in inactive conformations, but they must be capable of withstanding conditions that would otherwise trigger their activity. As examples, fusion proteins of enveloped viruses are made as precursor proteins or as inactive oligomers until they are correctly incorporated into the budded virus. Once within the envelope of extracellular viruses, the envelope proteins must bind specifically to the correct target membrane. This step gives specificity to fusion, but also permits initial close approach of donor and target membranes. Binding could then trigger the fusion process, or promote receptor-mediated uptake into endosomes where low pH could initiate the fusion events. When triggered, the fusion proteins must be capable of undergoing changes in conformation, oligomerization, etc., to the

active state. The active state must perturb the lipid bilayers and promote the unfavorable curvature and lipid packing intermediate structures. The fusion peptide is a common motif known to interact with and destabilize membranes, and is sufficient to induce membrane fusion in liposome systems. Whether fusion peptides are sufficient in biological fusion is questionable. The current analysis on enveloped virus fusion proteins focuses on whether additional attributes of different fusion protein conformations participate directly in the fusion event.

Figure 1.1 details the characteristics of different enveloped virus families. The sections that follow provide details about the fusion proteins of each family. In reiterating the general considerations mentioned above, I hope to present a clear picture of the enveloped virus fusion proteins and their mechanisms.

I.C. THE PROTOTYPICAL INFLUENZA HA AND OTHER SIMILAR FUSION PROTEINS.

Members of the orthomyxovirus, paramyxovirus, filovirus, retrovirus, arenavirus and coronavirus families share more common similarities within their fusion proteins than other enveloped virus families. They all encode trimeric glycoproteins, some of which initiate as precursors that require proteolytic cleavage to acquire membrane fusion potential. In the fusion proteins representative of each family, activation results in structural changes that expose

the previously buried fusion peptide and culminate with the formation of an α -helical trimeric coiled-coil trimer-of-hairpins (or six-helix bundle).

I.C.1. Trimer-of-hairpins structure

The formation of the stable trimer-of-hairpins conformation has recently become the predominant focus of many laboratories that study enveloped virus fusion proteins. This final conformation consists of antiparallel α -helical coiled-coils that position the membrane anchor and fusion peptides at the same end of the protein (Skehel and Wiley, 2000). Since the fusion peptide inserts into the target cell membrane while the transmembrane domain remains anchored in the donor membrane, the formation of the hairpin structure (with the N-terminal fusion peptide and C-terminus brought close to one another) would function to pull the donor and target membranes into close apposition (Weissenhorn et al., 1997; Hughson, 1997; Furuta et al., 1998; Chan and Kim, 1998). A cartoon depiction of the coiled-coil complexes formed by different viral family representatives was taken with permission from Eckert and Kim (2001) (Figure I.2A). A cartoon portrayal of the folding events predicted to follow fusion peptide insertion into the target membrane and the formation of the helical bundle is provided in figure I.2B. The following is a summary of enveloped viruses found to adopt the trimer-of-hairpins structure.

FAMILY +/- (GENUS)	VIRUS	REFERENCE(S)
Filovirus	Ebola	Weissenhorn et al., 1998
Retrovirus (Oncoretroviruses)	Mo-MLV and HTLV-1	Fas et al., 1996; Kobe et al., 1999
Retrovirus (Lentiviruses)	HIV-1 and SIV	Chan et al., 1997; Malashkevich et al., 1998; Weissenhorn et al., 1996, 1997; Tan et al., 1997; Caffrey et al., 1998
Paramyxovirus	SV5	Joshi et al., 1998, Baker et al., 1999
Orthomyxovirus	Influenza	Bullough et al., 1994; Chen et al., 1995, 1999

The LEARNCOIL-VMC computer program for detection of coiled-coil like regions has detected a large number of viral membrane fusion proteins with the potential to form trimer-of-hairpins structures (Berger and Singh, 1997). The SNARE proteins responsible for intracellular membrane fusion also contain coiled-coil structures that appose donor and target membranes (Sutton et al., 1998; Weber, 1998; Lin and Scheller, 1997; Hanson et al., 1997).

The trimer-of-hairpins structure is presumed to correspond to the final and most stable conformation that follows activation by triggers such as receptor binding or low pH (Weissenhorn et al., 1998). This assumption is based on comparison to the influenza HA for which the native state is known. Several fusion proteins were shown to be activated by heat, which could induce the conformational changes in the absence of the biological trigger. The thermostable conformation is, therefore, presumed to represent the final fusion protein state. It is distinct from the native metastable and nonfusogenic state.

The formation of coiled-coil structures was demonstrated to be essential for the fusion event. Mutations made within the heptad repeats of the gp41 of HIV, the F1 subunit of Newcastle disease virus (NDV), the GP2 subunit of Ebola virus and the S protein of murine coronavirus resulted in a loss of fusion (Bernstein et al., 1995; Cao et al., 1993; Chen et al., 1993; Chen, 1994; Dubay et al., 1992; Luo et al., 1999; Pountourios et al., 1997; Reitter et al., 1995; Weng and Weiss, 1998; Itoh et al., 1999; Watanabe, 2000). Peptides corresponding to the C-terminal and N-terminal helices involved in formation of the trimer-of-hairpins are also effective at blocking the fusogenic activity of Ebola virus GP2, HIV gp41, and the fusion protein (F) of several members of the Paramyxoviridae family (Chan et al., 1998; Jiang et al., 1993; Lambert et al., 1996; Rapaport et al., 1995; Wild et al., 1992; Yao and Compans, 1996; Kilby et al., 1998). These experiments confirmed that the exogenous peptides and/or amino acid substitutions did not prevent proper oligomerization of the fusion proteins. Figure 1.2B demonstrates how amino acid substitutions in- and peptides that bind- the heptad repeats would interrupt the association between C-terminal and N-terminal heptad repeats and the subsequent formation of the trimer-of-hairpins.

The helical coiled-coil bundles found within the different viruses do exhibit some variation. The loop between the N- and C- terminal heptad repeats formed by the antiparallel coiled-coils varies dramatically in size and composition. As an example, figure 1.2A shows that the intervening sequence between the heptad repeats is substantially longer in paramyxoviruses as compared to the remaining

viruses. The significance of the loop region for the different viruses remains to be assessed. Another difference is the length of the heptad repeats/ α -helices themselves. Again, figure 1.2A shows the gross variation in the length of the coiled-coils. Furthermore, some helical bundles are terminated by a 'cap' structure, designed to stabilize the terminal three-peptide amines that do not have hydrogen bond partners within the helix. In the cap structure, side chains from other amino acids provide the hydrogen bond partners. Interestingly, these interactions not only fix the N- and C- terminal coiled coils together, but also create bonds between oligomer subunits. The final distinction between the helical bundles of different virus families is the length of the spacer between the α -helices and the membranes. A long spacer would suggest flexibility between the rigid coiled-coils and the membrane imbedded fusion peptides and anchors. The flexibility may be important, perhaps to permit free peptide rotation within the bilayer and enhanced lipid rearrangements. On the other hand, the flexibility may suggest that linkage to the α -helices is unnecessary and that the fusion peptide and transmembrane anchor are functional alone. Short spacers suggest that a rigid linker may transfer the forces exerted during coiled-coils formation directly to the membrane, by 'tugging' on the anchor and fusion peptide. These specific differences will be highlighted in the sections to follow that discuss individual viral families.

Because variations do exist amongst the helical bundle structures of different viruses, there remains significant controversy as to the exact function of the

trimer-of-hairpins motif. Is the role of the helical bundle simply to bring the membranes into close apposition? Does it function to move the receptor-binding domain away from the fusion site? Does the conformational change to stable structures provide energy essential for the fusion reaction? Is the helical bundle conserved amongst many viral families because it is essential, or because it is highly efficient? Is the structure necessary at all, or would it suffice to express fusion peptide and transmembrane domain joined by a short linker to bring membranes together? Is it necessary for bilayer fusion, per se, or for regulation of fusion by permitting rapid, favorable and irreversible conformational change?

Interestingly, the formation of coiled-coil structures between the vesicle and target membrane SNARE proteins is also necessary for intracellular vesicle fusion (Skehel and Wiley, 1998; Hanson et al., 1997; Sutton et al., 1998; Poirier et al., 1998). In the case of SNAREs, four helices contribute to the stable bundle. The finding of coiled-coil structures in viral and cellular fusion mechanisms has led to the hypothesis that they are global motifs necessary to juxtapose the membranes. As we will see subsequently, the p10 proteins of ARV and NBV lack these coiled-coil structures implying that coiled-coils are not essential structures of all membrane fusion proteins.

The conformational transitions undertaken by these fusion proteins have created the foundation for current models describing the mechanism of viral membrane fusion (reviewed in Chan and Kim, 1998; Hernandez et al., 1996; Skehel and

Wiley, 1998). The general hypothesis is that the fusion protein six-helix bundle complex forms before membrane merger (Bullough et al., 1994; Chan et al., 1997; Weissenhorn et al., 1998b) or concomitant with the membrane merging (Baker et al., 1999). The formation of the highly stable six-helix bundle is speculated to overcome the forces that maintain two phospholipid bilayers apart including the curvature and hydrophobic effects (Carr and Kim, 1993; Bullough et al., 1994; Hernandez et al., 1996; Weissenhorn et al., 1997, 1998; Skehel and Wiley, 1998). This popular prediction, however, is refuted by the fact that the conformational changes that precede bundle formation (i.e. formation of the extended coiled-coil) release much more energy and should be expected to drive the reaction if energy released from conformational changes was optimally utilized (Bentz, 2000). The section that follows will describe the interpretations of studies on the prototypical influenza virus fusion protein. The existing models for enveloped virus-mediated fusion, including the role of the helical bundle, have sprung from investigations on influenza HA.

I.C.2. Orthomyxoviridae

The *Orthomyxoviridae* family includes the human influenza virus, the prototypic virus for studies on virus induced membrane fusion. Influenza contains a segmented ssRNA genome and is a rare example of an RNA virus that replicates within the nucleus of infected cells. Influenza virus enters cells through receptor-mediated endocytosis followed by low pH-induced fusion between the viral envelope and the endosomal membranes (Huang et al., 1981; Maeda and

Ohnishi, 1980; White et al., 1981). Influenza encodes one surface glycoprotein, the hemagglutinin (HA), responsible for attachment to- and fusion with host cells. HA forms trimers within the endoplasmic reticulum and undergoes standard delivery to the cell surface through the Golgi apparatus (Gething et al., 1986; Compeland et al., 1988; Hebert et al., 1995; Wilson et al., 1981).

The influenza HA is synthesized as a precursor, HA0, which is incapable of inducing membrane fusion even in the presence of low pH, the trigger for HA induced fusion. Cleavage of HA0 into HA2, the transmembrane subunit, and HA1, the receptor binding subunit, occurs at the cell surface or on released viruses (Figure 1.3A). The delayed proteolysis, required for producing functional HA molecules, ensures that the low pH of the Golgi apparatus does not prematurely trigger HA molecules. The HA molecules must remain functional to allow budded influenza particles to re-enter cells by fusion. The atomic structure of the uncleaved precursor HA0 (Chen et al., 1998) and the cleaved form consisting of HA1 and HA2 subunits at neutral pH (Wilson et al., 1981) have been confirmed. The proteolytic cleavage produces the N-terminal fusion peptide that buries into an adjacent cavity of charged and non-polar residues (Figure 1.3B, 1st conformation). The rearrangement produces HA molecules that are metastable and primed for activity. In the "spring-loaded" model of HA, cleavage creates intramolecular instability making HA molecules that are poised for conformational change under favorable conditions (Carr et al., 1997; Carr and Kim, 1993).

Critical irreversible conformational changes follow treatment with low pH (Figure I.3B, transition 1). A single-triple-stranded coiled-coil that includes two heptad repeats forms and relocates the fusion peptide to an exposed position towards the target membrane (Bullough et al., 1994; Chen et al., 1999). Once delivered to the target membrane, the fusion peptide can insert and induce local lipid destabilizing effects that would favor membrane merging (Figure I.3B, transition 2). Aggregation of several HA molecules are necessary for fusion, and will be discussed in further detail (Figure I.3B, transition 3). Presumably, the target and donor membranes are still too far from one another to promote efficient fusion, as a second refolding event occurs to bring them closer (Figure I.3B, transitions 4 and 5). The formation of the trimer-of-hairpins (or six helix bundle), the final and most stable conformation, functions to position the fusion peptide/target membrane near the membrane anchor/donor membrane and redirect the receptor-binding subunit (HA1) away from the fusion site (Weissenhorn et al., 1997).

Many studies support that the driving force for the conformational changes is the transition from metastable to stable states, prompted by conditions that further disfavor the metastable conformation. Studies on HA mutants that undergo pH-dependent conformational change at less acidic conditions than authentic proteins identified amino acid substitutions that would be expected to destabilize the pH 7 conformation (Daniels et al., 1984; Lin et al., 1997; Rott et al., 1984). The energy barrier for transition between the metastable native and stable final

conformations was reduced, permitting conformational change at increased pH values. Membrane fusion activity was also triggered by increased temperatures and chemical denaturants, again indicating that large changes in the stability of HA are provoked under protein structure destabilizing conditions (Ruigrok et al., 1986; Wharton et al., 1986; Car et al., 1997). Furthermore, when expressed in bacteria, the HA2 ectodomain devoid of the fusion peptide spontaneously folded into the trimer-of-hairpin structure even at neutral pH (Chen et al., 1995; Wharton et al., 1995). These studies confirm that the six-helix bundle is the lowest free-energy state of HA2. Interactions between HA1 and HA2 as well as the cleavage event create an energy trap that maintains HA within the metastable structure. Conversion from the metastable to the stable structure is presumed to involve intermediate conformations that are extremely unstable and, therefore, requires an activation energy that is lowered or overcome by decreased pH or increased temperature.

Several investigations suggest that multiple HA trimers cluster together to form a ring-like structure for the formation of a fusion pore (Danieli et al., 1996; Ellens et al., 1990; Betz, 1992, 2000; Blumenthal et al., 1996; Bentz et al., 1990; Zimmerberg et al., 1993). Studies that monitored the extent of fusion induced by varying surface densities of HA suggest that several HA trimers are necessary and that several fusion pores are opened during HA-mediated fusion (Dutch et al., 1998; Danieli et al., 1996). Furthermore, HA molecules at low pH appear as rosettes by electron microscopy (Fuigrok et al., 1992). Influenza HA-mediated

fusion, therefore, may necessitate the concerted effort of several trimers for fusion. Several predictions for the possible involvement of higher-order complexes have been proposed. Bentz (2000) suggests that upon triggering, the fusion peptides in aggregated HA trimers initially insert into donor membranes within the ring of HA transmembrane domains. The formation of the extended coiled-coil conformation that follows displaces several fusion peptides out of the donor membrane and into the target membrane and creates a hydrophobic void between the interacting transmembrane domains. The lipid flow between membranes is proposed to fill the hydrophobic void, resulting in fusion. The suggestion by Bentz (2000), if true, would prove an absolute requirement for protein aggregation during the fusion process. Apart from speculation, however, the role of HA trimer aggregation remains a mystery.

The hydrophobic void model described above is only one of several models suggested to describe the mechanism by which influenza HA induces lipid bilayer fusion. The "cast and retrieve" model identifies the shortening of the fusion peptide through secondary structure transitions as the driving force for membrane fusion (Zimmerberg et al., 1993). Another model suggests that fusion peptides function to dehydrate the space between opposing membranes during their exposure (i.e. by repelling water molecules) and thereby favor membrane merging (Bentz et al., 1990). Yet another model suggests that fusion peptide insertion into the donor membrane induces dimple formation that grows towards the target membrane until sufficient bending stress accumulates to favor

bilayer merger (Kozlov and Chernomordik et al., 1998). An alternative model suggests that fusion peptides alone function to overcome the barriers to fusion. The fusion peptides are the most important common denominator among enveloped virus fusion proteins (Hernandez et al., 1996). The binding of 8 to 16 fusion peptides would provide sufficient energy to stabilize membrane fusion process intermediates (Han and Tamm, 2000). This last model suggests that the fusion peptides, inserted deeply into the lipid bilayer of the target membrane in an oblique orientation during early stages of fusion (Durrer et al., 1996; Durell et al., 1997) perturb the lipid bilayer curvature and packing sufficiently to promote bilayer fusion despite the hydrophobic effect.

In addition to having an N-terminal fusion peptide, oligomeric structure, and conformationally distinct structures, the HA of influenza is palmitoylated at membrane proximal cysteines. The role of acylation in influenza HA-mediated fusion is controversial. Some studies have determined that acylation is not essential for fusion of HA-expressing cells with red blood cell ghosts or for syncytium formation (Philipp et al., 1995; Steinhauer et al., 1991). Other investigations have found that palmitoylation of HA is essential for various stages of the fusion process (Naeve and Williams, 1990; Fischer et al., 1998; Melikyan et al., 1997a). The controversy over the role of acylation for fusion protein function demonstrates further uncertainty with respect to the minimal requirements for protein-mediated fusion.

I.C.3. Paramyxoviridae

Measles virus, mumps virus, parainfluenza virus 2,3, and 4, Sendai virus, respiratory syncytial virus (RSV), simian virus 5 (SV5) and Newcastle disease virus (NDV) are members of the Paramyxoviridae family. The paramyxoviruses promote fusion at neutral pH allowing virus entry at the plasma membrane of cells (Stegmann et al., 1989). The fusion (F) proteins are suggested to have a trimeric structure based on chemical cross-linking analysis of paramyxovirus SV5 (Russel et al., 1994). The F proteins are synthesized as inactive precursors and require cleavage into two disulfide-linked subunits, F1 and F2 (Figure 1.4A) (Klenk and Garten, 1994). Interestingly, the F protein does not contain known receptor binding capability. In addition to the F protein, some paramyxoviruses encode a second viral glycoprotein, the hemagglutinin-neuraminidase (HN) protein or the hemagglutinin protein (HA) that is responsible for receptor binding (Hiebert et al., 1985), and a small hydrophobic (SH) protein that is dispensable for replication in tissue culture cells (He et al., 1998).

The requirement for HN during F-mediated fusion varies amongst the paramyxoviruses. For measles virus, human parainfluenza virus 3, Newcastle disease virus and mumps virus, both the homotypic HN protein and the F protein are required for fusion (Cattaneo and Rose, 1993; Ebata et al., 1991; Hu et al., 1992; Morrison et al., 1991; Tanabayashi et al., 1992; Wild et al., 1991). For these viruses, it is predicted that the binding of HN to sialic acid on the target cell

induces conformational changes that, through protein-protein interactions between HN and F, induce the conformational changes in the F protein necessary for fusion (Lamb, 1993; Sergel et al., 1993). The requirement of high surface densities of both F and HN proteins for efficient fusion suggest that they work cooperatively in the reaction (Dutch et al., 1998). The paramyxovirus SV5, however, can initiate fusion in the absence of HN and is hypothesized to be triggered by either close contact with the target membrane or binding to an unidentified receptor (Bagai and Lamb, 1995; Horvath et al., 1992; Lamb, 1993). The HN protein in SV5 only contributes to the fusion reaction by providing a binding function between membranes, as indicated by the independence of the extent of fusion on the amount of HN present and by an ability of other binding proteins to substitute for HN in promoting F-mediated fusion (Dutch et al., 1998). The slight enhancement of fusion in the presence of HN for SV5 suggests that HN may provide an optimal distance between target and donor membranes to permit favorable F protein interactions.

An interesting study compared the F proteins from two different strains of SV5, the HN-independent fusogenic W3A strain and the WR strain that required co-expression of HN for fusion. (Ito et al., 1997; Dutch et al., 1998). A single leucine to proline substitution in two possible locations within the WR F protein produced an HN-independent fusogenic protein (Figure 1.4)(Ito et al., 1997; Paterson et al., 2000). These studies help understand the transition involved in the conformational change from metastable to stable, active states. The

proline/leucine residues thought to be most essential are located within the F2 subunit (Figure 1.4A). The proline residues are predicted to destabilize the native conformation and make it more readily triggered for conformational changes that produce the stable, fusion active state.

Biochemical and structural studies of the SV5 F protein have confirmed the formation of the trimer-of-hairpins (Baker et al., 1999). Peptides corresponding to the N-terminal heptad repeat can form trimeric α -helical coiled-coil 'core' interactions, while added peptides that correspond to the C-terminal heptad repeats form antiparallel chains that surround the trimeric coil (Joshi et al., 1998; Dutch et al., 1999). The peptides confirm the formation of the six-helical bundle as a stable conformation that could locate the fusion peptide and transmembrane domain at the same end of the structure. Interestingly, the coiled-coil extends into the proposed fusion peptide region and suggests that flexibility between the hairpin and the membrane is not essential (Baker et al., 1999). The lack of flexibility may provide a means to couple the formation of the six-helix bundle with fusion by producing additional strain and negative curvature to the outer leaflet.

Fusion mediated by the SV5 F protein can be blocked by peptides that correspond to the heptad repeats (Joshi et al., 1998; Russel et al., 2001). Peptides that bind the N-terminal heptad repeat block fusion prior to the lipid mixing stage and suggest that a conformational change to produce an

intermediate or the six-helical bundle is necessary for hemifusion. Peptides that bind the C-terminal heptad repeat only block the steps after lipid mixing but before content mixing. Russel et al. (2001) suggest that the formation of the helical bundle is coupled directly to membrane fusion. One possibility is that the conformational change is necessary because it releases energy that is directed towards favoring fusion intermediates. On the other hand, the helical bundle may only serve to shorten the fusion protein thereby bringing the transmembrane domain and fusion peptide, and their respective membranes, in closer proximity.

The conformational changes undertaken by the F protein following activation have been successfully monitored with a panel of antipeptide antibodies (Dutch et al., 2001). These studies indicate that significant conformational changes within the heptad repeat regions are required for the transition from the precursor F protein to the six-helical bundle. By analogy to the influenza HA protein, the paramyxovirus F protein is predicted to exist in a metastable fusion-inactive state that maintains the fusion peptide buried (Baker et al., 1999; Lamb, 1993). The F protein is predicted to undergo conformational change to expose the fusion peptide. These refolding events that also involve the formation of the six-helical bundle are predicted to produce the fusion active form.

Similar to influenza, fusion by Sendai virus with liposomes (devoid of receptor proteins) can be induced by increased temperatures (Wharton et al., 2000). At an optimal temperature of 55°C, the F protein undergoes conformational change

as indicated by changes in susceptibility to proteolysis, is inactivated irreversibly in the absence of target membrane, and is capable of inducing membrane fusion suggesting that low pH and elevated temperature are interchangeable triggers for F protein function. These findings support that activation of fusion requires a conformational change from a metastable structure generated by precursor cleavage, to a stable trimer-of-hairpins structure.

Studies that monitor the progression of fusion under varying amounts of F protein suggest that multiple fusion pores are open as F protein concentrations and fusion rate increase (Dutch et al., 1998). Furthermore, similar to HA, it is suggested that accumulation of multiple F trimers are required to promote fusion, a process that requires time and results in the lag prior to initiation of fusion (Aroeti and Henis, 1991; Dutch et al., 1998). Interestingly, a low concentration of F protein on the surface results in an absence of fusion and cannot be compensated by increased time. This suggests that accumulation of active F proteins must occur rapidly and, therefore, that the intermediate fusion stages are not stable for long periods. The 'all or nothing' scenario suggested by Dutch et al. (1998) states that the initial fusion active conformation formed after fusion protein triggering is not tolerated for extended time and will quickly turn into the inactive final conformation if accumulation and fusion do not occur immediately. This scenario may provide an interesting 'safeguard' to erroneous fusion events and may be advantageous to an enveloped virus that would not desire an

inappropriately triggered conformational change to a single F protein to result in fusion.

The paramyxoviruses are proposed to have two fusion peptide sequences, a unique characteristic when compared to other fusion proteins. The newly formed hydrophobic N terminus, following cleavage of F into the F1 and F2 subunits, has been demonstrated to function as a fusion peptide (Gething et al., 1978; Richardson et al., 1986). Hydrophobic affinity labeling indicated that the N-terminal fusion peptide of the F protein inserts into target membranes (Asano and Asano, 1985; Novick and Hoekstra, 1988). Mutational analysis confirmed that the fusion peptide plays an essential role for fusion (Horvath and Lamb, 1992). Recently, however, a second internal fusion peptide was proposed for Sendai and measles viruses (Figure 1.4A). Synthetic peptides based on the region downstream of the N-terminal heptad repeat are capable of inducing membrane fusion of large unilamellar vesicles, and are even more efficient than the N-terminal fusion peptides (Peisajovich et al., 2000; Samuel and Shai, 2001). The internal fusion peptides were significantly more fusogenic towards vesicles with biological lipid composition. The synthetic internal peptides penetrate into the hydrophobic core of the membranes and adopt an oblique orientation within the membrane (Ghosh et al., 2000; Samuel and Shai, 2001). As mentioned previously, oblique insertion of N-terminal fusion peptides is widely interpreted to disturb the lipid packing and expand the interior of the membrane relative to the surface, thus inducing negative curvature strain that supports the formation of

membrane fusion intermediates (Erand et al., 1994). The internal paramyxovirus fusion peptides also showed high α -helical propensity despite the presence of prolines, and would exist within the loop formed within the six-helix bundle between the two antiparallel heptad repeats (Figure 1.4A and B).

An important distinction must be made between the internal fusion peptides found in fusion proteins that undergo coiled-coil conformations. The paramyxovirus internal fusion peptides are predicted to be located between the heptad repeats, and would therefore, be within a naturally occurring loop. The internal fusion peptides of the oncovirus avian sarcoma/leucosis virus (ASLV, retrovirus) and Ebola virus (filovirus) are located between the N-terminus and the N-terminal heptad repeat. For ASLV, the internal fusion peptide also forms a loop, but disulfide bonds and small local secondary structures must stabilize the loop (discussed in retrovirus section). Clearly these internal fusion peptides differ in their location relative to the folded protein, and cannot be assumed to play identical roles in the fusion reaction.

The importance of the internal fusion peptide in Sendai virus-induced fusion was demonstrated by the inhibitory effect of peptides corresponding to this region (Ghosh and Shai, 1998; Ghosh et al., 2000). Thus, paramyxoviruses may provide a unique example of multiple fusion peptides that function in concert to promote efficient fusion. Ghosh et al, (2000) suggest a new model of paramyxovirus induced fusion that accounts for the membrane interactions

between N- terminal and internal fusion peptides and the membrane (Figure I.4B, used with permission). Cleavage of the precursor F protein is speculated to produce a metastable conformation where fusion peptides are concealed (Figure I.4B, conformation A). Binding to the host receptor induces conformational changes presumed to expose the N-terminal fusion peptide, which inserts into the target membrane (Figure I.4B, conformation B). The trimer-of-hairpins is then formed, as visualized by crystallography. For paramyxovirus, an additional change in conformation is predicted to involve the relocation of heptad repeats towards the membrane and an “umbrella”-like opening of the coiled-coil (Ben-Efraim et al., 1999; Young et al., 1999). Ghosh et al (2000) predict that internal fusion peptide interactions with the acceptor membranes may drive the formation of the final open conformation (Figure I.4B, conformation E). The model in figure I.4B indicates that an alternative sequence of events may take place for paramyxovirus, steps that are unique from those proposed for orthomyxoviruses. In the alternative hypothesis, conformational change induced by the trigger exposes the N-terminal fusion peptide that inserts into the donor membrane (Figure I.4B, conformation C) but also exposes the internal fusion peptide (Figure I.4B, conformation C), which inserts into the target membrane (Figure I.4B, conformation D). A relocation of the N-terminal fusion peptide into the target membrane from the donor membrane would create the final umbrella structure (Figure I.4B, conformation E). Future studies that identify the pathway of paramyxovirus fusion may contribute to a broader perspective of the role of coiled-coil interactions.

In summary, the unique features of paramyxovirus fusion proteins in comparison to the fusion proteins of orthomyxoviruses and retroviruses include: The ectodomains are over twice the size, and presumed to have additional folding or functional properties, both internal and N-terminal fusion peptides are speculated to participate in fusion, and aside from the two heptad repeats common to all six-helical bundle motifs, the paramyxovirus F proteins have an additional leucine zipper motif (Ghosh et al., 1997). Studies on paramyxovirus fusion will expand the repertoire of conformational changes and membrane interactions that are involved in the fusion mechanism by fusion proteins that necessitate coiled-coil structures.

I.C.4. Retroviridae

The retroviridae family, which includes the human immunodeficiency virus (HIV), the simian immunodeficiency virus (SIV), the human T-cell leukemia virus (HTLV), and Rous Sarcoma virus, is characterized by a duplex of positive sense single stranded RNA molecules. Our understanding of the mechanisms of entry via fusion by HIV by far exceeds that of the remaining retroviruses. HIV enters cells at the cell surface in a pH-independent manner. The virus glycoprotein (env) is synthesized as a precursor (gp160) that requires proteolytic cleavage for fusion competency (Figure 1.5). The transmembrane subunit, gp41, is responsible for membrane fusion and contains the N-terminal fusion peptide. The surface subunit, gp120, is required for receptor binding, which functions to

trigger the fusogenic activity. Unlike HA, the subunits of the HIV fusion protein are held by noncovalent interactions.

At a molecular level, HIV utilizes a similar mechanism of fusion as influenza HA, but under the control of a different trigger (binding instead of low pH). For HIV, binding to cell receptors triggers a substantial conformational change in the fusion complex. Antibody recognition changes suggest that new epitopes are hidden or revealed (Thali et al., 1993). The native state is suggested to be a metastable (Moore and Klasse, 1992), inactive conformation that undergoes conformational rearrangement to the final stable trimer-of-hairpins structure. These conformational changes are required for the formation of fusogenic active intermediates where fusion peptides become exposed, integrated into membranes, and lipid mixing is favored. The details of this change have only started to be understood, and unlike HA, crystallographic images are only present for the final helical bundle conformation for gp41 (Chan and Kim, 1998).

The ability of peptides that bind to or near the predicted heptad repeat/ coiled-coil domains to prevent the formation of the trimer-of-hairpins structure and gp41 mediated fusion confirms that helical bundle formation is part of the fusion process (Lu et al., 1995; Jiang et al., 1993; Wild et al., 1992, 1993, 1994). These peptide studies also support the presence of a 'pre-hairpin' but post triggering intermediate, predicted to resemble the extended coiled-coil conformation of HA that exposes the fusion peptide (Weissenhorn et al., 1997; Furuta et al., 1998;

Munoz-Barroso et al., 1998). Recent studies with inhibitory peptides and lipids have shown that the gp41 bundle does not form until the fusion pore is created (Cohen et al., 2000), which suggests that bundle formation may be occurring simultaneously, or following, membrane merger. Despite exhaustive analysis, however, the precise role of the six-helix bundle remains unknown.

The fusion of HIV with target cells during entry has an interesting requirement for raft microdomains on target membranes (del Real et al., 2000). HIV-1 infection triggers redistribution and clustering of membrane microdomains following the interactions of the viral envelope with the cell surface receptors. The enlarged microdomains enable more rapid and frequent interactions between gp41 and receptors / co-receptors. Cholesterol depletion inhibits entry of HIV strains, although viral replication is unaffected. Although the present hypothesis is that rafts are involved in fusion indirectly, by concentrating fusion protein activity to localized sites, future experiments may find that other characteristics of rafts such as lipid composition play additional roles.

The avian sarcoma/leukosis virus (ASLV) is an avian retrovirus that contains an internal fusion peptide within the fusion protein and, therefore, differs from HIV and SIV. The fusion protein of ASLV is a trimer of two disulfide-bonded subunits, SU (gp85) and TM (gp37) formed by proteolytic cleavage of the pr95 precursor protein (Einfeld and Hunter 1997; Hunter et al., 1983). As for other retroviruses,

the SU is responsible for receptor binding while the TM functions to induce membrane fusion (Young et al., 1993; Hernandez and White, 1998).

Although N-terminal fusion peptides have been studied for decades, the function of internal fusion peptides has only recently emerged, in large part from studies on ASLV. The ASLV fusion peptide exists as a loop-structure stabilized by disulfide bonds, localized secondary structure, and the presence of secondary structure-perturbing residues such as glycines and prolines that promote loop formation (Delos et al., 2000, Delos and White, 2000; Balliet et al., 2000). Mutational studies also suggest that the loop structure is important for fusion, as amino acid substitutions that would affect loop formation produce nonfunctional proteins. Based on these findings, other internal fusion peptides including those within the fusion proteins of Ebola virus, tickborne encephalitis virus and viral hemorrhagic septicemia virus, have been modeled as looped structures (Delos et al., 2000, Delos and White, 2000; Gallaher, 1996; Gaudin et al., 1999b). Figure 1.6 provides a model of the six-helical bundle and fusion-active state of the ASLV TM subunit trimers. The looped internal fusion peptides are located in a similar position as the N-terminal fusion peptides with respect to the entire protein, and may be presumed to have similar involvement in the fusion process- to interact with and destabilize target membranes.

I.C.5 Filoviridae

The family Filoviridae contains some of the most virulent emerging pathogens including the Marburg and Ebola viruses. They are characterized as filamentous enveloped viruses containing nonsegmented negative-sense RNA genomes. The Ebola virus fusion glycoprotein (GP) has been most extensively characterized for filoviruses (Figure 1.7). The GP is cleaved into two disulfide-linked subunits (Sanchez et al., 1998; Volchkov et al., 1998). The GP2 subunit is responsible for receptor binding while the membrane anchored GP1 subunit results in membrane fusion following exposure to low pH (Takada et al., 1997; Wool-Lewis et al., 1998). The intracellular domain of GP2 is extremely short but is palmitoylated at two membrane proximal cysteines (Funke et al., 1995; Ito et al., 2000). The mature GP exists as a homotrimer within the membrane (Feldmann et al., 1991; Sauchez et al., 1998).

The Ebola virus GP2 is suggested to have a coiled-coil trimeric structure formed by interactions between two heptad repeat regions. An α -helical rod-like trimer is formed upon expression of GP2 polypeptides in bacteria (Weissenhorn et al., 1998a). Recent crystallographic analysis has confirmed the presence of a stable triple-stranded α -helical coiled-coil structure (Malaashkevido et al., 1999; Weissenhorn et al., 1998b) similar to the influenza HA conformation following low pH activation. Little is known about the native GP1/GP2 complex, but it is presumed to undergo significant changes in conformation in formation of the fusogenic state. The formation of coiled-coils was shown to be essential for

fusion since amino acid substitutions in both the N- and C- terminal heptad repeats or the addition of peptides corresponding to the heptad repeat regions inhibit Ebola virus GP mediated fusion (Watanabe et al., 2000)

The proposed fusion peptide present within the GP2 subunit is highly conserved among filoviruses and has been demonstrated to associate and fuse liposomes (Ruiz-Arguello et al., 1998). Studies with VSV particles pseudotyped with the Ebola virus GP protein containing mutations within the proposed fusion peptide strongly support that this region is essential for the fusion process (Ito et al., 1999). The fusion peptide is 22 residues away from the N-terminus of the GP2 subunit and is rich in glycine, alanine and proline residues suggesting that it may function as an internal fusion peptide in a loop conformation (Ito et al., 1999).

An interesting finding is that cell surface expressed Ebola virus GP does not induce polykaryon formation under variable pH conditions (Takada et al., 1997). Since fusion proteins of most enveloped viruses are transported to the surface of cells, they can be triggered to induce membrane fusion with neighboring cells. The inability of GP to induce syncytium may indicate that high concentrations are necessary in a localized environment, and that accumulation of GP only occurs during virus budding. If the propagation of Ebola virus would be hindered by cell-cell fusion, than a need for concentrated glycoprotein may provide an effective method of preventing syncytium formation. Another unique feature of the Ebola virus is that it is one of the smallest helical bundle-forming viral fusion proteins

and has minimal sequence intervening between the two heptad repeats. The GP of Ebola virus may contain the minimal requirements for fusion proteins that form trimer-of-hairpin structures.

I.C.6. Arenaviridae

Members of the Arenaviridae including Lassa fever virus, Machupo virus, Junin virus, Guanarito virus, and lymphocytic choriomeningitis virus (LCMV) are serious human pathogens. They all contain two ambisense genomic RNA segments. The only viral encoded glycoprotein present within the arenavirus envelope is GP C, which is cleaved into non-covalently linked GP1 and GP2 subunits (Buchmeier et al., 1987; Wright et al., 1990). The peripheral GP1 and membrane anchored GP2 subunits are responsible for viral receptor binding and membrane fusion activities respectively (Parekh and Buchmeier, 1986; Buchmeier et al., 1978; Borrow and Oldstone, 1994; Di Simone et al., 1994; Burns and Buchmeier, 1991). Unlike the fusion proteins of other enveloped viruses commonly found in trimeric or dimeric structures, the arenavirus fusion protein has been predicted to exist as a tetrameric complex by crosslinking, antibody binding, electron microscopy and other methods (Burns and Buchmeler, 1993). Under low pH conditions, the GP complex undergoes conformational changes concomitant with membrane fusion (Di Simone et al., 1994).

The GP2 subunit contains two extended α -helical heptad repeats that are proposed to stabilize the oligomeric structure. Binding by GP1 is necessary for

fusion as dissociation of GP1 from whole viruses results in noninfectious virions (Di Simone et al., 1994). Liposomes devoid of cellular receptors efficiently fuse with GP1/GP2 expressing membranes and suggest that the necessary conformational changes are triggered by low pH alone, and that receptor binding is likely essential for biological viral entry through fusion by permitting endocytosis (De Simone et al., 1994). Cryoelectron microscopy showed that the arenavirus glycoproteins are tightly packed within the envelope (Burns and Buchmeier, 1993). Three states are predicted for the arenavirus fusion protein based on antibody recognition and other methods: An inactive complex at neutral pH, a conformational change to the active state following exposure to acidic environment, and an irreversible conformational change that includes the dissociation of GP1 subunits (Di Simone et al., 1994; Di Simone et al., 1995). The dissociation of GP1 is thought to be necessary to permit close approach of the two merging membranes. The proposed model for a single subunit of GP2 in its final conformation (Figure 1.8) is based on high conservation of sequence and structural motifs with other viruses, especially filoviruses (Gallaher et al., 2001). If arenaviruses are in fact tetrameric then the final conformation would be an eight helix bundle (or tetramer-of-hairpins). If future studies confirm the tetrameric oligomerization of arenaviruses, they will provide yet another variation to the general themes emerging for fusion proteins that function through coiled-coil structures.

Six potential fusion peptides have been identified within the Lassa virus GP2 protein based on sequence analysis (Glushakova et al., 1990). Interestingly, a fusion peptide has not been located within the LCMV glycoprotein based on sequence analysis. The N-terminus of LCMV has the conserved G-X-F motif found in other N-terminal fusion peptides, but has a three-fold lower Eisenberg normalized consensus index of hydrophobicity as compared to the influenza HA fusion peptide (Di Simone et al., 1994). Another internal twenty-four residue sequence is highly conserved and is almost as hydrophobic as the influenza HA fusion peptide. The LCMV may, therefore, have a weak N-terminal fusion peptide, a strong internal fusion peptide, both, or none. As for filoviruses, the fact that LCMV does not induce syncytium formation at acidic pH may suggest that its fusogenic activity is weak and is only optimal under conditions of high protein concentration found within the envelope of arenaviruses (Di Simone et al., 1994). Alternatively, the weak fusion activity of arenaviruses may correlate with the absence of apparent strong fusion peptides.

I.C.7. Coronaviridae

The Coronaviridae family includes human pathogens such as human coronavirus 229E, OC43 and other viruses responsible for the common cold, upper respiratory infections and perhaps even pneumonia. The murine hepatitis virus (MHV) is most extensively utilized for understanding the Coronaviridae life cycle. Coronaviruses possess a large single-stranded positive-sense RNA genome. All coronaviruses contain the spike (S) protein responsible for attachment and fusion

during entry into host cells. Some coronaviruses also possess the hemagglutinin esterase protein (HE) and/or a small membrane protein (SM). The S protein is, however, necessary and sufficient for pH-independent membrane fusion. Surface expression of S results in extensive cell-cell fusion (Stauber et al., 1993; Taguchi et al., 1992).

The S protein is differentially processed for different coronaviruses. For some, cleavage by cell-dependent proteases produces two non-covalently associated 90 kDa subunits S1 and S2 (Figure I.9) (Frana et al., 1985; Sturman et al., 1985). The S1 subunit is responsible for binding to host cell receptors (Cavanagh et al., 1986; Taguchi, 1995), while the S2 subunit contains the domains thought to be involved in membrane fusion. Cleavage of coronavirus S protein enhances its fusogenic activity (Yamada et al., 1998; Gombold et al., 1993) but is not absolutely required for infectivity (Bos et al., 1997). For other fusion proteins that undergo proteolytic cleavage, the consequential production of an N-terminal fusion peptide makes cleavage essential (i.e. the N-terminal fusion peptide can only function if it is at the N-terminus, which is only produced following cleavage). In contrast, cleavage of the coronavirus S protein does produce an N-terminal fusion peptide and is, therefore, dispensable for fusion.

Using mutational analysis and structural predictions, three potential internal fusion peptides have been identified within MHV (Figure I.9) (Luo and Weiss, 1998). Potential fusion peptide 1 is located within the N-terminal heptad repeat,

an unlikely location for peptides expected to insert into the target membrane in view of the coiled-coil interactions between heptad repeats. The second predicted fusion peptide is between the heptad repeats, and would hence resemble the internal paramyxovirus fusion peptide in location. A third hydrophobic sequence located near the N-terminus where the internal fusion peptides of ASLV and Ebola virus are found, was eliminated as a potential fusion peptide for coronaviruses because multiple substitutions within this region had no effect on S protein-mediated fusion. The characteristics of the internal fusion peptide within the S2 subunit have not been determined.

The transmembrane domain and cytoplasmic tail of coronavirus S proteins are rich in cysteine residues that are highly conserved among most coronaviruses. Replacement of the cysteine residues abrogated the cell-cell fusion activity of S protein (Bos et al., 1995). The cysteine residues and other features of the membrane spanning domain and cytoplasmic tail could not withstand substitution without loss of fusion, suggesting that unlike many other viral fusion proteins, specific characteristics of the S protein domains that interact with the donor membrane are necessary for fusion (Chang et al., 2000). The S protein is known to be palmitoylated (Niemann and Klenk, 1991; Sturman et al., 1985; van Berlo et al., 1987) but a direct relationship between palmitoylation and fusion activity has not yet been demonstrated. The abundance of cysteine residues and palmitoylation within the S protein makes coronaviruses good candidates for

understanding potential contributions of the donor membrane-associated domain in the fusion mechanism.

The S protein exists as trimers in the virus envelope (Delmas and Laude, 1990). Three heptad repeat regions are present in the membrane-anchored S2 subunit (Figure 1.9)(Chambers et al., 1990) and are predicted to form helical coiled coils (Singh et al., 1999). Coiled-coil structures were first identified within the S protein in the late 1980's (de Groot et al., 1987). The heptad repeat region adjacent to the transmembrane domain consists of a leucine zipper motif bearing leucine residues on a single face of the α -helix. Amino acid changes within the leucine zipper motif affect proper oligomerization and fusogenic function of the coronavirus fusion protein (Luo et al., 1999). The S proteins of coronaviruses have been under-investigated in comparison to other viral fusion proteins. Although sequence analysis and mutagenesis has provided clues to the possible role of different domains, the conformations and conformational changes that follow activation are not known. Investigations unique to coronaviruses involve production of tissue culture-adapted mutants of the prototype JHM strain of MHV that vary in their requirement for receptor binding, and for high, neutral or low pH to trigger the fusogenic activity (Krueger et al., 2001). Future analysis may identify the structural changes within the wild type virus and these culture-adapted strains involved in the fusion mechanism.

I.D. FUSION PROTEINS THAT DIFFER FROM THE PROTOTYPE INFLUENZA HA FUSION PROTEIN

The fusion proteins of orthomyxoviruses, paramyxoviruses, retroviruses, filoviruses, arenaviruses, and coronaviruses share in common the formation of coiled-coil structures that permit conformational changes from inactive to active states. The coiled-coil structures are also predicted to play additional roles during the fusion reaction, such as directly providing energy for overcoming the barriers to membrane merger. One of the strongest arguments against the absolute need for coiled-coil structures and the formation of more stable states is that some enveloped virus families do not have heptad repeats and do not form irreversible stable structures upon activation. The next section demonstrates the diversity in structure and domain organization of enveloped virus fusion proteins by describing the fusion mechanisms proposed for togaviruses, flaviviruses and rhabdoviruses.

I.D.1. Togaviridae

The prototypical members of the togaviridae family are Sindbis virus (SIN) and Semliki Forest virus (SFV), which in addition to Ross River virus and Eastern equine encephalitis virus, belong within the *alphavirus* genus. These are small, enveloped positive-sense RNA viruses. Togaviruses have two glycoproteins essential for envelope-cell membrane fusion during entry; the 50kDa E1 and 50kDa E2 glycoproteins (Figure I.10A). The E2 subunit plays a regulatory role

and is responsible for binding target membranes, while the E1 subunit mediates membrane fusion (Klim-jack et al., 1994; Omar and Koblet, 1998).

X-ray crystallographic and cryo-electron microscope reconstruction of SFV revealed that the E1 and E2 proteins form head-to-tail heterodimers that "lie down" along the viral membrane surface (not 100% parallel, but close) at neutral pH (Figure I.10A)(Mancini et al., 2000; Rey et al., 1995; Rey et al., 2000). Each heterodimer is grouped into trimeric complexes forming a glycoprotein shell with icosahedral symmetry and triangulation $T=4$ (Cheng et al., 1995; Fuller et al., 1995; Paredes et al., 1993). The fusion peptide contained in the E1 subunit is buried between the E1/E2 trimeric interface (Figure I.10A) (Lescar et al., 2001). The association between E1 and E2 has several purposes. E2 is thought to act as a chaperone and aid the formation of metastable E1 and to ensure that the acidity within the Golgi complex does not alter the conformation of E1. The final E1-E2 complex conformation is primed for fusion activity upon encounter of the correct trigger (Ferlenghi et al., 1998).

The life cycle of togaviruses requires dissociation of the envelope shell during entry (reviewed in Garoff et al., 1994; Kielian, 1995; Strauss and Strauss, 1994). Entry into cells involves receptor-mediated endocytosis and acid-induced fusion of the viral envelope with the endosomal membrane (Smit et al., 1999; Glomb-Reinmund and Kielian, 1998; DeTulleo and Kirchhausen, 1998). Following E2-receptor binding and subsequent receptor-mediated endocytosis, exposure to

low pH induces a number of conformational changes to the E1-E2 dimer (Figure I.10B) (Fuller et al., 1995; Rey et al., 2000). The E1 and E2 subunits dissociate into monomers and E1, in its new conformation, interacts with the target membrane (Wahlberg and Garoff, 1992; Bron et al., 1993; Ahn et al., 1999; Justman et al., 1993; Salmaninen et al., 1992; Klimjack et al., 1994). Three E1 subunits then associate to form homotrimers that are extremely stable (Wahlberg et al., 1992; Wahlberg and Garoff, 1992; Gibbons et al., 2000; Kielian et al., 1996). Trimer formation only occurs following low-pH and in the presence of target membranes. Despite the fact that the trimeric form is most stable, heat or denaturants are not able to induce the formation of trimers from E1/E2 dimers since these treatments could not induce dissociation into monomers (Gibbons et al., 2000). These conformational changes do not involve the formation of extended α -helical coiled-coil structures found within paramyxoviruses, orthomyxoviruses, and retroviruses (Kielian, 1995). The model for togavirus fusion protein-mediated fusion presented in figure I.10B clearly demonstrates that significant differences exist in the fusion process as compared with the previously described mechanisms for HA-like fusion proteins.

Mutational analysis suggests that togaviruses such as SFV and Sindbis virus have internal fusion peptides approximately 80 residues from the N-terminus (Garoff et al., 1980; Kielian, 1995; Levy-Mintz and Kielian, 1991; Qiu et al., 2000; Yang et al., 1998; Shome and Kielian, 2001). The amino acid sequence of the putative fusion peptide of SFV suggests that it has a disulphide bond-stabilized

loop structure similar to the other internal fusion peptides recently proposed to insert into target membranes as loop structures (Delos et al., 2000; Rey et al., 1995). The central proline residues found in the fusion peptide of SFV are important for fusion by avian leukosis and sarcoma viruses and vesicular stomatitis viruses, and are believed to cause a kink or bend that favors loop formation (Delos et al., 2000; Fredericksen and Whitt, 1995; Zhang and Ghosh, 1994). Mutagenesis studies indicate that the glycine residues are important for the fusion activity of SFV E1 (Shome and Kielian, 2001). Liposomes fusion assays revealed that synthetic peptides designed according to the SFV fusion peptide are capable of destabilizing membranes at low pH (Agirre et al., 2000). Despite the significant difference in the structures, oligomerization, and conformational changes between togavirus- and the HA-like fusion proteins, the importance of a fusion peptide motif and extensive conformational changes appears to be conserved.

Togaviruses have a unique requirement for cholesterol and sphingolipid in the target membrane (Kielian et al., 2000; Wilschut et al., 1995; Nieva et al., 1994; Phalen and Lielian, 1991; Vashishtha et al., 1998). These lipids are believed to promote E1-membrane association and rapid and efficient conformational changes within E1 that culminate with homotrimer formation. Cholesterol appears to permit binding of the low-pH-treated virus to liposomes while sphingolipid appears to directly catalyze the fusion event (Chatterjee et al., 2000; Smit et al., 1999).

I.D.2. Flavivirus

The Flaviviridae family of viruses includes dengue virus, hepatitis C virus, West Nile virus, yellow fever virus, and the commonly studied tick-borne encephalitis virus (TBE). Similar to the togaviruses, they are also small, positive-sense RNA viruses. Instead of having two glycoproteins however, flaviviruses use only the E glycoprotein to create a proteinaceous envelope shell. Upon synthesis, the E protein forms a heterodimer with prM, which presumably functions similar to the E2 of togaviruses to permit correct folding and/or prevent premature conformational changes. Flaviviruses bud into the endoplasmic reticulum and enveloped viruses exit the cell through exocytosis. During the exocytic pathway, prM is proteolytically cleaved and the fusogenic potential of E is activated (Stadler et al., 1997). The E proteins form homodimers that lie parallel with the membrane surface similar to togaviruses (Figure I.11A). The crystal structure of the TBE E protein dimer showed that the proposed internal fusion peptide forms a loop structure and buries within a hydrophobic pocket formed between the subunits at the interface of dimerization (Rey et al., 1995; Allison et al., 2001). Lateral interactions between 90 E-E dimers create an icosahedral protein shell with triangulation $T=3$ (Furlong et al., 2001). Comparison of figure I.10A (togavirus) and figure I.11A (flavivirus) shows the subtle differences between the organization of their envelope glycoproteins. While togavirus E1/E2 heterodimers do not bury the fusion peptide (Figure I.10A, dimer) but do so in the 'Y' shaped E1/E2 trimer (Figure 10A, trimer), the flavivirus E/E dimers do bury

the fusion peptide (Figure I.11.A, dimer) and form a triangular shaped trimer (Figure I.11.A, trimer).

Flaviviruses enter cells through endocytosis and low-pH-induced fusion (Rice, 1996; Heinz et al., 1994). At low pH, irreversible conformational changes are triggered in the E protein. E dimers dissociate and expose the fusion peptide that interact with the target membrane (Stiasny et al., 1996; Heinz et al., 2000). E monomers then rearrange into homotrimers (Allison et al., 1995). The trimeric conformation likely brings the fusion peptide and transmembrane domain of E subunits into closer proximity (Ferlenghi et al., 2001). Overall, the changes in the flavivirus E dimers following activation resemble those described for the togavirus E1/E2 dimers (Figure I.10B). The involvement of different domains within the TBE-E protein in changes to the oligomeric structure of protein E were predicted and are summarized in figure I.11B (Allison et al., 1999).

As already mentioned, the internal fusion peptide of TBE was predicted through the X-ray crystal structure of TBE (Rey et al., 1995) and has been confirmed through mutational analysis (Allison et al., 2001). Crystallographic analysis showed that the TBE fusion peptide has a highly conserved loop structure at the distal tip of each subunit. Mutational analysis indicated that changes to the fusion peptide prevented membrane interactions with liposomes and impaired or abolished fusion activity while not affecting the structural rearrangements from dimer to trimer structures. As for ASLV, the internal fusion peptide of TBE

requires further investigation to assess whether it functions similar to the extensively studied N-terminal fusion peptides.

TBE virus has the fastest and most efficient fusion machinery of all enveloped viruses analyzed to date along with an absence of a lag phase (Heinz et al., 2000; Corver, 2000). It is presumed that an optimal arrangement exists within the envelopes of flavi- and togaviruses allowing rapid interactions between fusion proteins. For the remaining viruses with envelopes that do not form icosahedral symmetry, organization of fusion peptides to localized areas in correct arrangement would require time. The lag phase that precedes the initiation of fusion caused by influenza virus HA (Clague et al., 1991), vesicular stomatitis virus G protein (Clague et al., 1990), and Sendai virus (Hoekstra et al., 1985) is predicted to represent the time required for accumulation of active-state fusion oligomers at the site of fusion. Interestingly, the Semliki Forest virus E2/E1 that also forms icosahedral symmetry within the envelope has a lag time preceding the onset of fusion (Bron et al., 1993). The difference in lag time between togaviruses and flaviviruses may be because the E protein of flaviviruses contains both fusion peptide and receptor binding motifs. Thus, while togaviruses require time to relocate E2 subunits away from the newly forming E1 trimers, every subunit within flaviviruses becomes incorporated into a functional fusion active complex. Another hypothesis proposed by Corver et al. (2000) is that the most essential and time-limiting conformational change necessary for membrane fusion is the exposure of the fusion peptide. For the E proteins of

TBE, dissociation of dimers would be the only time-limiting step. For fusion proteins that have coiled-coil structure, complex conformational changes formed by unzipping and refolding of heptad leucine repeat motifs would require more time.

Differences between the coiled coil fusion proteins and those that don't form coiled coils has lead to the formation of two classes of enveloped virus fusion proteins. The type I class includes the viruses discussed in section I.A that have at least two heptad repeats capable of forming the six helical bundle. The fusion proteins of togaviruses and flaviviruses are classified as type II fusion proteins. They are not proteolytically cleaved during maturation but instead, associate with a second protein that promotes correct folding into dimeric structures. Acidic environments induce the dissociation of dimers and exposure of internal fusion peptides, followed by the formation of the highly stable trimer structures. Even though the final trimeric conformation appears more stable than the dimeric structure, denaturants and heat are unable to induce the same transition brought upon by low-pH, likely because these treatments do not support the formation of monomeric intermediate required for trimerization (Stiasny et al., 2001). No coiled-coils are predicted to be involved in the activation of type II fusion proteins. Slight changes do exist between togaviruses and flaviviruses. For example, TBE virus also does not require cholesterol or sphingolipids in target membranes for fusion (Corver et al., 2000), unlike SFV and SIN. Nonetheless, type II fusion proteins do dispute the necessity of coiled-coils and helical bundles for

membrane fusion and question whether the paradigm of HA-mediated fusion applies to all fusion proteins.

I.D.3. Rhabdoviridae

Rhabdoviruses are characterized by their typical bullet shape and single-stranded negative sense RNA. Rabies virus and vesicular stomatitis virus (VSV) are the best characterized rhabdoviruses with respect to the mechanism of membrane fusion used for virus entry. The lipid envelope contains a single viral-encoded glycoprotein (G) in a trimeric structure (Whitt et al., 1991; Gaudin et al., 1992). Low-resolution structural assessment of G has been obtained using electron microscopy and contributed to the head and stalk model of the G glycoprotein structure (Figure I.12). The trimeric G protein is necessary and sufficient to induce membrane fusion under acidic conditions (Coll, 1995; White et al., 1981; Riedel et al., 1984; Whitt et al., 1991; Gaudin et al., 1993). Rhabdovirus G-mediated fusion does not require a specific membrane lipid composition (Gaudin et al., 1991; Yamada and Ohnishi, 1986; Hermann et al., 1990).

The rhabdovirus G protein undergoes conformational changes and moves to the ends of the bullet-shaped particles in response to low pH (Brown et al., 1988; Gaudin et al., 1993, 1995). Using electron microscopy (Gaudin et al., 1991, 1993), kinetic fusion analysis (Clague et al., 1990; Puri et al., 1992), proteolytic cleavage sensitivity analysis (Gaudin et al., 1995a), and antibody recognition

analysis (Gaudin 1997), the rhabdovirus G has been shown to adopt at least three conformations (Figure 1.12) (reviewed in Gaudin et al., 1995b; Gaudin, 1999). The native state (N) is detected above neutral pH. An equilibrium between the N state and the activated state (A) falls in favor of the A state as the pH is reduced. Finally, the rhabdovirus G undergoes further structural changes resulting in the inactive state (I). In the A state, the fusion peptide is exposed from a previously buried location and aggregation of several G proteins can be seen. A minimal fusion complex consisting of several G trimers in their active states is predicted to be essential for promoting membrane fusion (Bundo-Marita et al., 1988; Gaudin et al., 1993). Interestingly, mutant viruses delayed in their transition from the activated to the inactivated state showed a hexagonal lattice (composed of 6 G trimers) that is stabilized at acidic pH corresponding to prefusion conditions (Gaudin et al., 1996). The present model of rabies virus-induced fusion proposes a hexagon of G trimers as a minimal prefusion complex (Gaudin et al., 1999). Despite the absence of coiled-coil structures and the reversibility of structural changes suggesting that substantially more stable forms are not formed, rhabdovirus- and other viral fusion proteins share in common the presence of a fusion peptide and the need for aggregation. Thus, both exposure of the fusion peptide and complex formation during the active conformation may drive the fusion reaction. The conformational changes of rhabdovirus G protein are suggested to compensate for the high energy costs of membrane fusion (Gaudin, 2000).

A region within the VSV G protein has been defined that controls the low pH-induced conformational changes leading to fusion (Gaudin et al., 1996; Li et al., 1993; Shokralla et al., 1998; Shokralla et al., 1999). Because the conformational changes of the G protein are not predicted to be driven by rearrangement of coiled-coils common to some other viruses (Figure 1.1), it would be interesting to identify the additional structural elements that allow conformational changes necessary for fusion peptide exposure and the subsequent fusion reaction.

Unique to the G protein of rhabdoviruses is a reversibility of the three states. The reversibility of conformational changes suggests that the native G protein may not be in a metastable state. The reversibility promotes the thermodynamic model (where the optimal state shifts depending on the environmental conditions) rather than the kinetic model (where proteins are produced in a metastable poised state like a mouse trap awaiting the trigger to spring into the stable conformation). The reversibility between the three known states of protein G has also contributed to the hypothesized function of the inactive state. Rhabdoviruses containing mutated G protein that were delayed in their transition to the inactive state had no deficiency in their fusogenic activity suggesting that the inactive state was unrelated to the fusion reaction. Immunofluorescence studies of protein G during its intracellular transport was effective using antiserum specific to the inactive conformation of G and suggested that the reversible inactive state permits efficient transport of G protein through the acidic Golgi environment without detrimental effects such as fusion peptide function or

aggregation prior to surface localization (Gaudin et al., 1995a). Rhabdoviruses have, therefore, evolved a unique mechanism to ensure that their fusion proteins remain inactive during intracellular transport, yet are rapidly activated to expose their fusion peptides under restricted conditions.

The internal fusion peptide of vesicular stomatitis virus and rabies virus was predicted by Ohnishi (1988) and has been identified by mutagenesis and direct labeling experiments (Fredericksen and Whitt, 1995; Whitt et al., 1990; Li et al., 1993; Zhang and Ghosh, 1994). The internal fusion peptide is unique, however, because it is not rich in hydrophobic residues but instead contains glycine, proline, aspartic acid, and neutral amino acids. Hydrophobic photolabelling (photoactivatable lipids that covalently modify protein residues inserted within the core of lipid bilayers) confirmed the location of the internal fusion peptide and demonstrated that fusion peptide exposure and membrane association was only associated with the low pH, active conformation of rhabdovirus G protein (Durrer et al., 1995; Gaudin et al., 1993). Furthermore, no fusion peptide insertion into the viral membrane was determined, which differs from fusion proteins such as the influenza HA whose fusion peptides have been demonstrated to insert into the donor membrane as well as the target membrane (Weber et al., 1994). The association of the G fusion peptide with the membrane was also reversible (Pak et al., 1997), again suggesting that there may not be a large energy barrier to the transition between active and native states and that favorable interactions

between differentially charged residues due to changes in pH may be sufficient to favor the changes in conformation.

I.E. SUMMARY OF ENVELOPED VIRUS FUSION PROTEINS

The enveloped virus-encoded fusion proteins have become a focus of investigation in hopes of understanding the mechanisms of membrane fusion for all essential biological processes. The rationale for the focus on enveloped virus fusion proteins is that viruses must minimize their genome size and protein complexity and may provide the simplest model of protein-mediated fusion. Furthermore, since enveloped viruses enter cells via fusion, they must carry all the machinery essential on donor membranes for bilayer fusion, and can not be burdened with 'extra baggage'. Yet, despite over three decades of intensive analysis on the fusion proteins of enveloped viruses, many questions remain as to whether they are essential for the fusion process. As described in previous sections for individual virus families, the structures and motifs involved in fusion are beginning to emerge, yet their exact role in the fusion process remains uncertain. The complexities of enveloped virus fusion proteins can be attributed to a requirement for fulfillment of the following:

1. Fusion induction
 - a. Bilayer destabilization
 - b. Membrane apposition
2. Specificity
 - a. Receptor binding potential

3. Regulation

- a. Multiple states (conformations, oligomerization, etc.) that bury or expose the fusion peptide
- b. Maintaining an inactive state throughout transport to the surface
- c. Optimal transition from inactive to active states in response to specific triggers
- d. An inactivation method to eliminate prematurely activated proteins

4. Efficiency, and rapidity

- a. Optimal organization of fusion proteins

If the fusion proteins of enveloped viruses have evolved to meet all of these requirements in an efficient and rapid process, distinguishing among the roles of different features will be extremely difficult in biological assays. How would one then decipher whether a step in the activity of enveloped virus fusion proteins is essential for regulation, or directly for membrane fusion?

I.F. COMMON MEMBRANE INTERMEDIATES IN DIVERSE FUSION PROCESSES

I.F.1. Lipid intermediates in the fusion process

Although different virus families differ in their oligomeric structure, the triggers required for activation, and their structural intermediates during membrane fusion, a common model for changes to the lipid bilayers has been proposed

(summarized in Melikyan and Chernomordik, 2000). The lipid fusion intermediates have been predicted based on several studies using different lipid components or viral fusion protein mutants to arrest the fusion process at various stages such as before and after outer leaflet mixing, inner leaflet mixing, and content mixing.

Normally, the outer leaflet of biological membranes has positive curvature while the inner leaflet has the reverse/ negative curvature. Membrane lipids with greater head group volume with respect to the acyl chain volume would promote positive curvature, while a low lipid head group: acyl chain volume ratio would promote negative curvature (Figure I.13A). Inverted cone-shaped lysolipids such as lysophosphatidylcholine (LPC) promote positive curvature. Experiments have shown that LPC can inhibit fusion when incorporated into the outer leaflet, but support the fusion process if present in the inner leaflet (Chernomordik et al., 1993, 1995; Vogel et al., 1993; Yeagle et al., 1994). These experiments demonstrate that during the fusion process, the outer leaflet must acquire negative curvature while the inner leaflet requires positive bending. One of the first transformations to the membrane during fusion is, therefore, speculated to involve the formation of a stalk intermediate where the outer leaflets of opposing bilayers are connected through negative curvature (Figure I.13B) (Siegel, 1993; Chernomordik et al., 1995). The stalk intermediate would be prevented by LPC but promoted by cone-shaped lipids such as oleic acid (OA).

The second predicted membrane fusion intermediate involves the formation of a hemifusion diaphragm. The lipid diffusion between membranes is initially restricted, possibly by a ring-like aggregate of fusion proteins around the hemifusion diaphragm (Chernomordik et al., 1998) but becomes unrestricted and permits the transfer of lipid dyes from target to donor membranes participating in membrane fusion (Kemble et al., 1994; Melikyan et al., 1995).

Merging of the inner leaflets creates a pore. Electrophysiological measurements can detect a small flickering pore (Chanturiya et al., 1997). Enlargement of pores is indicated by the transfer of small organic molecules (Melikyan et al., 1993; Blumenthal et al., 1996), and subsequently leads to complete fusion (Melikyan et al., 1997b; Chernomordik et al., 1998). Because fusion requires positive curvature of the inner leaflet, OA but not LPC in the inner leaflet would favor membrane fusion at this stage. Inner leaflet localization of LPC and OA can be promoted by using shorter acyl chains, as the transbilayer migration of lipids with smaller acyl chain length is increased (Fujii et al., 1985). The effect of LPC and OA on pore formation has been interpreted to suggest that pores formed during membrane fusion are lipid and not proteinaceous (Zimmerberg et al., 1993; Kemble et al., 1994; Melikyan et al., 1997b; Chernomordik et al., 1998, 1999). The mixing of contents enclosed by donor and target membranes has been used to monitor complete fusion.

The model for membrane fusion is mostly founded on investigations of influenza HA induced fusion (Chernomordik et al., 1997, 1998) but is proposed to be universal. The effect of lipids with varied head group-to-acyl chain ratios on fusion supports the existence of similar intermediates induced by fusion proteins of other enveloped virus families (Chernomordik et al., 1995; Gaudin, 2000). Furthermore, energy calculations of the lipidic structures support this model (Siegel, 1993). The insertion of fusion peptides into the target and perhaps also the donor membrane has been generally supposed for all fusion proteins (Stegmann et al., 1991; Tsurudome et al., 1992; Durrer et al., 1995; Weber et al., 1994; Wharton et al., 1995). The formation of stalk intermediates is common not only to fusion proteins such as baculovirus, Sendai virus, rabies virus and influenza virus, but also to calcium triggered exocytosis and GTP-dependent microsome fusion (Chernomordik et al., 1993, 1995; Yeagle et al., 1994). Hemifusion has been demonstrated for influenza, and paramyxovirus SV5 (Bagai and Lamb, 1996; Kemble et al., 1994; Qiao et al., 1999). Lastly, the fusion pore also appears to be common to all biological fusion events (Monck and Fernandez, 1992). Thus, despite obvious differences between the fusion machineries of fusion proteins encoded by enveloped viruses from different families, a similar pathway of membrane changes is presently advocated. Viral protein mediated membrane fusion is predicted to proceed through the same membrane structures as fusion of protein-free bilayers (Chernomordik et al., 1995).

I.F.2. Other examples of membrane fusion

Poly (ethylene glycol) (PEG) brings membrane vesicles into close inter-vesicle contact by removing intervening water molecules (dehydration), a process that induces aggregation and fusion (Lentz and Lee, 1999). Studies that define changes in lipid structure that occur during PEG mediated fusion show similarities with structural changes facilitated by protein fusion machinery (Lentz, 2000). The series of lipid structural changes that accompany PEG-mediated fusion include the mixing of outer leaflets, hemifusion, and inner leaflet fusion. The lipid changes and intermediates are dependent on membrane composition, curvature, and lipid packing, consistent with the mechanism of protein-mediated fusion. A biological lipid composition (similar to viral and vesicle fusion membranes) optimizes the fusion capacity of membrane fusion mediated by PEG. Furthermore, compounds thought to “fill the void” created during hemifusion such as alcohols and/or cholesterol, promote PEG-mediated fusion.

Fusion of lipid vesicles can also be induced by phospholipases and sphingomyelinases (Nieva et al., 1989; Basanez et al., 1997; Ruiz-Arguello et al., 1998b; Villar et al., 2000). Phospholipases C and D degrade phospholipids in the outer surface of the bilayer, catalyzing the removal of the head groups (Figure I.14). The products of the reaction are usually destabilizing to the bilayer because they produce a very high acyl chain: head group volume ratio and, therefore, negative curvature. As mentioned previously, negative curvature favors the formation of membrane fusion intermediates (outer leaflet fusion/

hemifusion). Interestingly, similar to the requirement for several viral fusion proteins in one location to induce rapid fusion, localized “hotspots” of locally produced diacylglycerols are required to trigger the fusion reaction. The fusion induced by phospholipase activity depends on temperature and lipid composition, and follows similar kinetics as enveloped virus-mediated fusion. Phospholipase induced fusion is also affected by exogenously added lipids of different shapes in a similar manner as protein-mediated fusion.

Intracellular vesicular membrane fusion requires cognate vesicle- and target membrane- associated SNARE (SNAP receptors) proteins. SNAREs are integral membrane proteins that undergo specific and stable associations and are predicted to be the minimal machinery required for intracellular membrane fusion events (Weber et al., 1998). The v- and t- SNAREs, when correctly matched, form a four-helix bundle reminiscent of the orthomyxo, paramyxo and retroviral fusion proteins. The formation of this complex is predicted to drive membrane fusion, perhaps by providing energy to help overcome the barrier to fusion (Jahn and Hanson, 1998; Hanson et al., 1997). Additional proteins such as the ATPase NSF (NEM-Sensitive fusion protein) and SNAPs (Soluble NSF Attachment Proteins) are recruited to the SNARE complex and, upon ATP hydrolysis, dissociate the SNARE complex (Rothman, 1994). The brevity of this description underscores the present debates that attempt to decipher which events are truly essential for fusion, specificity, regulation, or recharging of fusion machinery.

I.G. THE OUTCAST FUSION PROTEINS: P10 ENCODED BY THE NON-ENVELOPED AVIAN AND NELSON BAY REOVIRUSES

Avian and Nelson Bay reoviruses (ARV and NBV, respectively) are members of the Reoviridae family of viruses characterized by the absence of a lipid envelope and the presence of a segmented double stranded RNA genome. Members of the *orthoreovirus* genus have ten segments of dsRNA and include the fusogenic ARV, NBV, baboon reovirus (BRV), snake reovirus (SRV) in addition to the nonfusogenic prototype mammalian reovirus (MRV). The life cycle of nonenveloped viruses does not involve membrane fusion and therefore most nonenveloped viruses, including MRV, do not encode fusion proteins. The production of fusion proteins by the fusogenic reoviruses is atypical for nonenveloped viruses.

The fusion proteins (p10) encoded by the nonenveloped avian (ARV) and Nelson Bay reovirus (NBV) may provide new insights into protein-mediated fusion because they have evolved under unique limitations and requirements. Nonenveloped viruses do not enter cells by membrane fusion and, therefore, do not carry fusion proteins. Avian and Nelson Bay reoviruses share a similar life cycle as other nonenveloped viruses, yet, they encode fusion proteins within infected cells. The fusion proteins are not incorporated into newly made virions, and, therefore, induce membrane fusion only between neighboring cells. Specificity and regulation are predictably not essential because every infected cell encodes p10 proteins and undergoes membrane fusion. Efficiency and

rapidity are also not as critical because the virus is already safe within the cell and instantaneous fusion has no significant selective benefit. The absence of these requirements may explain why p10 is the smallest viral fusion protein, with only forty ectoplasmic residues presumed to interact with the target membrane. This thesis describes the identification and characterization of p10. The motifs found in p10 may reflect the direct minimal requirements for bilayer fusion.

The first chapter describes the identification of p10 as a type I (N-terminus out) surface localized integral membrane fusion protein. The direct involvement in fusion is suggested by inhibitory effects of antibody binding on p10-mediated fusion. These characteristics are shared in common with the enveloped virus fusion proteins. The first chapter also provides a sequence-based analysis of the motifs found within p10, and a preliminary substitution analysis. By keeping in mind the basic structures of fusion proteins from enveloped viruses, the uniqueness of p10 will become evident.

The second chapter addresses the different requirements on p10 versus the fusion proteins of enveloped viruses. Data suggest that p10 could rely on cell-cell interactions for membrane apposition since these interactions would already exist for infected cells. Additional cell binding for membrane apposition may be redundant and, therefore, not necessary for p10-mediated fusion. Furthermore, chapter two will discuss why specificity and regulation would not be necessary for p10. Like enveloped virus fusion proteins, p10 may require a method to prevent

inactivation/ aggregation throughout intracellular transport to ensure functional p10 on the surface of cells.

Chapter three focuses on the ectodomain of p10 and our identification of motifs involved in interactions with the target membrane. The presence of an internal fusion peptide predicted to have a looped conformation will be demonstrated by mutagenesis and liposome fusion assays. Evidence will be provided to suggest that the fusion peptide may initiate in the exposed conformation. The 'fully loaded and poised' model of p10 suggests that unlike the fusion proteins of enveloped viruses, p10 initiates in an active conformation and does not require coiled-coil structures or gross conformational changes to function.

Chapter four identifies motifs within the transmembrane and intracellular domains of p10 that are necessary for fusion. Interactions with the donor membrane may play a greater role for p10 than the fusion proteins of enveloped viruses because the latter cannot afford to destabilize their envelope. All together, a fusion peptide, triglycine motif within the transmembrane anchor, palmitoylation, and basic intracellular residues participate in p10-mediated fusion. These motifs may represent the minimal requirements for membrane fusion in the absence of additional restrictions and requirements.

The last chapter (chapter five) describes multiple strategies that provide evidence for a monomeric structure of p10. If p10 does exist as a monomer, it is not only

the smallest fusion protein in peptide sequence, but in tertiary structure. Perhaps the oligomeric structure of other fusion proteins is essential to provide a method to bury the fusion peptide, undergo multiple changes in activity, and concentrate fusion activity. As mentioned previously, the life cycle of ARV suggests that p10 may not require instantaneous fusion activity, and may afford to aggregate and accumulate fusion activity slowly.

Together, the chapters will hopefully demonstrate our present understanding of p10 and the rationales for the hypothesized mechanisms for p10-mediated fusion. Although future studies will likely refute some of the present concepts of p10-mediated fusion, p10 has and will continue to contribute to the understanding of the requirements for membrane fusion due to its obvious divergence from the typical enveloped virus fusion protein. One of the privileges of studying p10 is that interpretations and investigations require unorthodox approaches and prevent dogmatic thinking.

I.H. INTRODUCTION FIGURES

Figure 1.1. Summarized characteristics of fusion proteins encoded by different enveloped virus families. The characteristics of different enveloped fusion proteins are summarized from references provided within the text, and are only assumed to apply to all examples provided for the family members.

FAMILY	PROTOTYPE(S)	FUSION PROTEIN COMPLEX ¹	MW (KDa)	pH OF FUSION	FUSION PEPTIDE	FUSION PROTEIN CLEAVED	CONFORMATIONAL CHANGE	FORMATION OF MORE STABLE STATES	REVERSIBLE	HEPTAD REPEAT or COILED-COIL
<i>Orthomyxoviridae</i>	Influenza virus A, B, C	[HA1/HA2] ₃	58/28	low pH (<6.4)	N-terminal	yes	yes	yes	no	yes
<i>Paramyxoviridae</i>	Measles, mumps, parainfluenza 2,3, and 4, Sendai virus, Respiratory syncytial virus (RSV), new castor disease virus (NDV)	[F1/F2] ₃	~10/40	neutral	N-terminal and internal	yes	yes	yes	no	yes
<i>Retroviridae</i>	Human immunodeficiency virus (HIV), Simian immunodeficiency virus (SIV)	[gp120/gp41] ₃		neutral	N-terminal	yes	yes	yes	no	yes
<i>Filoviridae</i>	Marburg virus, Ebola virus	[GP1/GP2] ₃	50/20	low pH (<6.3)	internal	yes	yes	yes	no	yes
<i>Arenaviridae</i>	Lassa, Machupo, Junin, lymphocytic choriomeningitis virus (LCMV)	[GP1/GP2] ₄	~40/35	low pH	?	yes	yes	?	no	yes
<i>Coronaviridae</i>	Murine hepatitis virus (MHV), human coronaviruses	[S] ₃ or [S1/S2] ₃	180 or 90/90	neutral	internal	no or yes	?	?	?	yes
<i>Togaviridae</i>	Sindbis virus, Semliki Forest virus (SFV)	[E1/E2/E3] ₃ → [E1] ₃	51/51/(11)	low pH (<6.2)	internal	no	yes	yes	no	no
<i>Flaviviridae</i>	Dengue virus, Hepatitis C, West Nile virus, Yellow fever virus, tick borne encephalitis (TBE)	[E] ₂ → [E] ₃	50	low pH (<6.8)	internal	no	yes	yes	no	no
<i>Rhabdoviridae</i>	Rabies virus, vesicular stomatitis virus (VSV)	[G] ₃	~50	low pH (<6.2)	internal	no	yes	no	yes	no

1. [Binding subunit, fusion peptide bearing subunit]₃ oligomeric structure

Figure 1.1

Figure 1.2. (A) Trimer-of-hairpin structures demonstrated for the membrane-anchored subunits of enveloped virus fusion glycoproteins. The domain organization linear cartoon depicts the fusion peptide (red), N-terminal heptad repeat (blue), C-terminal heptad repeat (yellow), and the transmembrane anchor (black). The coiled-coil bundle formed between the heptad repeats is viewed from the side and top. Taken with consent from Eckert and Kim, 2001 (B) **Formation of the helical bundle after fusion peptide insertion into target membranes.** The legend describes the coloration of each domain. Peptides that bind to the heptad repeats, or mutations made within the heptad repeats, are expected to prevent the final conformational change.

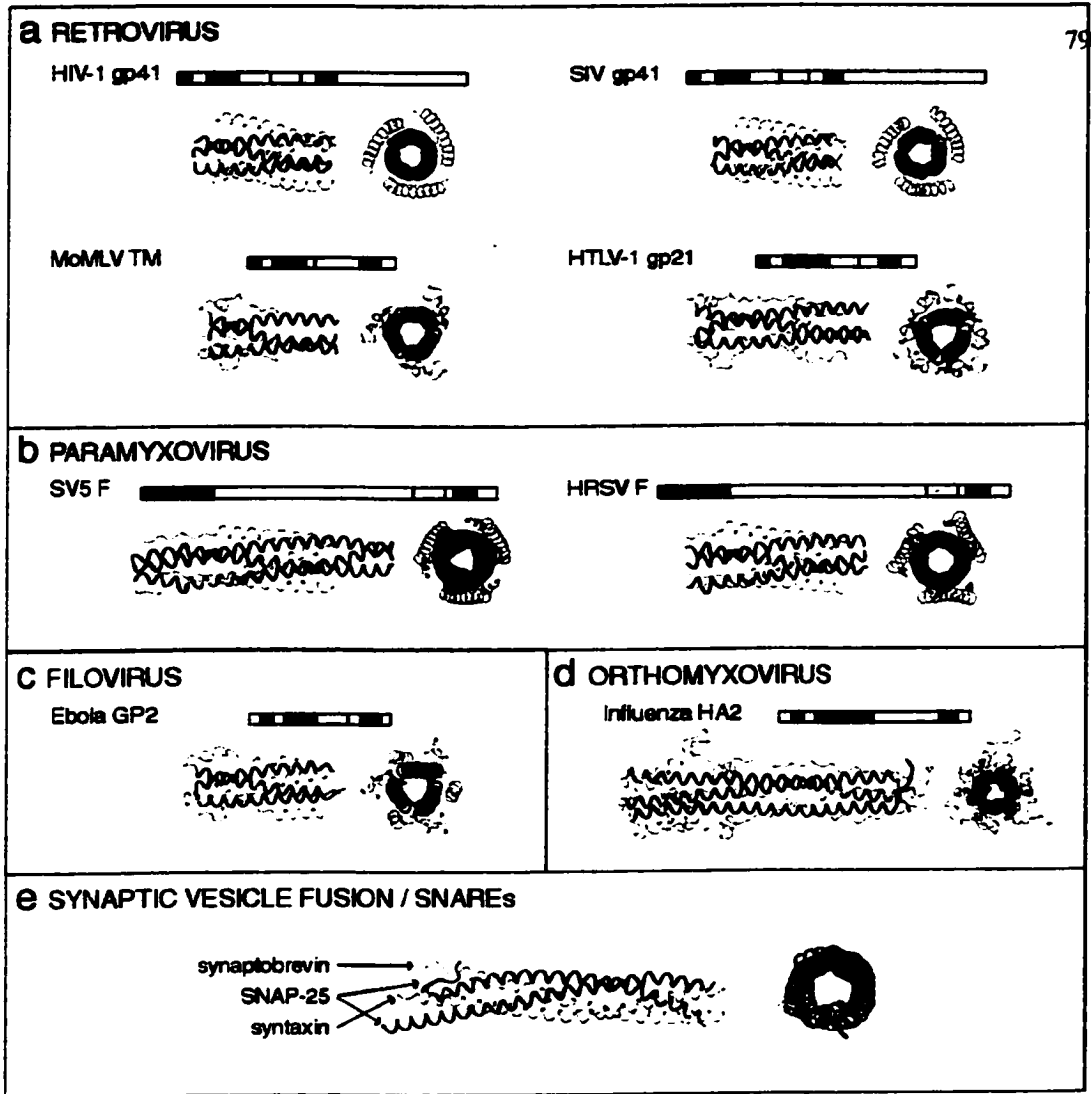
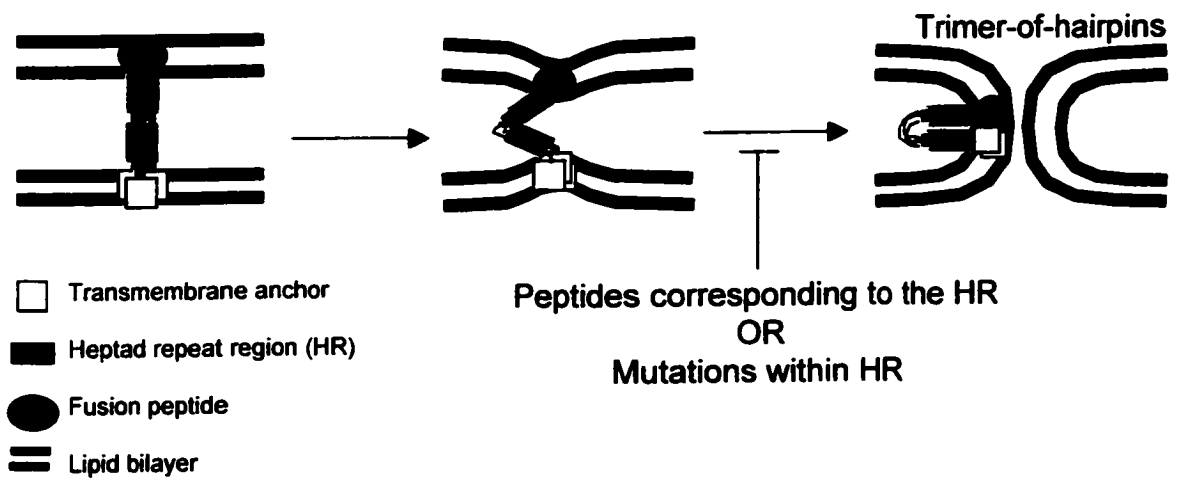
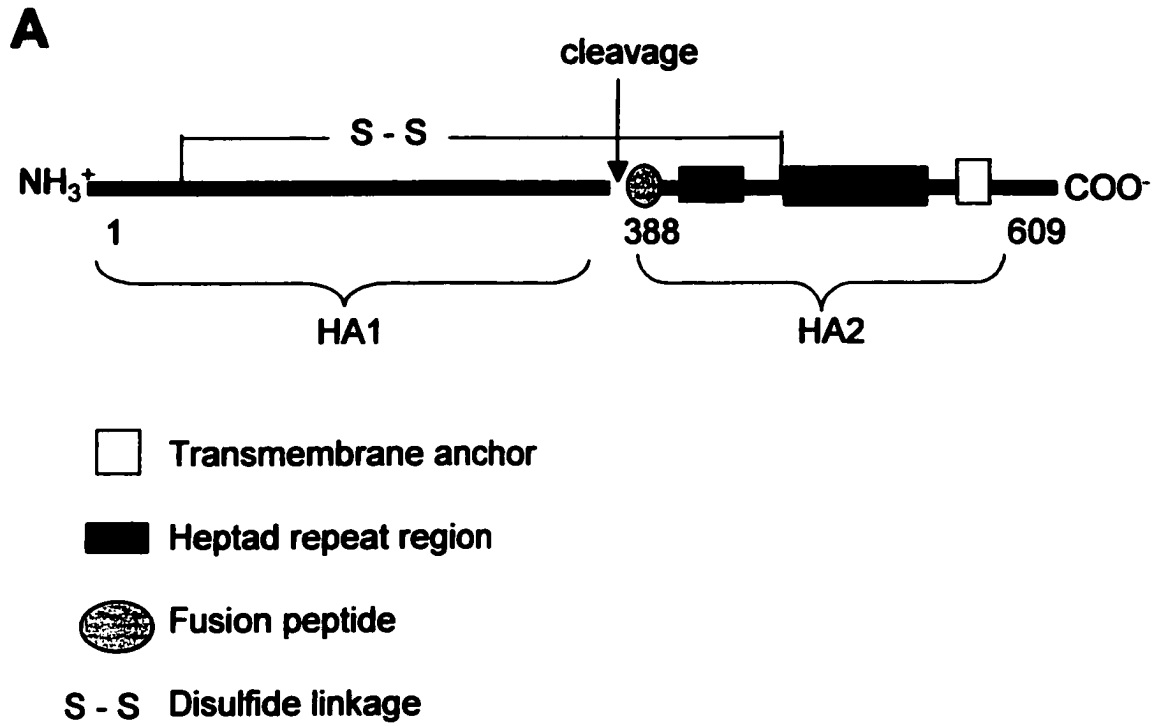
A**B**

Figure I.2

Figure 1.3. (A) Cartoon depiction of the influenza virus (orthomyxovirus) fusion glycoprotein (HA). Legend details assignment of domains. (B) Model for low pH induced membrane fusion of HA. The fusion peptides (red) are initially buried in the native conformation. A loop-to-helix transition (blue) induced at low pH repositions the fusion peptide near the target membrane (1). The fusion peptide inserts into the target membrane (2). Aggregation of the HA trimers results in a lag time prior to fusion (3). The tilting of HA and formation of the coiled-coil six helix bundle forces fusion peptide / target membrane and transmembrane anchor / donor membrane into close apposition (4,5,6) during which dimpling (4), outer leaflet fusion (5) and pore formation and dilation (6) culminate in complete fusion. Taken directly from the homepage of the Dr. Judith White (<http://www.people.Virginia.EDU/~jag6n/model.html>).

**B**

White Lab HA Fusion Model 1998

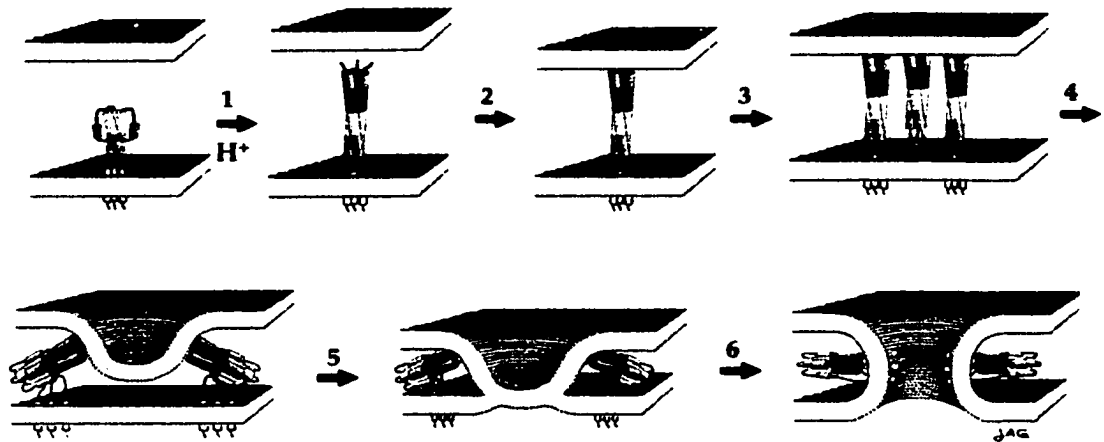


Figure I.3

Figure 1.4. (A) Cartoon depiction of the paramyxovirus fusion glycoprotein (F). Legend details assignment of domains. (B) Model for membrane fusion induced by paramyxovirus F. Upon binding to the cellular receptor, the native state (A) where fusion peptides are buried undergoes a conformational change that results in the exposure of the N-terminal (squiggly arrow) or internal fusion peptide (green). In one hypothesized pathway (A, B, E), the N-terminal fusion peptide inserts into the target membrane and the six-helix bundle is formed (B). The affinity of the internal fusion peptide results in the opening of the coiled-coil (E). In the second hypothesis (A, C, D, E), the N-terminal fusion peptide inserts into the donor membrane, the six-helical bundle is formed, and the internal fusion peptide is thereby localized near the target membrane (C). The internal fusion peptide inserts into the target membrane (D) followed by the relocation of the N-terminal fusion peptide from donor to target membrane and the opening up of the helical bundle (E). Taken with permission from Ghosh et al., 2000.

Figure 1.5. Cartoon depiction of the HIV (retrovirus) fusion glycoprotein (env). Legend details assignment of domains.

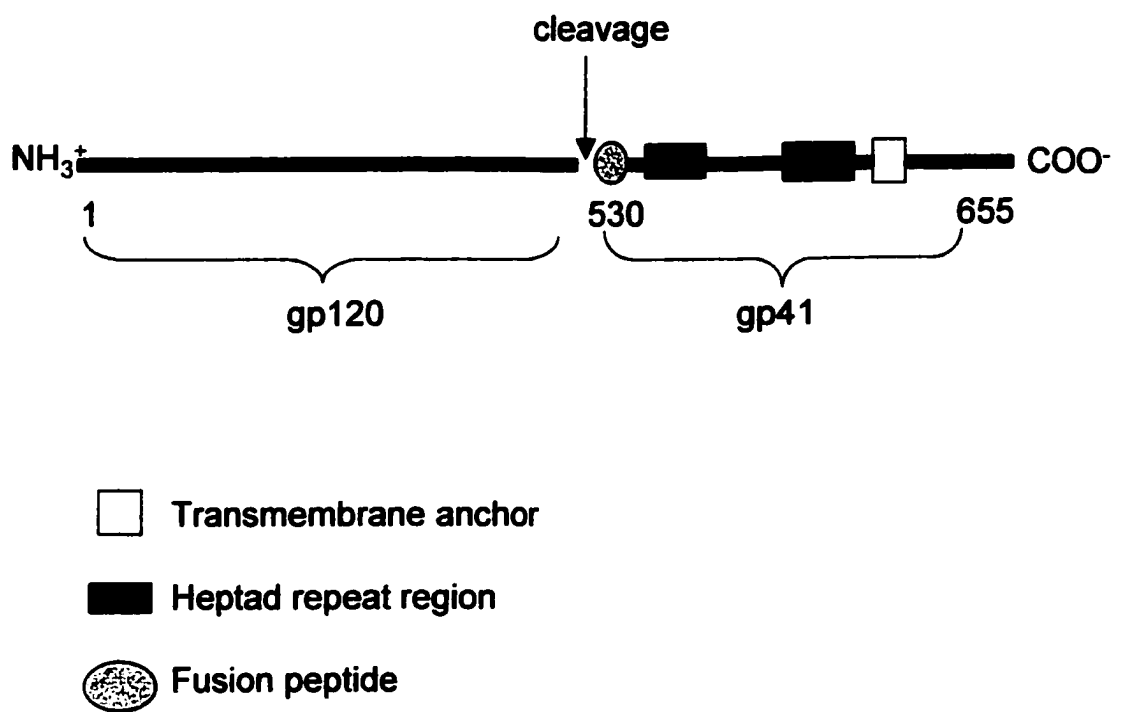
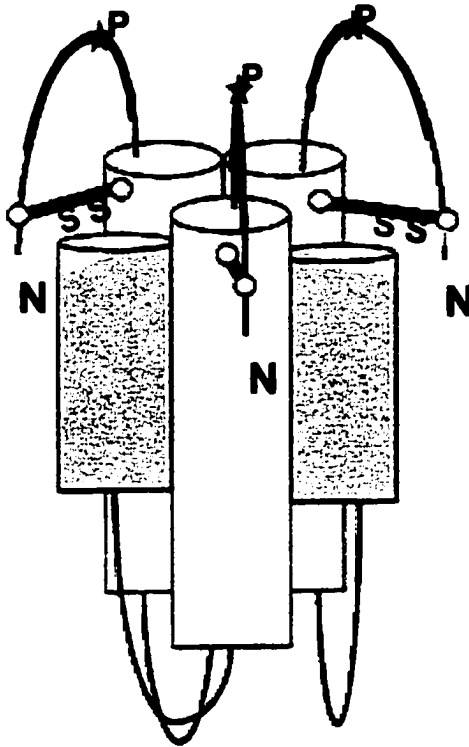


Figure I.5

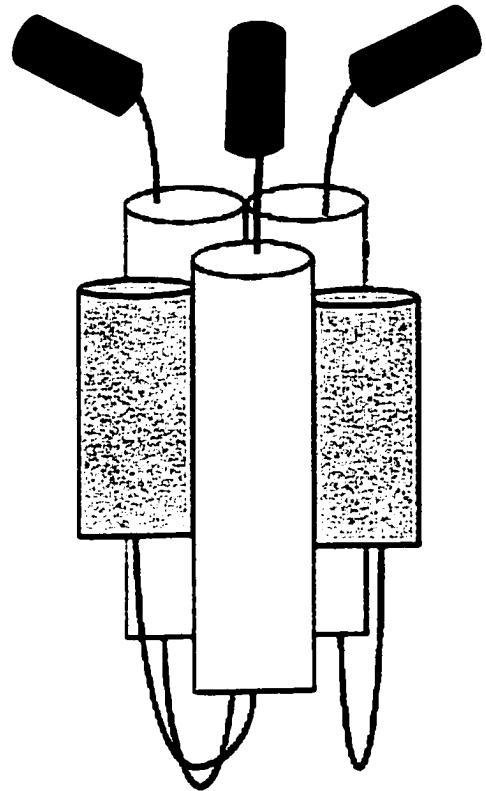
Figure 1.6. Cartoon depiction of the six helical bundle for viruses containing an internal fusion peptide (A) or an N-terminal fusion peptide (B).

A.



ASLV looped- structure
Internal fusion peptide

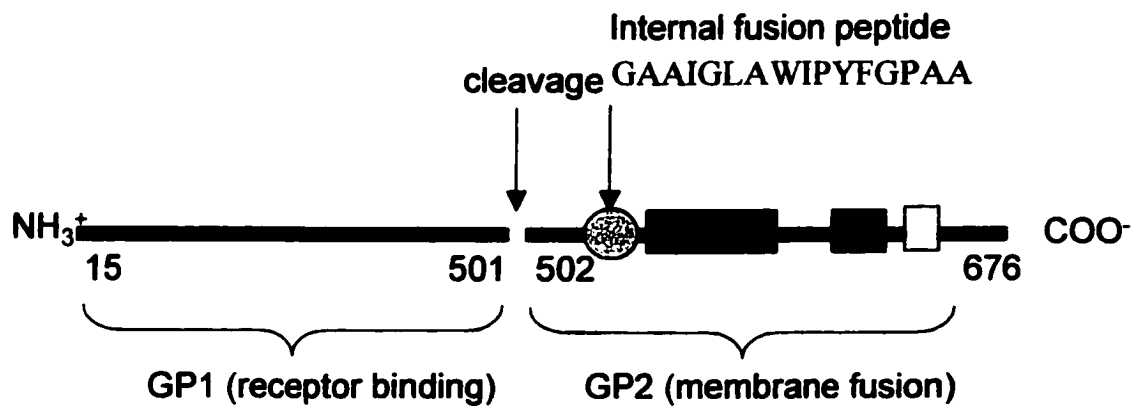
B.



α -helical N-terminal
fusion peptides

Figure 1.6

Figure 1.7. Cartoon depiction of the Ebola virus (filovirus) fusion glycoprotein (gpC). Legend details assignment of domains.



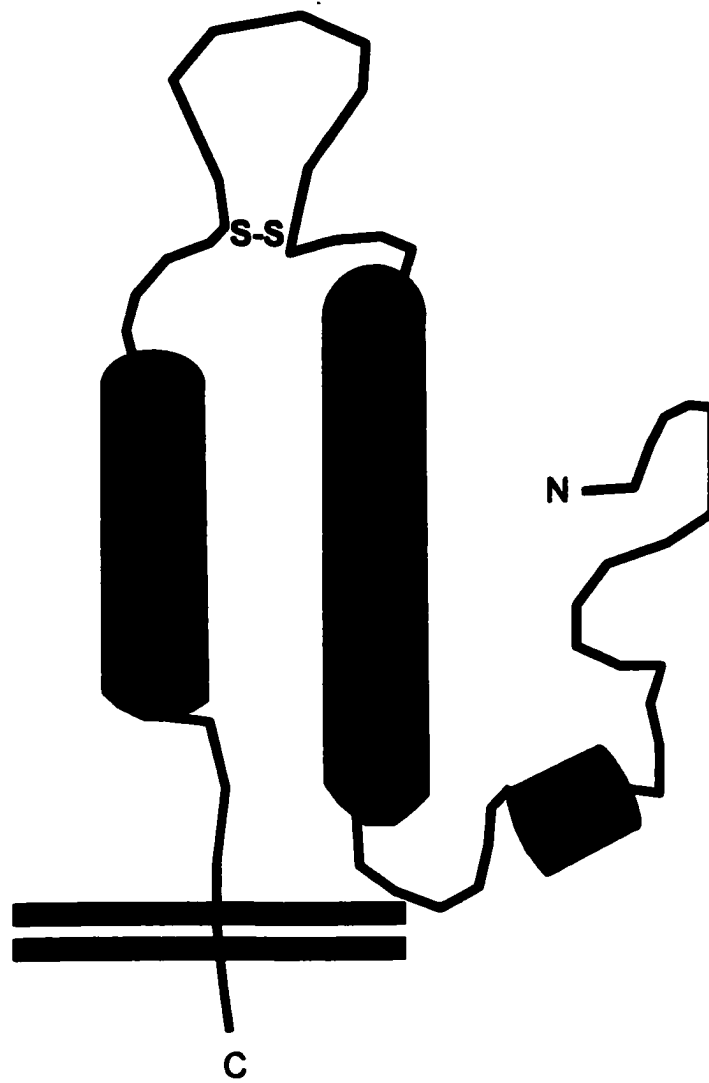
□ Transmembrane anchor

■ Heptad repeat region

● Fusion peptide

Figure I.7

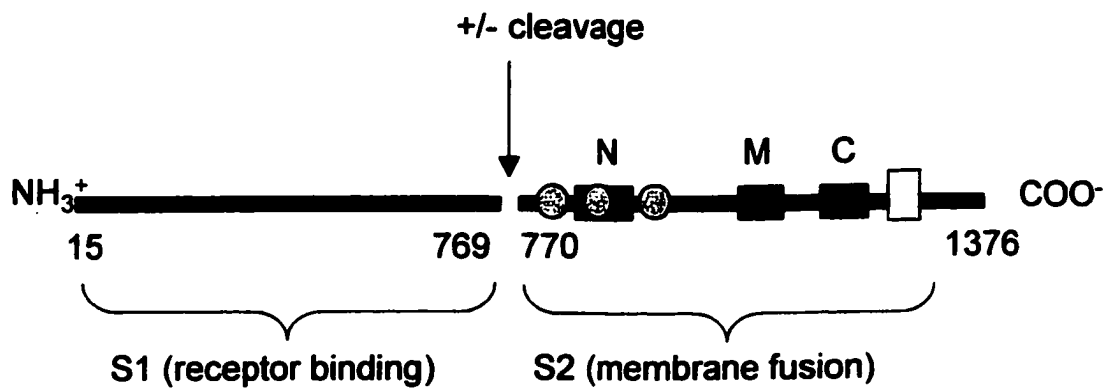
Figure 1.8. Cartoon depiction of a single monomer of the arenavirus fusion glycoprotein membrane anchored subunit (GP2). The two heptad repeats (black) predicted to form coiled-coil interactions are depicted.



GP2 of Lassa virus

Figure I.8

Figure 1.9. Cartoon depiction of the coronavirus fusion glycoprotein (S).
Legend details assignment of domains.






-  Transmembrane anchor
-  Heptad repeat region
-  Potential internal fusion peptide

Figure I.9

Figure 1.10. (A) Cartoon depiction of the togavirus fusion glycoproteins (E1/E2). One E1/E2 dimer is shown as well as the trimeric organization of E1/E2 dimers that form the final icosahedral symmetry. **(B) Model for low pH induced togavirus fusion with target membranes.** The E1/E2 dimers initially bury the fusion peptide, but low pH results in the dissociation into monomers and subsequent fusion peptide exposure and insertion into target membranes. The E1 subunits organize into trimers and induce membrane fusion.

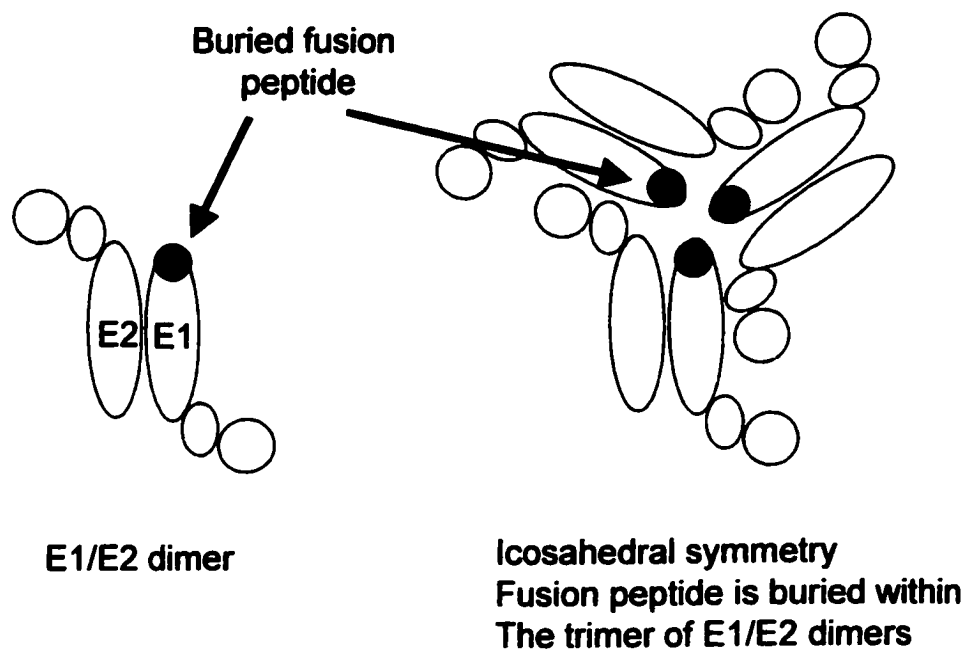
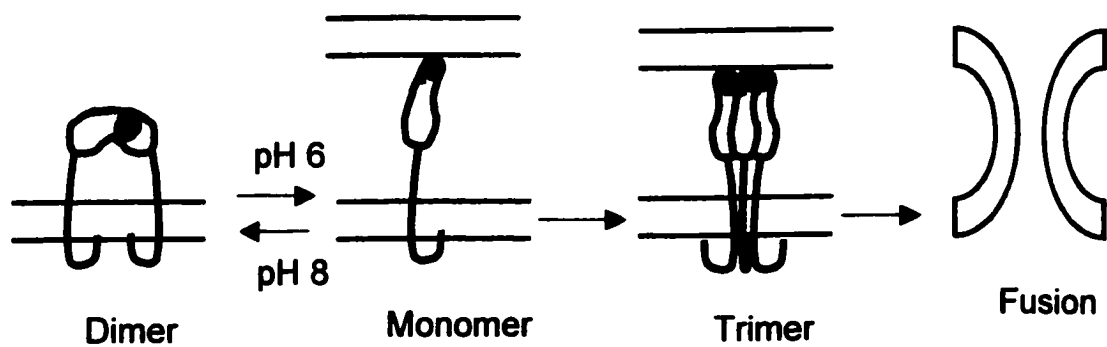
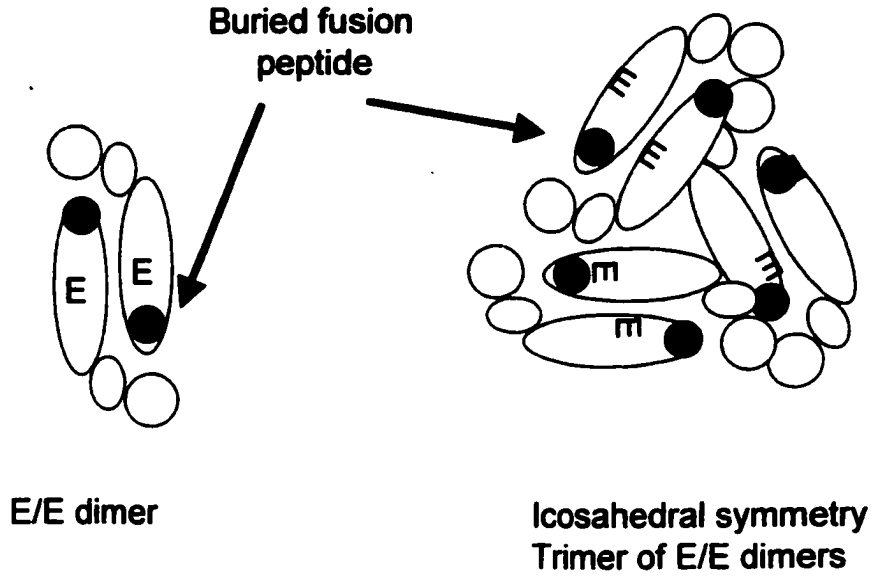
A**B**

Figure I.10

Figure I.11. (A) Cartoon depiction of the flavivirus fusion glycoproteins (E). One E homodimer is shown as well as the trimeric organization of homotrimers that form the final icosahedral symmetry. **(B) Assignment of roles to different domains of the E protein.** The predicted roles of each domain of the flavivirus E protein are summarized.

A



B

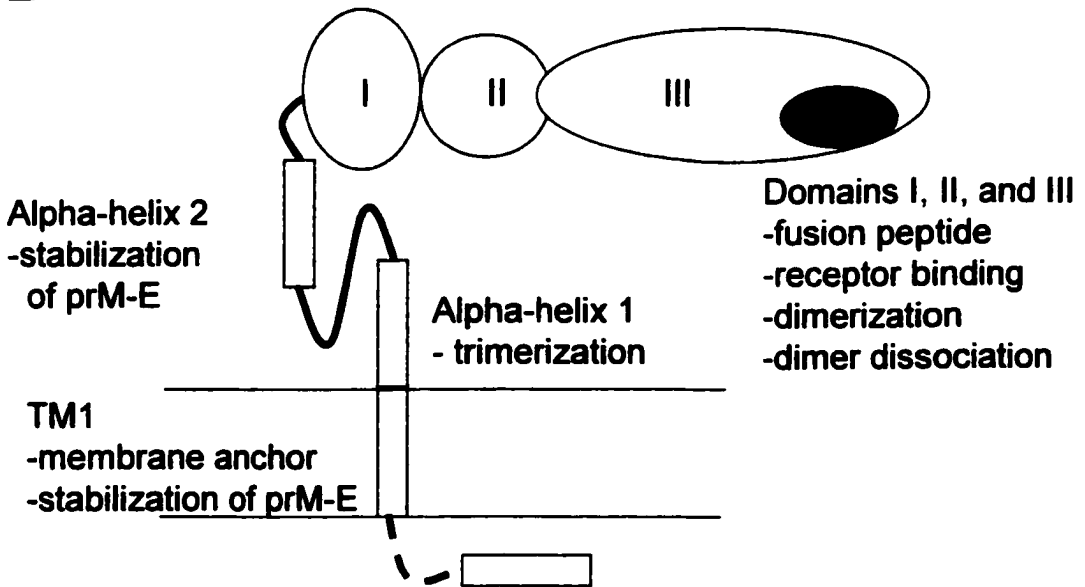


Figure I.11

Figure 1.12. Cartoon depiction of the low pH induced conformational changes within the rhabdovirus fusion glycoproteins (G) that mediate membrane fusion. In the native state, the fusion peptides are buried within the G homotrimer structure. Low pH induces conformational change resulting in fusion peptide exposure. Aggregation of several activated trimers can result in fusion. Alternatively, in the absence of aggregation the G protein undergoes further conformational change to the inactive state. All steps excluding the induction of complete fusion are reversible.

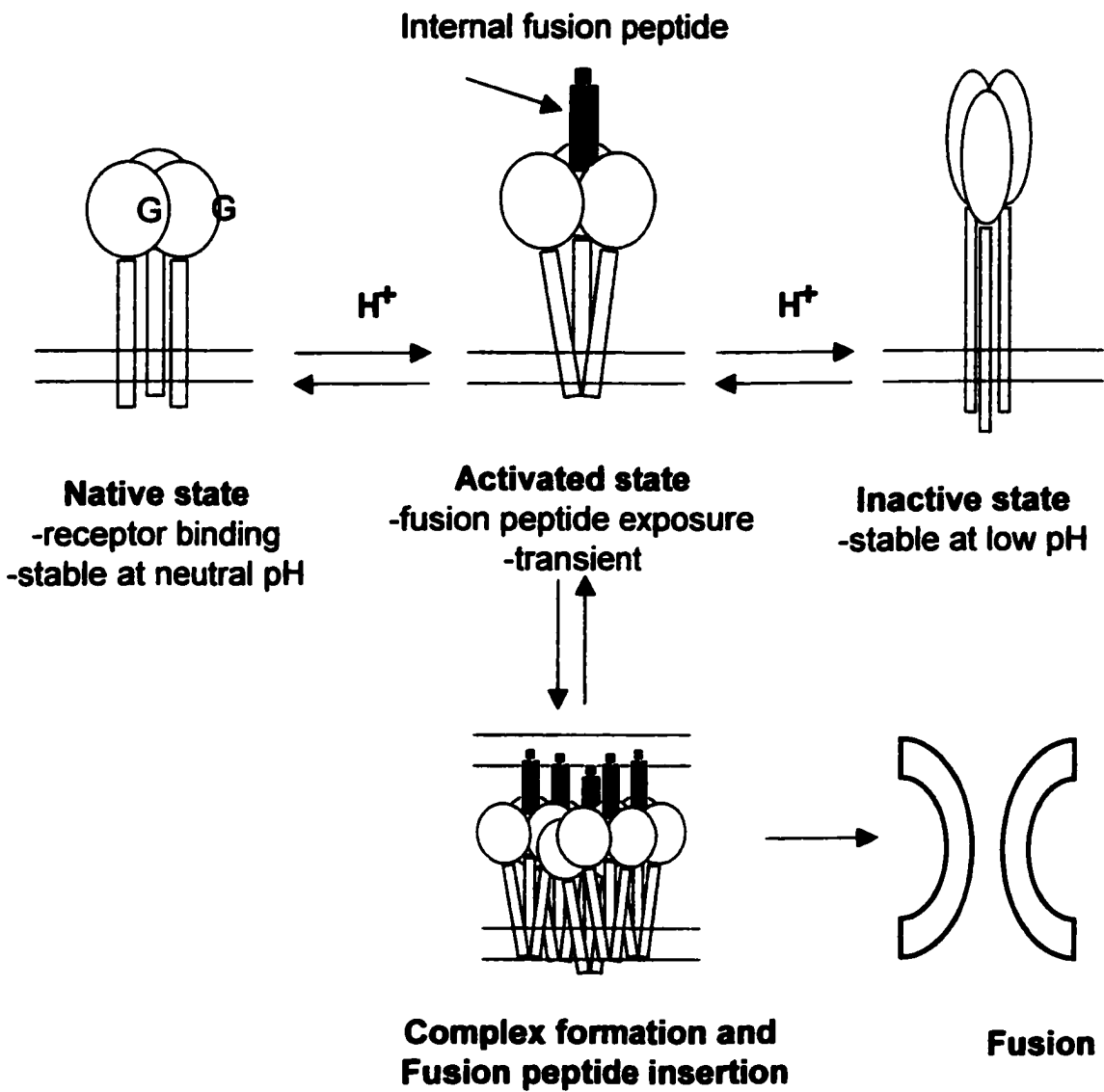


Figure I.12

Figure I.13. (A) Effects of different lipid shapes on membrane curvature. (B) Lipid rearrangements proposed to accompany membrane fusion.

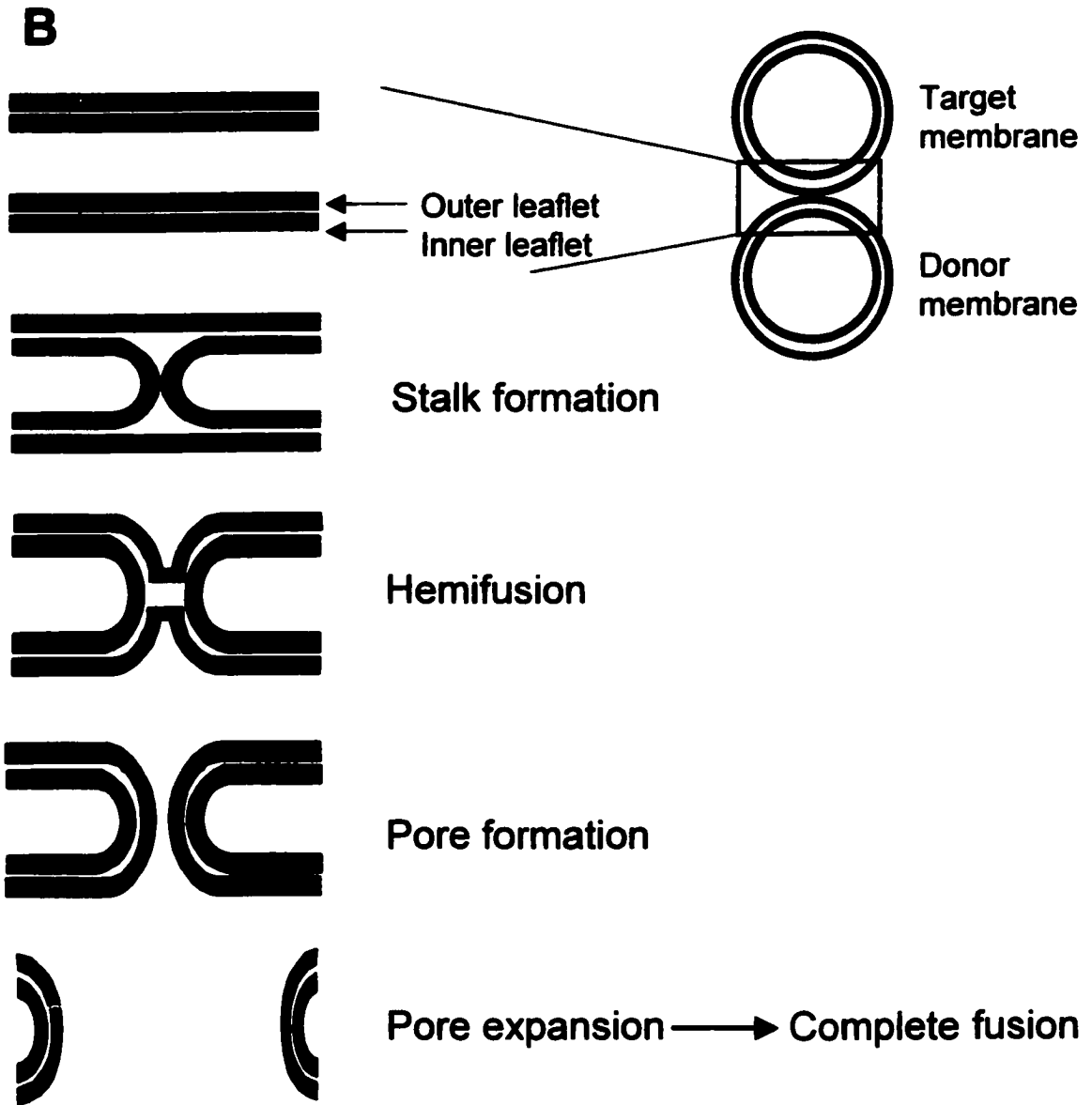
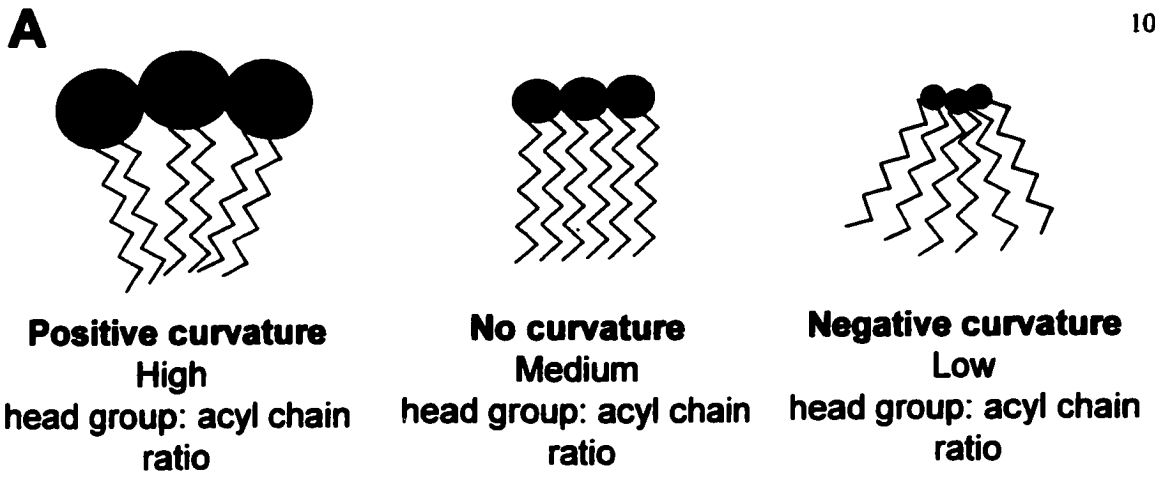


Figure I.13

Figure 1.14. Cartoon depiction of a single phospholipid with sites of cleavage catalyzed by phospholipids.

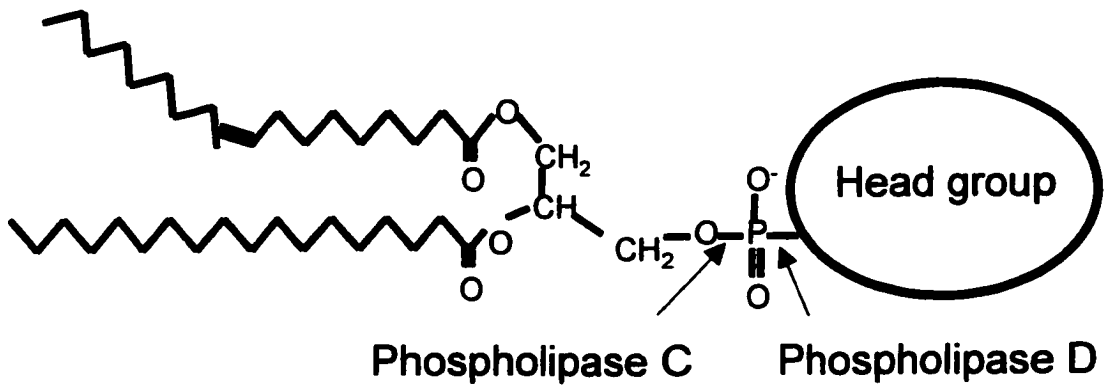


Figure I.14

**CHAPTER 1. A new class of fusion-associated small transmembrane
(FAST) proteins encoded by the nonenveloped fusogenic reoviruses**

1.A. ABSTRACT

The nonenveloped fusogenic avian and Nelson Bay reoviruses encode homologous 10-kDa nonstructural transmembrane proteins. The p10 proteins localize to the cell surface of transfected cells in a type I orientation and induce efficient cell-cell fusion. Mutagenic studies revealed the importance of conserved sequence-predicted structural motifs in the membrane association and fusogenic properties of p10. These motifs included a centrally located transmembrane domain, a conserved cytoplasmic basic region, a small hydrophobic motif in the N-terminal domain, and four conserved cysteine residues. Functional analysis indicated that the extreme C-terminus of p10 functions in a sequence-independent manner to effect p10 membrane localization, while the N-terminal domain displays a sequence-dependent effect on the fusogenic property of p10. The small size, unusual arrangement of structural motifs, and lack of any homologues in previously described membrane fusion proteins suggests that the fusion-associated small transmembrane (FAST) proteins of reovirus represent a new class of membrane fusion proteins.

1.B. INTRODUCTION

Structural and functional studies of enveloped virus fusion proteins, primarily the influenza virus HA fusion protein, has been instrumental in the development of a model for protein-mediated membrane fusion (Gaudin et al., 1995; Ramalho-Santos and de Lima, 1998). Similarities in the structural arrangement of the transmembrane-anchored polypeptides of several enveloped virus fusion proteins suggests that the working model for HA-mediated fusion may extend to many enveloped virus fusion proteins (Gaudin et al., 1995; Weissenhorn et al., 1997; Hughson, 1997; Joshi et al., 1998; Ben-Efraim et al., 1999). Furthermore, the studies of enveloped virus fusion proteins have been complemented by recent investigations of SNARE-mediated intracellular vesicular fusion events (Sollner et al., 1993; Sutton et al., 1998; Weber et al., 1998). The convergence of these two lines of investigation has suggested that the mechanism of action of both SNAREs and viral fusion proteins may be similar. In both cases, the energy required to overcome the thermodynamically unfavorable process of lipid leaflet mixing may be contributed by rearrangements of extended heptad repeats to generate coiled coil structures in membrane-anchored proteins (Weber et al., 1998; Skehel and Wiley, 1998). Although the generation of coiled coils is clearly an essential step in membrane fusion mediated by these viral and cellular proteins, the precise function of this interaction in the actual fusion reaction remains unclear (Ungermann et al., 1998; Otter-Nilsson et al., 1999). Furthermore, structural analysis of certain enveloped virus fusion proteins indicates that the paradigm of extensive coiled coil rearrangements is not

universal (Kielian, 1995; Rey et al., 1995). In spite of considerable study, the nature of the minimal fusion machinery and the precise sequence of events that regulate and mediate protein-mediated membrane fusion has not been discerned.

We have been investigating an unusual example of exoplasmic fusion - the induction of syncytium formation by a group of nonenveloped viruses, the fusogenic orthoreoviruses (Duncan et al., 1995, and references therein). The orthoreoviruses are one of nine genera in the family *Reoviridae*, a large diverse family of nonenveloped viruses with segmented dsRNA genomes (Nibert et al., 1996). The majority of the members of the *Reoviridae* do not induce cell fusion, a typical phenotype for nonenveloped viruses that do not require fusion proteins to facilitate virus entry or egress from cells. However, within the genus *Orthoreovirus*, all of the avian reovirus (ARV) isolates induce syncytium formation in cell culture (Kawamura et al., 1965). There are also two atypical mammalian reoviruses that share the syncytium-inducing properties of ARV, Nelson Bay virus (NBV) and baboon reovirus (BRV) (Gard and Compans, 1970; Duncan et al., 1995). The nature of the viral protein responsible for reovirus-induced cell fusion, and its mechanism of promoting membrane fusion, have not been determined.

We have previously shown that, unlike enveloped virus-induced membrane fusion, the mechanism responsible for ARV-induced cell fusion is not directly

related to either the viral entry or exit pathways (Duncan, 1996; Duncan et al., 1996). The primary purpose of the ARV fusion protein may be to direct cell-cell fusion, a process that contributes to a rapid lytic response and enhanced rate of virus release (Duncan et al., 1996). Since the ARV fusion protein is not required for virus entry or egress, it is conceivable that this accessory viral protein may not be subject to the mechanisms (i.e. ligand binding and/or low pH) that regulate the fusion activity of enveloped virus fusion proteins. Such a fusion protein might offer a simplified system for investigating the minimal determinants required for protein-mediated membrane fusion.

Using transfection studies, we have now identified the homologous fusion proteins of ARV and NBV. These 10-kDa nonenveloped virus fusion proteins are the smallest known viral or cellular fusion proteins. Moreover, the p10 proteins lack any extended heptad repeat structures or obvious fusion peptide motif typical of many enveloped virus fusion proteins. These simple fusion proteins appear to represent a new class of membrane fusion proteins whose structural features indicate that they mediate membrane fusion through a coiled coil-independent pathway.

1.C. RESULTS

1.C.1. ARV and NBV encode 10-kDa nonstructural proteins that are responsible for cell fusion

Previous genetic studies implicated the S1 genome segment in the fusogenic activity of ARV (Duncan and Sullivan, 1998). The genetic implication of the ARV S1 genome segment in reovirus-induced syncytium formation was confirmed by expressing the full length cloned S1 cDNA in transfected cells (Figure 1.1, panel a). Similar results were obtained by expression of the S1 cDNA of the related NBV (Figure 1.1, panel b). None of the other cloned S-class genome segment cDNAs of ARV or NBV were capable of inducing cell fusion in transfected cells (data not shown), indicating that a S1-specific gene product was responsible for syncytium formation.

The S1 genome segment of ARV and NBV contains three sequential, overlapping open reading frames (ORFs) (Kool and Holmes, 1995). Only the 3'-terminal ORF has previously been shown to be functional. This ORF encodes the receptor-binding protein of ARV, σ C (Varella and Benavente, 1994; Shapouri et al., 1996; Martinez-Costas et al., 1997), that was previously implicated in syncytium formation (Theophilos et al., 1995). However, expression of the σ C ORF of either ARV or NBV in transfected quail cells, as revealed by immunostaining (Figure 1.1, panels e and f), failed to induce syncytium formation. Identical results were obtained in σ C-transfected COS-7 and Vero cells (data not shown). Conversely, expression of the 5'-terminal S1 ORF alone

(which encodes a predicted 10-kDa protein) resulted in cell-cell fusion (Figure 1.1, panels c and d), implying that a previously unidentified reovirus protein (p10) was responsible for the fusogenic property of the virus. Interestingly, the polyclonal antisera raised against purified virus particles failed to stain syncytial foci induced by transfection of the p10 ORF alone (Figure 1.1, panels c and d), suggesting that the predicted p10 protein might be a nonstructural protein of the virus.

We confirmed that the first ORF of the S1 genome segment encodes a 10-kDa protein responsible for cell-cell fusion using specific antiserum. Polyclonal antiserum was raised against the C-terminal half of the predicted ARV p10 protein by expression in *E. coli* as a chimeric maltose-binding protein (MBP)/p10 construct. The p10 antiserum precipitated a 10-kDa protein from radiolabeled transfected and infected cell lysates (Figure 1.2A). The specificity of the p10 antiserum was evident from the lack of significant cross reaction with other ARV structural, or nonstructural, proteins (Figure 1.2A, lane 6). The low level of ARV structural proteins precipitated by the anti-p10 antiserum reflects non-specific trapping of radiolabeled virus particles, as evident by a similar degree of trapping when using normal rabbit serum (Figure 1.2A, lane 6 vs. lane 13). These results confirmed that the 5'-terminal ORF of the reovirus S1 genome segment is indeed functional, and encodes a 10-kDa protein that is responsible for virus-induced cell fusion. The ARV p10 antiserum did not cross react with the NBV protein

(data not shown), therefore, all subsequent experiments were performed with ARV alone.

As added proof that the ARV p10 protein is responsible for cell fusion, the sub-optimal translation start site for the p10 ORF was modified to an optimal context (from CGUCAUGC to CCACCAUGG), which resulted in both enhanced syncytium formation (Figure 1.2B) and increased p10 expression (Figure 1.2A, lane 4 vs. lane 5). The level of p10 expression from the optimized construct was still less than that observed in cell lysates infected with limiting virus dilutions that generated approximately equivalent numbers of syncytial foci as observed in transfected cells (Figure 1.2A, lane 5 vs. lane 6, and Figure 1.2B, panels b and c), indicating that cell fusion mediated by the p10 ORF alone was not an artifact of protein over-expression. These results conclusively demonstrated that ARV, and by inference, NBV, encode the smallest known viral fusion-associated proteins.

The inability of polyclonal antiserum specific for ARV structural proteins to immunostain p10-transfected cells (Figure 1.1, panels c and d), or to precipitate radiolabeled p10 from infected or transfected cell lysates (Figure 1.2A, lanes 8-10) suggested that, unlike all enveloped virus fusion proteins (Bentz, 1993), the reovirus p10 protein might be a nonstructural protein of the virus. This speculation was confirmed by the inability of the p10 antiserum to detect p10 in radiolabeled virus particles. Virus particles were disrupted with SDS and heat (to

expose inner, as well as outer, capsid proteins), and the solubilized proteins were immune precipitated using the p10-specific antiserum. Contrary to the ability of the polyclonal anti-ARV serum to recognize the known λ -, μ -, and σ -class virus structural proteins, the p10 antiserum failed to precipitate any protein present in the virus pellets (Figure 1.3). The absence of p10 in the virus pellets was apparent even after extended autoradiographic exposure of the gels (Figure 1.3, lanes 7-9), whereas the minor σ C receptor-binding protein of the virus, present at only 36 copies per virus particle (Strong et al., 1991; Shapouri et al., 1996), was clearly detected. In addition, the ability of the p10 antiserum to precipitate SDS-denatured p10 obtained from whole cell lysates (Figure 1.3, lane 5) indicated that the inability of this serum to precipitate p10 from solubilized virus pellets was not the result of p10 epitope destruction due to SDS-denaturation. The cumulative evidence strongly implies that p10 is not only the first nonenveloped virus protein capable of promoting fusion-from-within, it is also the first nonstructural virus protein capable of inducing cell-cell fusion.

1.C.2. Sequence-predicted structural motifs in the p10 fusion proteins

Assuming that ARV p10 initiates from the first in-phase methionine codon (there are two methionine codons at residue one and four in the predicted p10 ORF of ARV; both exist in a sub-optimal initiation consensus sequence), then the aligned ARV and NBV p10 proteins possess an overall sequence identity of only 33%, with an obvious clustering of conserved residues in the N-proximal domain of p10. Both proteins are small (98 or 95 amino acids for ARV and NBV,

respectively), hydrophobic, and basic ($pI = 8.8$). A gapped BLAST search failed to identify any known homologues of p10. The p10 proteins possess no identifiable N-terminal signal peptide but they do have a predicted 19 residue transmembrane (TM) domain in the center of each sequence (Figure 1.4) that could serve as a signal/anchor sequence (Zheng and Gierasch, 1996; Martoglio and Dobberstein, 1998). This highly hydrophobic 19 amino acid sequence was identified as a transmembrane (TM) domain using the TMAP algorithm of Persson and Argos (1994). The majority of the basic residues reside in a conserved basic region on the C-proximal side of the TM domain, suggesting that the p10 proteins assume a type I orientation (N-terminus out) based on the positive-inside rule (Matlack et al., 1998).

The ARV and NBV p10 proteins contain four cys residues in conserved locations, two immediately adjacent to the C-terminus of the TM domain, and the other two located in the N-proximal domain of p10 (Figure 1.4). The two conserved cys residues in the N-proximal domain reside adjacent to the most conserved region of the ARV and NBV p10 proteins. These cys residues lie near the ends of a 16 amino acid region (residues 9-24 in ARV) that can be modeled as a short, moderately hydrophobic helix. This small hydrophobic region is the only portion of the p10 proteins that bears any resemblance to a fusion peptide motif. However, the overall hydrophobicity of this region is considerably less than that of the N-terminal fusion peptides of enveloped viral fusion proteins as determined using the normalized consensus scale of Eisenberg (1984) (0.29 for ARV and

0.41 for NBV, vs. an average of 0.61 for enveloped virus fusion peptides) (White, 1990). Moreover, the p10 hydrophobic helix does not display an obvious amphipathic nature or preference for bulkier amino acids on one side of the helix, common features of enveloped virus fusion peptides (reviewed in White, 1990). If this region does function as a fusion peptide by directly interacting with the phospholipid bilayer of target membranes, then it represents an unusual fusion peptide motif.

1.C.3. The reovirus p10 protein is a surface-localized type I transmembrane protein

If the reovirus p10 fusion-associated proteins directly contribute to membrane fusion, then one would expect that these proteins should be surface-localized transmembrane proteins. Immune precipitation of the membrane fraction from ARV-infected cells clearly indicated that p10 exists exclusively in the membrane pellet (Figure 1.5). As a control, antiserum specific for a major outer capsid protein of the virus, $\mu 2$, was used to demonstrate that this soluble viral protein resided entirely in the supernatant fraction (Figure 1.5, lanes 10 and 11), indicating that the membrane pellet was devoid of detectable contamination with the soluble fraction of the cell lysate. Moreover, the removal of proteins peripherally associated with the membrane fraction by extraction with high salt and pH did not remove p10 (Figure 1.5, lane 7) indicating that p10 is an integral membrane protein, consistent with the presence of a predicted central transmembrane domain (Figure 1.4).

To assess the membrane orientation and surface localization of p10, the N- and C-termini of the ARV p10 protein were tagged using the influenza virus HA epitope, and an anti-HA monoclonal antibody was used for immunofluorescent-staining of permeabilized and non-permeabilized transfected cells (Figure 1.6). The N- and C-terminal epitope tags had no significant effect on p10-induced syncytium formation (see Figure 1.7). Immunofluorescent staining of permeabilized transfected cells revealed a diffuse punctate staining pattern in the cytoplasm of syncytial cells generated by transfection with either modified p10 construct (Figure 1.6, panels a and b), indicating that both proteins were expressed in transfected cells. Staining of nonpermeabilized cells transfected with the N-tagged p10 construct revealed fluorescent staining of the periphery of syncytial foci (Figure 1.6, panel c), clearly indicating the presence of cell surface-localized p10. Conversely, no specific fluorescence was detected in nonpermeabilized cells expressing the C-terminal tagged p10 construct (Figure 1.6, panel d), confirming the surface-specificity of the fluorescence observed with the N-terminal tagged p10 construct. These results indicated that p10 localizes to the cell surface in a type I (N-out) orientation.

Although surface immunofluorescence successfully detected p10 on the cell surface, the inability to detect p10 by other methods including cellular enzyme linked immunoadsorbant assay (CELISA), complement lysis, and flow cytometry suggested that surface localized p10 was scarce. Cytopins made from p10-

expressing immunostained cells showed that surface localization was detectable by optimized microscopic capture despite being below detectable limits for flow cytometry (Figure 1.6B). FITC-conjugated secondary antibody alone or an isotype control secondary antibody used along with anti-HA antibodies showed no background immunofluorescence. The anti-HA antibodies did result in low nonspecific staining of untransfected cells (white arrows), although fluorescence from p10-transfected cells was clearly stronger (yellow arrows). Titration of primary and secondary antibodies was attempted to optimize fluorescence for flow cytometry, and clearly showed differences when cells were photographed by microscopy (Figure 1.6C). Despite the ability to detect p10 by microscopy and the expert assistance of Dr. Marshall's laboratory, detection by flow cytometry failed (data not shown). The low level of p10 on the cell surface contrasts with the enveloped virus fusion proteins, which accumulate in abundance on the surface of infected cells.

As further evidence of the surface localization and membrane orientation of p10, we used the anti-HA monoclonal antibodies in a syncytial inhibition assay. Both the N- and C-terminally tagged p10 constructs induced syncytium formation in transfected cells (Figure 1.7, panels a and b). Addition of the anti-HA monoclonal antibody to the medium on transfected cells abrogated syncytium formation induced by the N-terminally modified p10 construct (Figure 1.7, panel c), but had no effect on syncytium formation induced by the C-terminally tagged p10 construct (Figure 1.7, panel d). These results confirmed the type I surface

orientation of p10, and provided evidence that p10 might be directly involved in the fusion reaction.

1.C.4. Mutational analysis of the reovirus p10 fusion proteins

In order to assess the significance of the sequence-predicted structural motifs we identified in p10, a series of amino acid substitutions were engineered into the ARV p10 protein, and the fusogenic activity and membrane association of the altered proteins was determined. The results obtained from HA-tagging indicated that alteration of the termini of p10 had no effect on the fusogenic activity of the protein (Figure 1.8). However, deletion of the extreme N- or C-terminus of p10 abrogated the fusion-inducing ability of the protein (Figure 1.8). Deletion of the N-terminal domain did not affect p10 membrane association, indicating that the N-terminal domain influences the functional structure of the protein independent of its membrane association. Interestingly, while deletion of the ARV C-terminus eliminated both the membrane association and fusogenic capability of p10, substitution of the ARV C-terminus with the non-conserved C-terminus of NBV (Figure 1.8), or with the HA tag (Figure 1.8) restored both properties. The C-terminal domain of p10 apparently functions in a sequence-independent manner to effect p10 membrane association.

Since the N- and C-terminal domains of p10 are physically separated in distinct subcellular environments by the intervening TM domain and are likely to fold independently, it might be expected that alterations in the N-terminal domain

should not affect the folding of the C-terminal domain. In conjunction with the extensive sequence conservation between the ARV and NBV p10 N-terminal domains, and the presence of conserved structural motifs in this region, we anticipated that the N-terminal domains of ARV and NBV should be interchangeable. However, this was found not to be the case; substitution of the ARV N-terminal domain with that of NBV eliminated the fusogenic property of p10 but did not influence membrane association (Figure 1.8). This somewhat surprising result suggested that the N-terminal domain of p10 functions in a sequence-dependent manner, and in concert with the TM and/or C-terminal domains of p10, to influence p10 structure or function.

To evaluate the role of the conserved cys residues in p10 membrane localization and fusion, site-specific substitutions were engineered into the p10 protein. Alteration of the N-terminal cys residues (C9A and C21S constructs) ablated the fusogenic property of p10 (Figure 1.8). The substitution of cys21 by ser, an alteration that conserves both hydrophobicity and mass, suggested an essential requirement for a cys residue in this location. These cys residues are unlikely to mediate disulfide-stabilized dimer formation of p10 since the electrophoretic mobility of p10 was not altered under non-reducing conditions (data not shown). Similarly, the importance of the conserved di-cysteine motif adjacent to the TM domain was confirmed by substitution analysis. A single substitution (C63S) of the di-cysteine motif reduced, but did not abrogate, p10-induced cell fusion while alanine substitution of both cys residues (C63/64A) eliminated the fusogenic

properties of p10 (Figure 1.8). Alteration of these cys residues did not affect p10 membrane association.

Site-directed substitutions were also engineered into the conserved basic region and TM domain of p10 (Figure 1.8). Conservative substitution of basic residues in the C-terminal basic region (K69R and R79K) had no effect on p10 membrane fusion, while a non-conservative substitution (K69M) eliminated p10-induced fusion (Figure 1.8). A conservative substitution in the predicted TM domain (V55F) had no effect on p10 function, while conservative substitutions in the conserved polyglycine region of the TM domain (G49A and G49/50A) eliminated p10-induced syncytium formation. Interestingly, the substitutions in the polyglycine region and the non-conservative substitution in the basic region, all of which eliminated the fusogenic activity of p10, did not affect p10 membrane association. These results indicated that minor alterations to the TM domain and basic region in p10 alter protein structure or function and affect the fusogenic property of p10 independent of the influence of these regions on p10 membrane association.

1.D. DISCUSSION

The analysis of the influenza virus hemagglutinin, and of the cellular SNARE proteins involved in constitutive vesicle transport and regulated exocytosis, has contributed to the development of a model for protein-mediated membrane fusion (Carr and Kim, 1993; Rothman, 1994; Pfeffer, 1996; Weimbs et al., 1997; Weber et al., 1998; Skehel and Wiley, 1998). Structural and functional studies suggest that the rearrangement of extended heptad repeat structures in membrane-anchored fusion proteins may function to supply the energy required to overcome the thermodynamic barriers that prevent spontaneous membrane fusion. This current model is unlikely, however, to be the complete story since certain viral fusion proteins do not conform to the current paradigm of membrane fusion induced by enveloped virus fusion proteins. For example, although the 14 kDa fusion protein of vaccinia virus contains a coiled coil motif, this small atypical fusion protein lacks an identifiable fusion peptide and is anchored in membranes not through a transmembrane domain, but via interactions with another vaccinia-encoded protein (Vazquez et al., 1998). Furthermore, structural analysis of the fusion proteins of various alphaviruses and flaviviruses indicates that a requirement for extensive rearrangements mediated by heptad repeats is not universal (Kielian, 1995; Rey et al., 1995). Consequently, alternative models of protein-mediated membrane fusion need to be developed.

The unusual properties of the ARV and NBV fusion proteins described in this report are without precedent amongst the viral and cellular proteins implicated in

membrane fusion. In conjunction with the absence of any identifiable homologues, the unique structural features of the reovirus p10 proteins suggests that these fusion-associated small transmembrane (FAST) proteins represent a new class of membrane fusion-inducing proteins, the first example of nonstructural proteins encoded by a nonenveloped virus that are capable of inducing fusion-from-within. The FAST proteins contain only a small 39-43 amino acid ectodomain that lacks an extended heptad repeat, therefore, the extensive conformational changes that accompany membrane fusion induced by certain enveloped virus fusion proteins are unlikely to be possible in these simple fusion-inducing proteins. How such a simple protein could overcome the thermodynamic barriers to membrane fusion is presently unknown, although it seems clear that the FAST proteins are likely to use a novel mechanism to promote membrane fusion.

Our results indicate that the FAST proteins are the only reovirus proteins required to promote syncytium formation. It is not possible, however, to state that the FAST proteins function independently to induce membrane fusion and, hence, are true fusion proteins *per se*. It is conceivable, for example, that the FAST proteins might function indirectly to effect cell-cell fusion, possibly through the auspices of an unidentified host factor. However, the ability of a viral protein to indirectly trigger a cellular fusion response has never been reported. Furthermore, actinomycin D inhibits host cell transcription, but has no effect on reovirus transcription or on virus-induced syncytium formation (Ni and Ramig,

1993; Duncan, unpublished). Consequently, the FAST proteins would need to modulate the activity of a pre-existing host factor that never functions independently to promote exoplasmic fusion, but is capable of doing so only in the presence of p10. Such a scenario seems unlikely. It seems more probable that the FAST proteins are, in fact, fusion proteins that directly contribute to lipid bilayer mixing. This contention is supported by the cell surface localization of p10, and by the ability of HA monoclonal antibodies to abrogate syncytium formation induced by the N-terminally tagged p10 construct. Direct evidence that p10 alone is sufficient to induce membrane fusion will require demonstrating that purified p10 promotes fusion of pure phospholipid bilayers. Such studies are currently underway.

Our preliminary sequence and functional analyses of the reovirus fusion-associated proteins provide the basis for a working model of p10 structure and function (Figure 1.9). The p10 proteins are surface-localized type I transmembrane proteins. Our observation that brefeldin A, an inhibitor of vesicular transport, abrogates ARV-induced cell fusion (Duncan et al., 1996) is consistent with p10 transport through the ER-Golgi pathway (Einfeld and Hunter, 1991). The deletion and substitution analysis of the C-terminus of p10 suggests that p10 localization to the ER most likely occurs via a signal-recognition particle (SRP)-dependent targeting mechanism, mediated by the TM domain serving as a signal-anchor sequence (Pugsley, 1990; Zheng and Gierasch, 1996; Wilkinson et al., 1997; Matlack et al., 1998). This conclusion is based on the absence of a

cleavable N-terminal signal peptide in p10 (Martoglio and Dobberstein, 1998), and on the fact that deletion of the nonconserved C-terminus eliminates p10 membrane association while substitution of this region with the NBV C-terminus or with an HA epitope restores both p10 membrane association and fusion. The fact that substitution of the C-terminal portion of p10 with heterologous sequences restores membrane association indicates that this region functions in a sequence-independent manner to effect targeting of p10 to the membrane fraction of cells. Since the SRP only recognizes nascent signal peptides, approximately 30-40 amino acids (the length of polypeptide protected by a translating ribosome and the approximate length of the p10 C-terminal domain) must lie on the C-proximal side of the signal/anchor peptide to allow it to be exposed on the surface of the ribosome for interaction with the SRP particle (Pugsley, 1990). Therefore, we suggest that the C-terminal tail of p10 may serve as a "stuffer" to permit SRP-dependent ER insertion. To confirm our prediction, an N-terminal signal peptide could be added to p10 devoid of the C-terminal non-conserved residues. If addition of the signal peptide restores membrane insertion and orientation and, therefore, substitutes for the function of the C-terminal non-conserved region, then the "stuffer" hypothesis would be strengthened.

Additional mutagenic analyses demonstrated the importance of several conserved motifs present in the ARV and NBV p10 proteins. A conservative substitution in the predicted TM domain (V55F) had no effect on syncytium

formation, while a single alteration to the polyglycine region in the TM domain (G49A) eliminated cell fusion but did not affect p10 membrane association. The ability to disrupt the fusogenic property of p10 without altering p10 membrane association suggests that the TM domain may serve as more than just a signal-anchor, either by destabilizing the donor membrane, as suggested by studies with GPI-anchored HA which promote hemifusion but not complete fusion (Kemble et al., 1994), or by promoting functional p10 folding or multimer formation as occurs with several integral membrane proteins (McGinnes et al., 1993; Lemmon et al., 1994; Shai, 1995; Mingarro et al., 1996; Burke et al., 1997). Chapter four describes more detailed analysis of the transmembrane domain and suggests a direct role in the mechanism of p10-mediated fusion.

The majority of the basic residues present in p10 reside in the cytoplasmic domain, immediately adjacent to the predicted TM domain (Figure 1.4). These basic residues likely contribute to the type I orientation of the protein. However, the presence of a basic domain adjacent to a TM domain is a hallmark feature of a large group of small membrane proteins, the viroporins (also referred to as holins in bacteriophages), encoded by numerous enveloped and nonenveloped viruses (Carrasco, 1995; Young, 1992). Viroporins appear to contribute to cellular membrane destabilization, possibly as a means to promote virus exit from cells (Tollefson et al., 1996; Tiganos et al., 1998). Our preliminary mutagenic analysis implicates the basic region in p10 function independent of any role it might have on p10 membrane association. Conservative changes in the p10 cytoplasmic

basic domain had no effect on p10 function, while a single nonconservative substitution (K69M) eliminated the fusogenic activity, but not p10 membrane association. Since the cytoplasmic, TM, and extracellular domains of transmembrane proteins generally fold independently (Doms et al., 1993), it is likely that a single substitution in the basic domain of p10 would have only local effects on p10 structure. It is conceivable, therefore, that the p10 basic domain may not only influence the membrane orientation of the protein, but may also contribute to destabilization of the donor lipid bilayer, analogous to the viroporins. A concerted mutagenic analysis of the basic region in the context of the N-terminal HA tagged construct is presented in chapter four and reveals the influence of this region on the relationship between p10 membrane localization and membrane fusion.

Alteration of the conserved cys residues in p10 reduced, or eliminated, the fusion-inducing property of p10 but did not affect p10 membrane association. The two conserved cys residues in the predicted cytoplasmic domain of p10, immediately adjacent to the TM domain (Figure 1.9), may be palmitoylated, similar to the situation with the adenovirus death protein (Hausmann et al., 1998). Although several enveloped virus fusion proteins are also palmitoylated on membrane-proximal cys residues, the role for palmitoylation in the fusion activity of enveloped virus fusion proteins is variable (Yang et al., 1995; Veit et al., 1996; Ryan et al., 1998; Fisher et al., 1998). Chapter four provides a more thorough analysis of these conserved intracellular cys residues. Similarly, alteration of

either of the two cys residues flanking the small hydrophobic region in the N-terminal domain (Figure 1.9) abrogated p10-induced cell fusion. This is the only region of p10 that bears any resemblance to a fusion peptide motif, containing a moderately hydrophobic short heptad repeat structure that might exist in a membrane-seeking helical conformation. However, the biophysical properties of this region are quite distinct from any previously characterized fusion peptides from enveloped virus fusion proteins. Chapter three provides evidence for identification of this region as a fusion peptide and provides rationale for the unique characteristics of the p10 fusion peptide.

The FAST proteins of the fusogenic reoviruses are clearly distinct from any previously identified fusion-inducing proteins, and may offer a minimalist model for investigating the mechanism of protein-mediated membrane fusion. The FAST proteins are not directly involved in virus entry or exit from cells, and appear to be nonessential proteins of the virus whose sole, or primary, purpose is to promote membrane fusion (Duncan, 1996; Duncan et al., 1996). The accessory nature of the FAST proteins may have afforded these nonstructural viral fusion-inducing proteins the ability to evolve a simplified structure with a specialized purpose. In addition, since they do not directly contribute to virus entry or exit, their fusion activity may not be subject to the triggering mechanisms that regulate the fusogenic activity of enveloped virus fusion proteins. The absence of a requirement for regulated fusion would further permit these novel fusion proteins to simplify their domain organization to include the minimal

determinants required to direct membrane localization, destabilization, and fusion. The next chapter (chapter two) will discuss the rationale for, and data indicating, that p10 has evolved under unique limitations and will provide a more direct means of identifying the minimal fusion machinery aside from the complexities associated with regulation, specificity, efficiency and rapidity of the membrane fusion process.

1.E. CHAPTER 1 FIGURES

Figure 1.1. The p10 open reading frame of the S1 genome segment is sufficient for fusion. A schematic representation of the S1 genome segments of ARV and NBV is presented at the top of the figure. The three sequential overlapping open reading frames encoding the 10-kDa fusion protein, a putative 17-kDa protein, and the cell attachment protein, σ C, are indicated, along with the nucleotide positions corresponding to the first position of the start codon and the last position of the open reading frame. The lower part of the figure represents transfected QM5 cells immunostained using antibodies raised against the structural proteins of ARV (a, c, e) or NBV (b, d, f). The monolayers were transfected with expression plasmids encoding the ARV or NBV S1 segment (a and b, respectively), the ARV or NBV p10 protein (c and d, respectively), or the ARV or NBV σ C protein (e and f, respectively). Arrows in panels a and b indicate syncytial foci that stained antigen-positive due to the presence of the σ C protein expressed from the full-length S1 cDNA, while those in panels (c) and (d) indicate the location of syncytia induced by transfection of the p10 open reading frame alone that failed to react with the polyclonal antisera against virus structural proteins. Cells were photographed at 100x magnification.

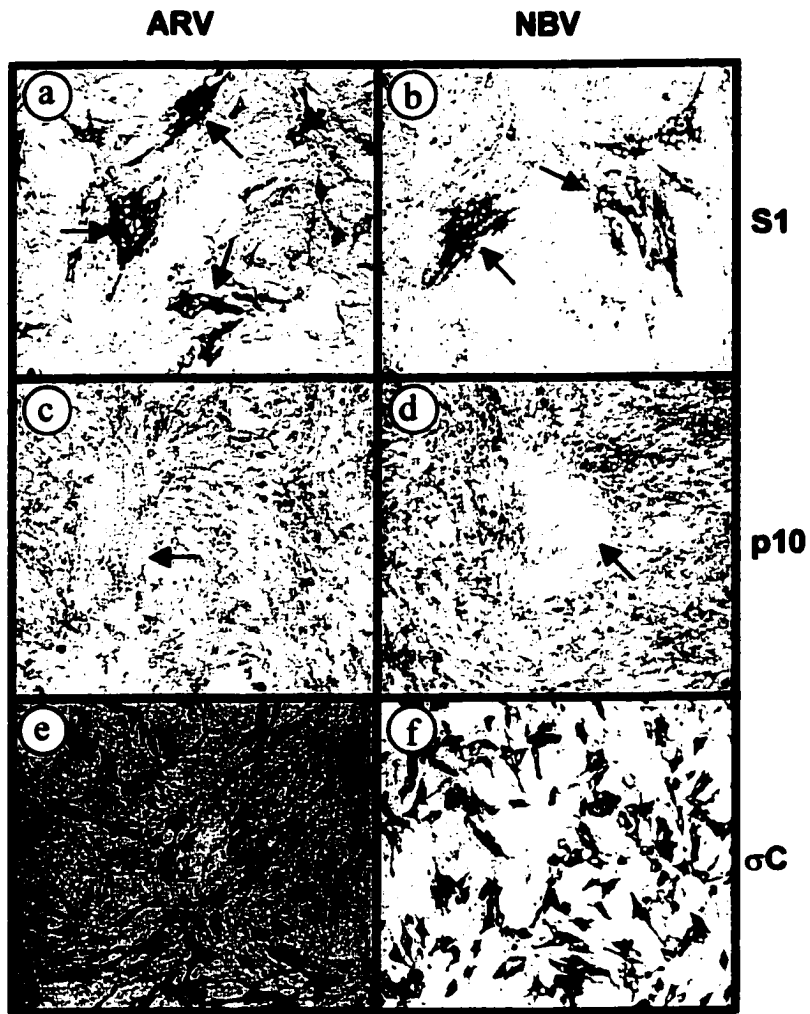
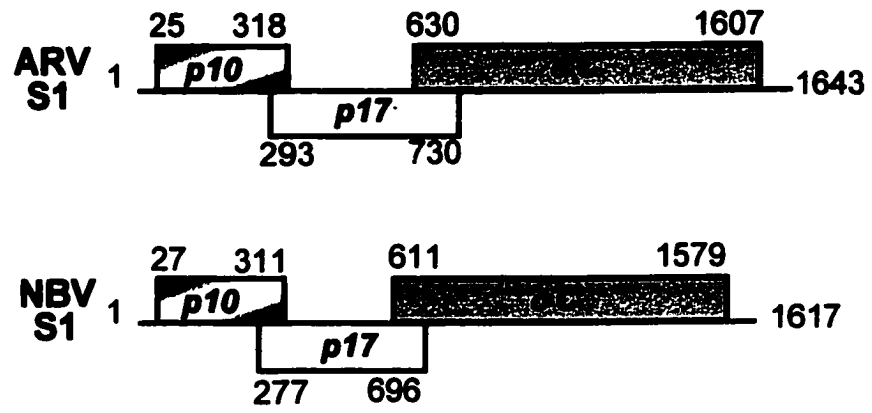
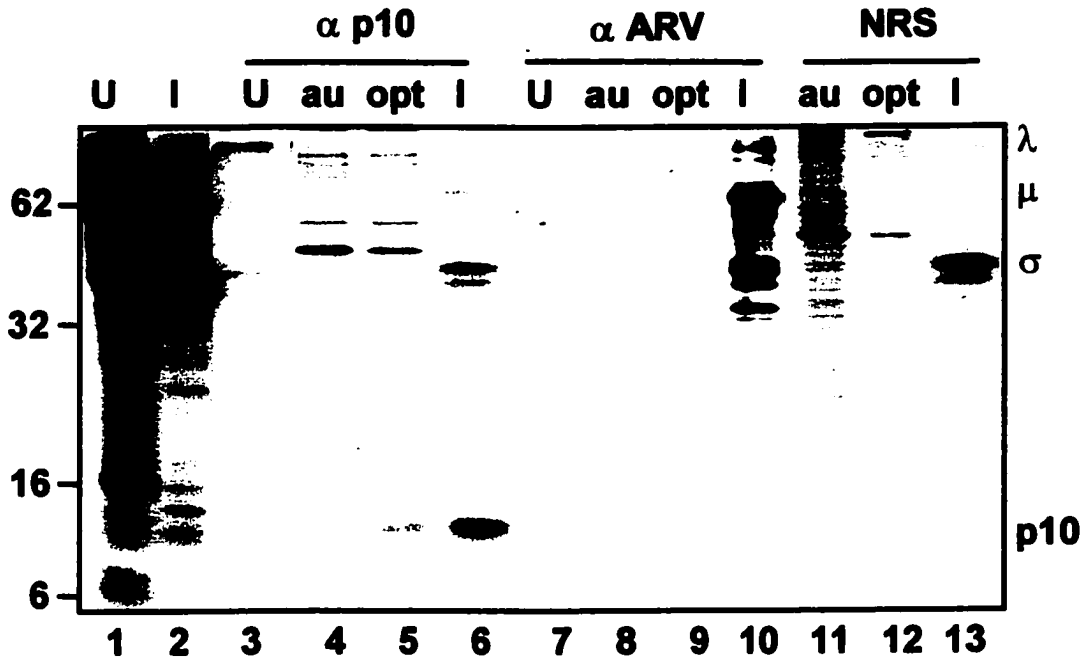


Figure 1.1

Figure 1.2. Increased p10 expression corresponds with enhanced fusion.

(A) Cells were transfected with the authentic p10 gene (au) or with p10 containing an optimized translation initiation sequence (opt), or were infected with ARV (I) or mock-infected (U). Radiolabeled cell lysates were immune precipitated using anti-p10 (α p10), polyclonal anti-ARV serum (α ARV), or normal rabbit serum (NRS), and the cell lysates (lanes 1 and 2) or immune complexes were resolved on a 15% acrylamide gel and detected by fluorography. Numbers on the left indicate the location of molecular weight markers. The locations of the major λ -, μ -, and σ -class viral proteins, and of p10, are indicated on the right. (B) QM5 cells were transfected with an expression plasmid encoding p10 containing the authentic translation start site (a), with p10 containing an optimized translation initiation sequence (b), or infected with ARV (c), and syncytium formation was detected by Wright-Giemsa staining. Arrows indicate multinucleated syncytia. Cells were photographed at 100x magnification.

A



B

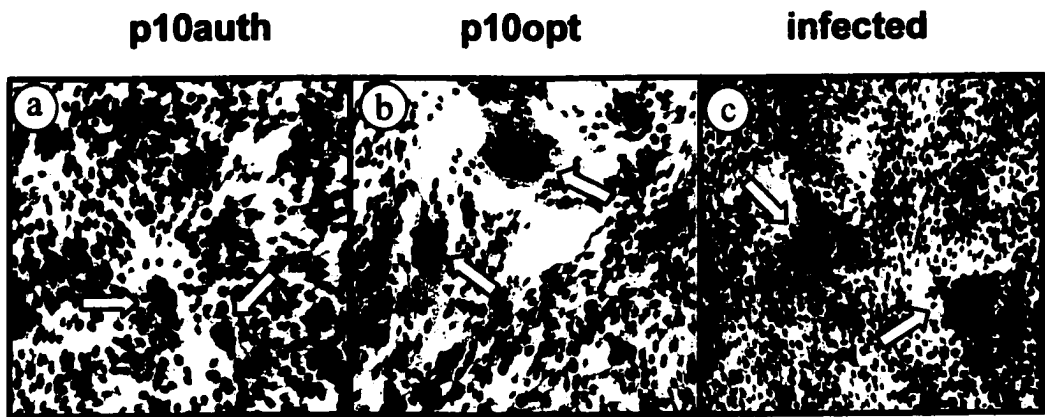


Figure 1.2

Figure 1.3. The p10 protein is a nonstructural viral protein. Detergent-solubilized, radiolabeled virus pellets (Virus), or detergent-solubilized virus-infected cell lysates (Inf. Lysate), were immune precipitated with polyclonal anti-ARV serum (α ARV), anti-p10 (α p10), or normal rabbit serum (NRS), and the immune complexes were resolved on 15% acrylamide gels and detected by fluorography. The locations of the major λ -, μ -, and σ -class viral proteins, and of p10, are indicated on the left, while the location of the minor virus structural protein σ C is indicated on the right.. Lanes 7-9 are an extended exposure of lanes 1-3.

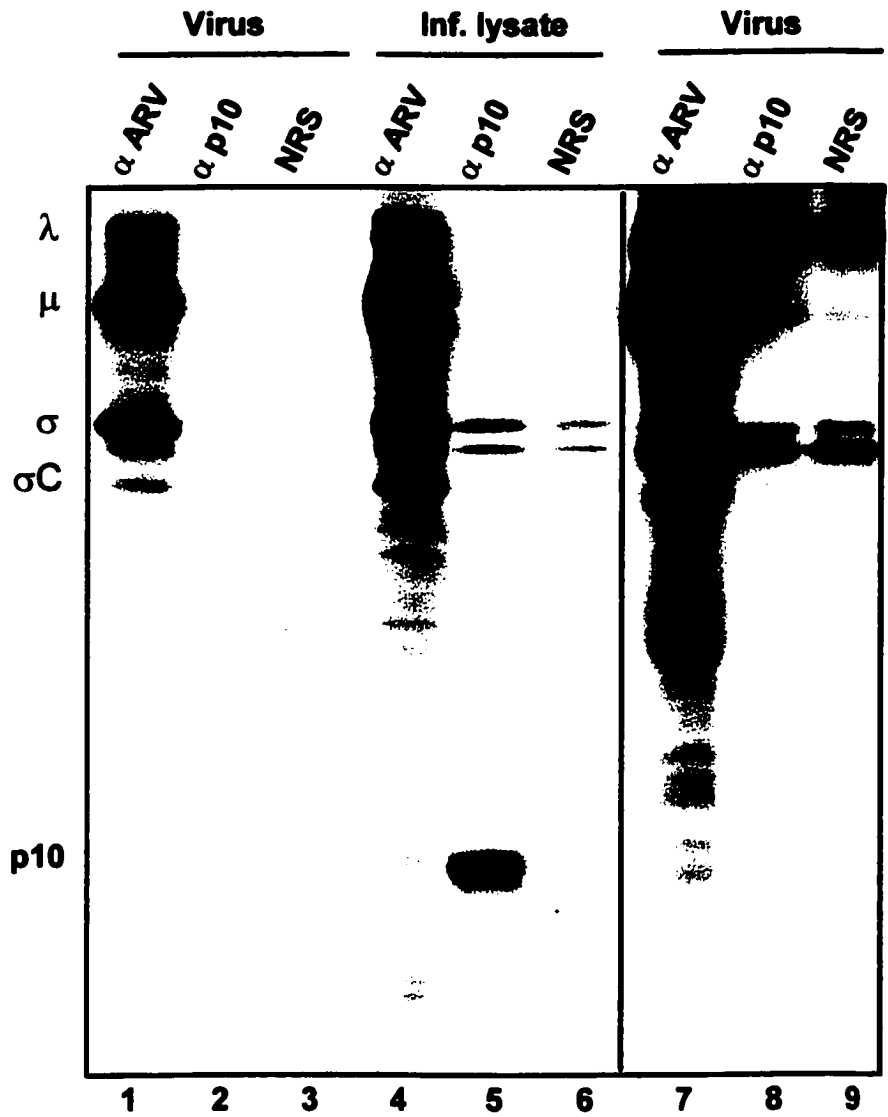


Figure 1.3

Figure 1.4. Sequence and structural conservation in the p10 proteins of ARV and NBV. The top panel indicates the locations of conserved structural motifs, and the first and last amino acid of each motif in the ARV sequences. The centre panel shows the aligned p10 amino acid sequences of ARV strains 176 (first line) and 138 (second line), and of NBV (third line). The locations of conserved identical amino acids is indicated (fourth line), along with the locations of the four conserved cysteine residues (diamonds) and the conserved basic residues (+). The overlining corresponds to the locations of the conserved structural motifs identified in the top panel. The bottom panel represents a hydropathy profile of the ARV p10 protein according to the algorithm of Kyte and Doolittle, averaged over a window of seven residues.

Figure 1.5. The p10 protein is an integral membrane protein. Uninfected (U) or ARV-infected (I) cell lysates (- RIP) were immune precipitated using anti-p10 (α p10) or anti- μ 2C (α μ 2C). The infected cell lysates were also fractionated into the membrane pellet (P) or membrane supernatant (S) fractions before immune precipitation, either without (mem) or with (int) prior extraction of peripheral membrane proteins to reveal the integral nature of p10 membrane association. Samples were resolved by SDS-PAGE using a 15% acrylamide gel, and detected by fluorography.

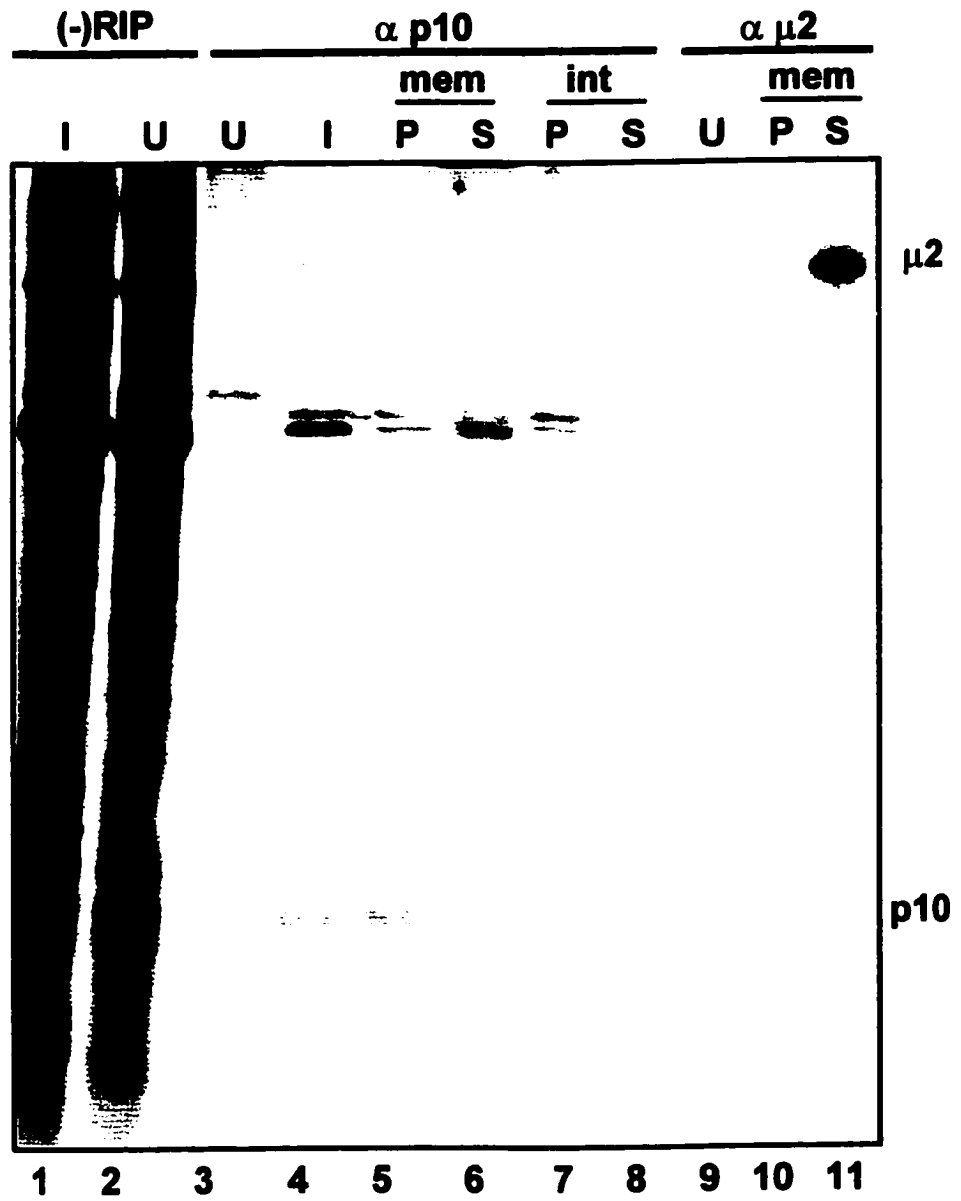


Figure 1.5

Figure 1.6. p10 is a surface localized type I transmembrane protein. (A) Cells were transfected with the N-terminal (panels a and c) or C-terminal (panels b and d) HA-tagged p10 constructs. The transfected cells were stained using anti-HA monoclonal antibody and FITC-conjugated secondary antibody. The cells in panels a and b were permeabilized prior to incubation with the antibody to reveal intracellular expression of the tagged p10 constructs. Cells in panels c and d were stained without permeabilization of the cells to reveal surface-localized p10. The arrows indicate the membrane boundaries of a single syncytial foci. The nuclei present within a syncytium in the permeabilized cells are indicated (N). The bars represent 10 μ m. (B) Cells were transfected with the N-terminal HA-tagged p10 (HA-p10) constructs or pcDNA vector alone (mock). Cells stained for flow cytometry using combinations of primary (anti-HA antibodies, 1:50), secondary (FITC-conjugated anti-mouse antibodies, 1:25), or isotype control secondary (IgG2b, 1:25) were viewed under fluorescence (FITC) or DIC to demonstrate proportion of positively stained cells (yellow arrows) over background staining (white arrows). (C) Cells transfected with N-terminal HA tagged p10 (HA-p10) were stained for flow cytometry using primary (anti-HA) and secondary (FITC-conjugated) antibodies at concentrations shown. Yellow arrows depict strongly fluorescent p10-expressing cells while white arrows point to background stained cells.

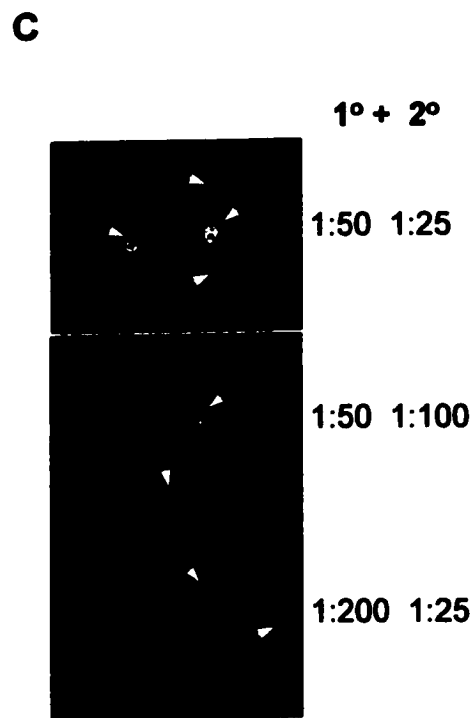
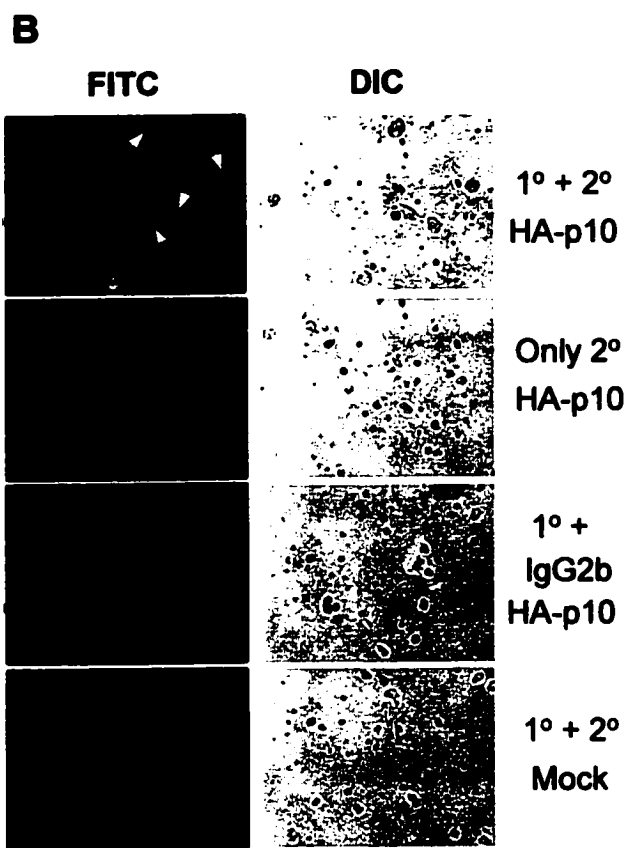
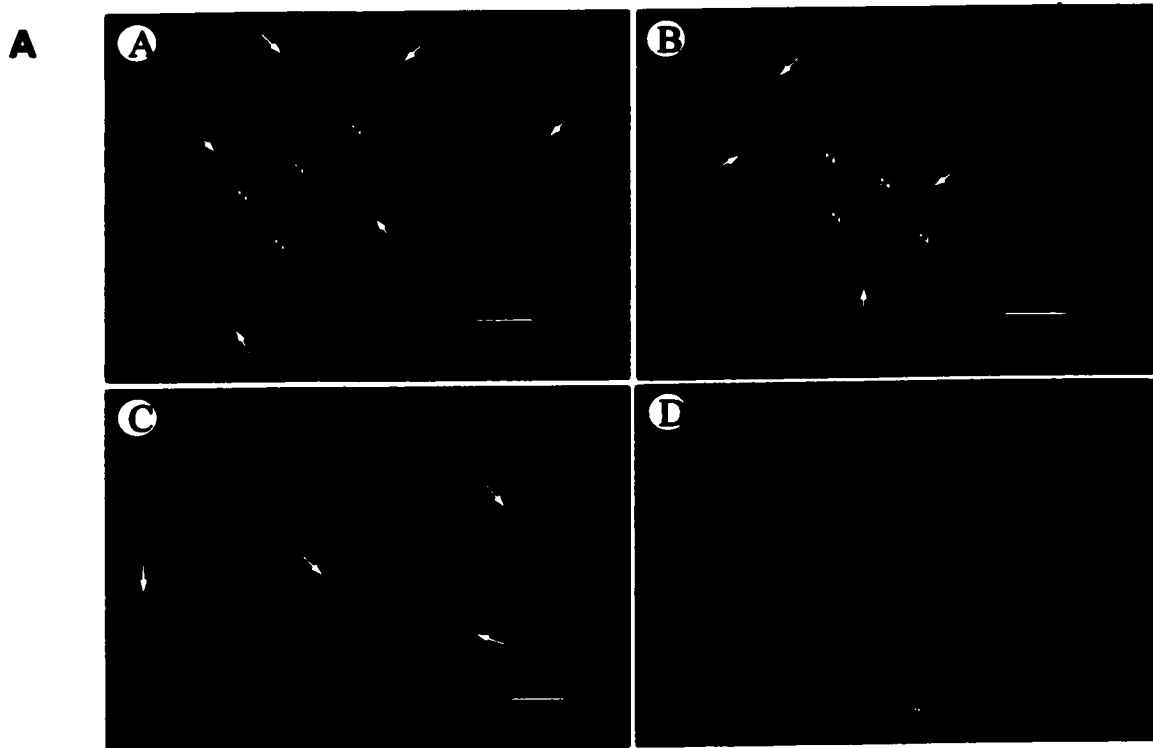


Figure 1.6

Figure 1.7. Anti-HA monoclonal antibody inhibits syncytium formation by the N-terminal tagged p10 construct. Quail cells were transfected with plasmids expressing the N-terminal (panels a and c) or C-terminal (panels b and d) HA-tagged p10 constructs, and incubated in the absence (panels a and b) or presence (panels c and d) of anti-HA monoclonal antibody. Monolayers were fixed and Wright-Giemsa stained to reveal the presence of multinucleated syncytial foci.

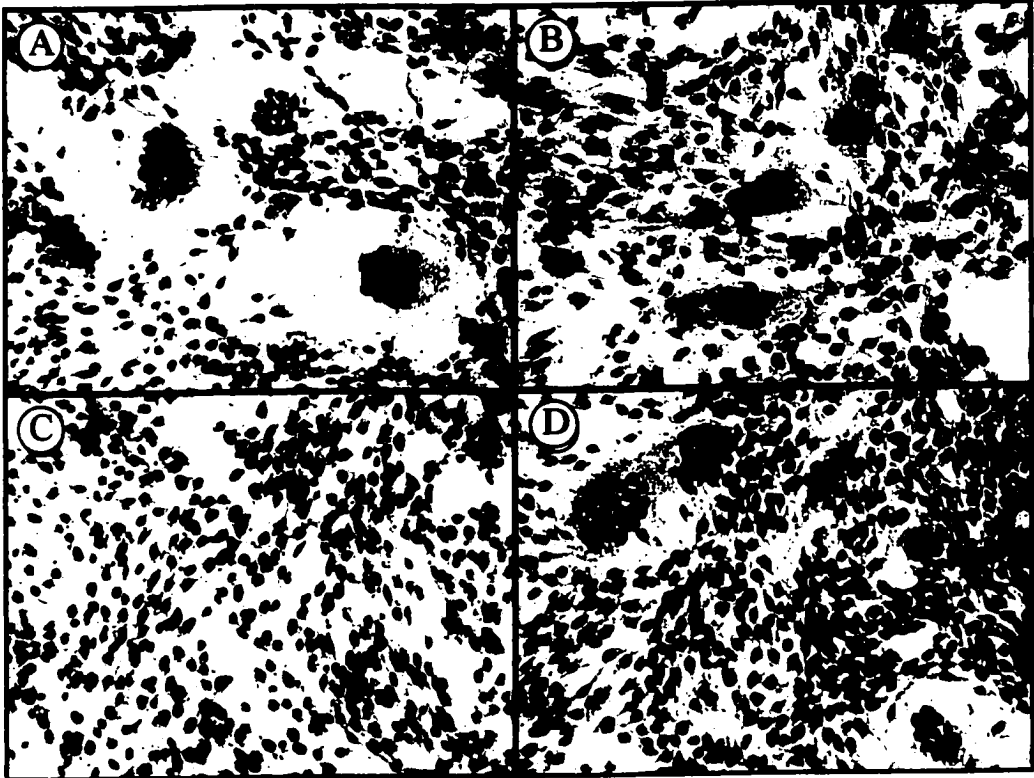


Figure 1.7

Figure 1.8. Deletion and substitution analysis of p10. Various deletions or site-specific substitutions were constructed in the ARV p10 protein, the deleted proteins were expressed in transfected cells, and the ability of the proteins to induce cell fusion, or to localize to the membrane fraction was assessed. Similar approaches were used to assess the effects of chimeric constructs of the ARV and NBV p10 proteins (filled rectangles indicate NBV sequences). The identities and approximate locations of the site-specific substitutions are indicated, using the single letter amino acid code to indicate the identity of the authentic amino, its position, and the identity of the substituted residue. The C21S and V55F substitutions were constructed in the NBV p10 protein. ND- not determined.






	Fusion	Membrane
	++	+
	-	+
	-	-
	-	+
	++	ND
C9A	-	+
C21S	-	ND
G49A	-	+
V55F	++	ND
C63S	+	+
C63/64A	-	+
K69R	++	+
K69M	-	+
R79K	++	+

Figure 1.8

Figure 1.9. Structural model of the reovirus p10 fusion protein. The membrane orientation of the p10 protein is diagrammed, along with the locations of the conserved cys residues, hydrophobic heptad, basic region, transmembrane domain, a short stretch of completely conserved residues, and the nonconserved C-terminal region.

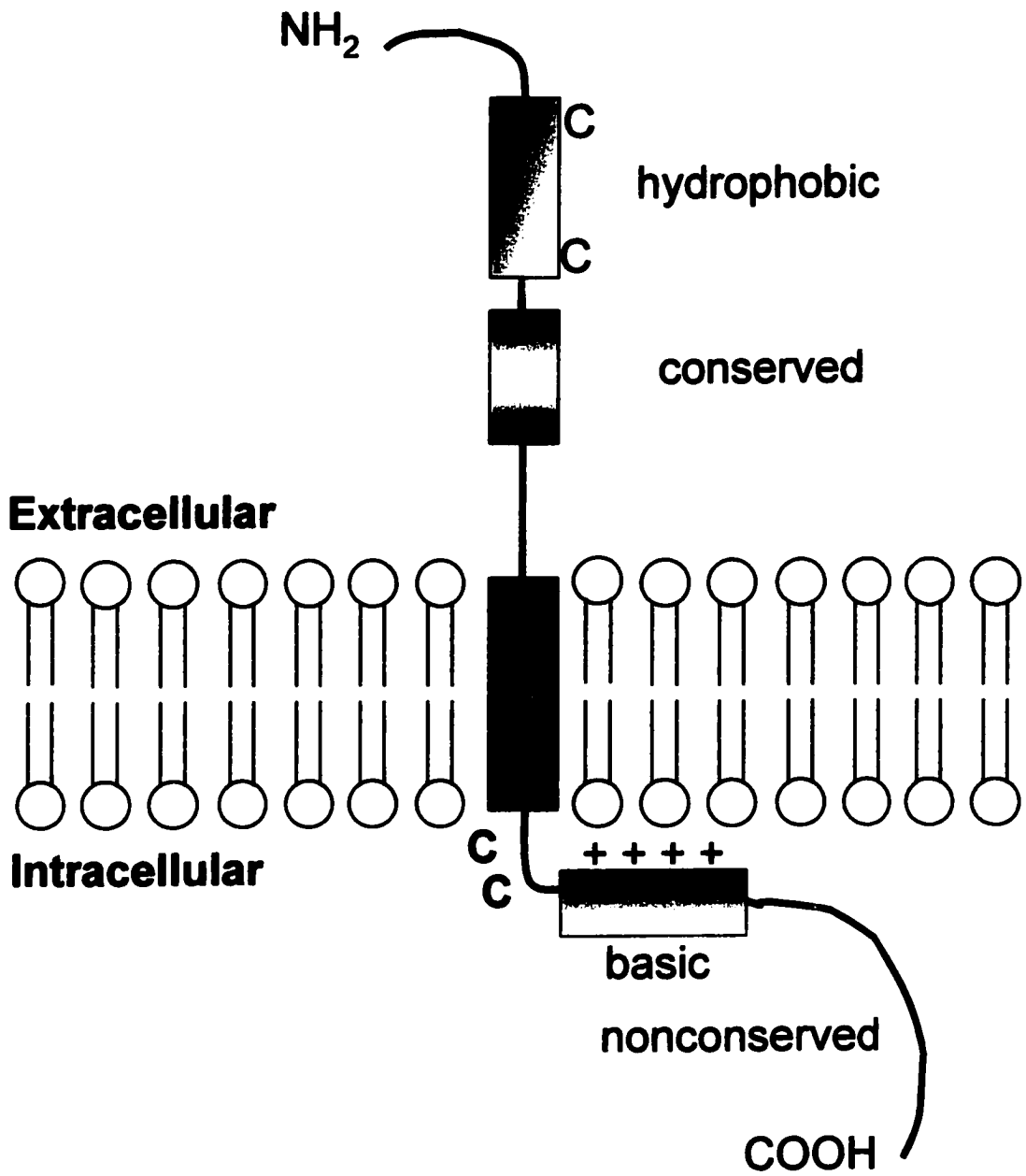


Figure 1.9

1.F. MATERIALS AND METHODS

Plasmids, virus and cells

Avian reovirus (ARV) strain 176, and Nelson Bay virus (NBV) have been previously described (Duncan et al., 1995), and were grown and plaque-purified in a continuous quail cell line, QM5 (Duncan and Sullivan, 1998), or in Vero cells, respectively. All cells were maintained in growth medium consisting of medium 199 supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth, and penicillin/streptomycin (50U/ml and 50ug/ml, respectively). The QM5 cells were used for most of the transfection assays due to their high transfection efficiency.

The eukaryotic expression vector pcDNA3 (Invitrogen) was used for expression of viral genes in transfected cells. pMAL-c2 (New England Biolabs) was used for expression of the maltose-binding protein (MBP)-p10 chimeric protein in *E. coli*.

Cloning, site-directed PCR mutagenesis, and epitope tagging

The full length cDNAs corresponding to the S1 genome segments of ARV and NBV were cloned into pcDNA3, as previously described (Duncan, 1999). The sequences of the ARV-176 and NBV S1 genome segment cDNAs are deposited in GenBank (accession numbers AF218358 and AF218360, respectively). These clones were used as templates for PCR subcloning, using Vent polymerase (New England Biolabs), to generate fragments corresponding to the p10 gene alone,

the p10 gene with an optimised translational start sequence, p10 harboring site specific mutations, and p10 containing the HA epitope at the N- or C-terminus of p10. The sequence of all constructs was confirmed. To synthesize the p10 gene, and the p10 gene containing an optimized translation initiation sequence, forward primers 5'-TACTACTAAGCTTGCTTTTTCAATCCCTTGTTTCG-3', and 5'-TACTACTAAGCTTGCTTTTTCAATCCCTTGTTCCACCATGGTGCGTATGCC-3' were used, respectively, along with the reverse primer 5'-TGAAGAAGCGGCCGCGAAGTGATAGCGGAC-3'. Primers annealed to the noncoding sequences (underlined) flanking the 5'- and 3'-ends of the p10 open reading frame, and added Hind III and Not I sites to the 5' and 3' ends, respectively. Primers containing the HA nonapeptide sequence (5'-TACCCATACGATGTTCTGACTATGCG-3') and sequences complementary to the 5'- or 3'-ends of the p10 open reading frame were used to introduce the HA epitope as a nine residue amino-terminal extension, or C-terminal replacement, of the p10 open reading frame. The final PCR reaction consisted of 1 X Vent polymerase buffer, 2mM MgSO₄, 0.2mM each dNTP, 0.05pmol template, 40pmol each forward and reverse primer, and 0.5ul of Vent polymerase in a final volume of 100ul. Samples were heated at 94°C for 4 min and then cycled 30x at 94°C for 1 min, 52°C for 30 sec, then 72°C for 30 sec. Products and vector (pcDNA3) were digested with Hind III and Not I, and gel purified using β -agarase (New England Biolabs) and GeneClean (BIO101) according to the manufacturer's instructions. The purified vector and insert were ligated, and transformed into Top-10 cells according to standard protocols .

All site-directed mutations were made using a rapid PCR-based technique. Internal primers were synthesized which incorporated the desired mutation flanked by extended sequence complementary to the template. In the first round of PCR, the forward primer containing the optimized translational start sequence (see above) was used along with the internal mutagenic primer to synthesize a fragment containing the mutation near the 3' end. The original primers were removed using Qiaquick (Qiagen), and the first round PCR product was used as the primer for a second round of PCR, along with the reverse p10 primer (see above) producing a fragment corresponding to the entire p10 gene containing the mutation. Touch down PCR was used for better product specificity and yield, which involved 5 cycles of 94°C for 1 min, 52°C for 40 sec, 72°C for 40sec, 5 cycles of 94°C for 1 min, 50°C for 40 sec, 72°C for 40sec and 25 cycles of 94°C for 1 min, 48°C for 40 sec, 72°C for 40sec. The products were then cloned into pcDNA3 as above. Using this method, a mutation can be specifically made and inserted into a vector in a single day using only one additional primer.

p10 antiserum production

Polyclonal antiserum was raised against the C-terminal domain of p10. To synthesize the MBP-p10 recombinant protein construct, the C-terminal portion of p10 (amino acids 63-98) was cloned in frame with the maltose-binding protein in

the pMAL-c2 vector. PCR was performed as above using the forward primer 5'-TACTGTTGTAAGGCTAAGGTC-3' and the reverse primer 5'-CGCGGATCCTCAGTTACGTCGTATGGCGGAGC-3' (underlined sequences are complementary to the p10 open reading frame), cloned into the Xmn I and Bam HI sites of pMAL-c2 (New England Biolabs), and transformed into *E. coli* Top10 cells. The chimeric MBP-p10 protein was induced with IPTG, purified from 1 litre cultures on amylose affinity columns, and used to inject rabbits. The rabbits were immunized at three sites (two intramuscular and one subcutaneous) using 300-500 µg of the chimeric protein in Freund's complete adjuvant, then repeatedly boosted using a similar regime with Freund's incomplete adjuvant. Animals were exsanguinated when the antibody titer plateaued after seven injection series. A similar protocol failed to obtain an immune response against the N-terminal domain of p10.

Cell staining

Monolayers of QM5 cells were transfected with the p10-expressing pcDNA3 constructs using Lipofectamine (Life Technologies Inc.) and incubated for 24-36 hours. Cell monolayers were also infected with ARV at a low multiplicity of infection to generate syncytial foci after 16 hr. Transfected or infected cell monolayers were stained with Wright-Giemsa stain (Diff-Quik) according to the manufacturer's instructions (VWR Scientific) to visualize cell nuclei and polykaryon formation. Viral proteins were detected immunocytochemically using primary antiserum raised either against virus structural proteins (Duncan et al.,

1996), or against p10. The p10 antiserum and the polyclonal anti-ARV serum were diluted 1:400 in antibody blocking buffer (2% BSA, 10% normal goat serum, 20mM Tris, pH 7.5, 150mM NaCl, 0.1% NP-40) and adsorbed to fixed monolayers for 60 min at room temp. The monolayers were extensively washed before and after antibody additions with PBS containing 2% BSA. Foci were visualized using a secondary goat anti-rabbit IgG conjugated with alkaline phosphatase (Life Technologies, 1:600 dilution) according to standard protocols (Harlow and Lane, 1988). Cells stained with alkaline phosphatase were visualized and photographed on a Nikon Diaphot inverted microscope at 100x magnification.

Fluorescent staining and syncytial inhibition using HA monoclonal antibodies

The HA-tagged p10 constructs were expressed in transfected cells growing on multiwell chamber slides (Nunc) as described above. The medium was removed from the transfected cells 28 hr post-transfection, the monolayers were washed twice with Hank's balanced salt solution, and preblocked with antibody blocking buffer for 30 min at room temperature. The HA monoclonal antibody was prepared from 12CA5 hybridoma cell culture supernatants by ammonium sulfate precipitation (50% saturation) and dialysis against phosphate-buffered saline. The antibody suspension (5 mg/ml protein) was diluted 1:100 in antibody blocking buffer and incubated with the unfixed cell monolayers for one hr at room temperature to detect cell surface localized p10. For visualization of intracellular

p10 expression, cells were fixed and permeabilized using methanol prior to addition of a 1:200 dilution of the monoclonal antibody. Primary antibody was removed by four washes with Hank's balanced salt solution containing 2% fetal bovine serum at room temperature for 30 min. A secondary rabbit anti-mouse FITC-conjugated antiserum (Life Technologies Inc.) was diluted 1:20 in antibody blocking buffer, and incubated with the monolayers for one hr. Monolayers were washed extensively as above, fixed with 4% paraformaldehyde for 10 min at room temperature, and the slides mounted for examination by confocal microscopy. The cells were visualized and photographed on a Zeiss LSM510 scanning argon laser confocal microscope with appropriate filter sets using the 63x or 100x objectives.

For antibody inhibition of syncytium formation, the HA monoclonal antibody was diluted 1:400 in tissue culture medium and added to transfected cells four hr post-transfection. At 36 hr post-transfection, the cells were methanol fixed and Giemsa stained as described above, and examined for the presence of multinucleated syncytia.

Staining for flow cytometry

One million cells transfected with the N-terminal HA-tagged p10 constructs or mock transfected cells (pcDNA vector alone) were suspended with Hanks balanced salt solution (HBSS)/5mM EDTA and washed with HBSS to remove media (2 minutes 3000rpm in microcentrifuge). Suspended cells were stained

with monoclonal anti-HA antibodies in 100µl volume (dilution ratios according to figure legend) for 45 minutes on ice in PBS/0.02% azide/2%FCS. Azide was added to prevent endocytosis of antibodies. Cells were washed three times and incubated with secondary antibody according to figure legend for 45 minutes on ice. Following three washes, cells were fixed in cold PBS/2% paraformaldehyde and stored at 4 degrees Celsius protected from light.

Analysis of virus structural proteins

The analysis of virus structural proteins was essentially as previously described (Duncan, 1996). QM5 cells grown in 175 cm² flasks (3.6×10^7 cells) were infected at an m.o.i of 0.1, labeled at 14 hours, and again at 17 hours, post-infection with [³⁵S]methionine (50µCi/ml), and the infection was allowed to proceed until cell lysis. Cell lysates were frozen and thawed three times to disrupt virus aggregates, centrifuged at 10,000g for 20 min to remove cell debris, then centrifuged at 100,000g for 1 hour through a 30% (w/v) sucrose cushion to obtain the virus particles. The virus pellet was resuspended in 1% SDS, and the virus particles were disrupted by heating at 37°C for 30 min to liberate all of the structural proteins. The disrupted virions were diluted in RIPA to a final concentration of 0.1% SDS before proceeding to immunoprecipitation.

Immunoprecipitation

Immunoprecipitation was performed as previously described (Duncan and Sullivan, 1998). QM5 cells were transfected or infected with ARV at an m.o.i of

0.1 and labeled with [³⁵S]methionine (50 μ Ci/ml) for 1 hour at 24, or 14, hours post-transfection/infection, respectively. Cells were lysed on ice in RIPA buffer containing protease inhibitors, and cell lysates were centrifuged at 100,000g for 25 minutes to remove virus particles. The supernatant was precipitated for 60 min on ice using rabbit antiserum raised against viral structural proteins, p10, or normal rabbit serum (all diluted 1:250). Immune complexes were recovered using IgG-sorb (The Enzyme Center), washed extensively with RIPA, and released by boiling in SDS protein sample buffer (Laemmli, 1970) before SDS-PAGE using 15% acrylamide gels.

Membrane fractionation of infected and transfected cells

QM5 cells, in 12 well cluster plates, were infected with ARV at a multiplicity of infection (m.o.i) of 0.1, or cells were transfected using LipofectAMINE reagent (Life Technologies) according to product instructions, using 3 μ l of LipofectAMINE and 0.75 μ g of DNA on 70% confluent cell monolayers in 12 well cluster plates. Infected/transfected cells were labeled with [³⁵S]methionine (50 μ Ci/ml) for 1 hour when extensive syncytium was observed, washed twice with PBS, harvested by scraping into 1ml of PBS, then passed through a 30-gauge needle ten times. Nuclei and cell debris were removed by centrifugation at 600g for 3 min, and the membrane fraction was recovered by centrifugation at 100,000g for 25 min. The membrane pellet was either dissolved in electrophoresis sample buffer (Laemmli, 1970) for direct analysis by SDS-PAGE using 15% acrylamide gels, or was dissolved in RIPA buffer (50mM Tris, pH 8, 150mM NaCl, 1mM EDTA, 1% NP-

40, 0.5% NaDeoxycholate, 0.02% NaN₃) containing protease inhibitors (1µg/ml each of aprotinin, leupeptin, and pepstatin) for subsequent immunoprecipitation analysis. For removal of peripheral membrane associated proteins, pellets consisting of membranous material were treated with 100mM Na₂CO₃, pH 11.3, for 30 min on ice, followed by centrifugation at 100,000g for 25 min to recover the membrane fraction and associated integral membrane proteins.

CHAPTER 2. The fusion proteins (p10) of nonenveloped avian and Nelson Bay reovirus have evolved as minimal machinery for catalysis of biological lipid bilayer fusion devoid of complexities necessary for specificity, regulation, efficiency or rapidity of membrane fusion.

2.A. ABSTRACT

The life cycle of the nonenveloped avian reovirus (ARV) and the unique characteristics of the ARV-encoded membrane fusion protein (p10) suggest that p10 may induce membrane fusion in the absence of regulatory triggers, specific target membrane interactions. In further contrast to the fusion proteins of enveloped viruses, characteristics of the ARV life cycle and p10 support that p10 induced membrane fusion is inefficient and slowly accumulates during the infection. P10 production increases during the viral life cycle similar to all other ARV-encoded proteins. Relative to other ARV-encoded proteins, however, p10 and sigma C are produced in least amounts and are expressed from a single polycistronic transcript. We strongly suspect that p10 activity is not regulated by environmental triggers, protein-protein interactions or changes in conformation. Instead, the slow expression of p10 correlates with the late onset of cell-cell fusion and has likely evolved as the method of synchronizing p10-induced fusion with the release of new virus particles.

2.B. INTRODUCTION

This chapter provides the rationale and preliminary evidence for the absence of regulation, specificity, efficiency and rapidity in p10-mediated membrane fusion. Recognition that p10 is a rudimentary fusion protein will be important in the interpretation of data pertaining to the mechanism of p10-mediated membrane fusion that follows in chapters three, four and five. In comparison to other proteins that mediate membrane merger, p10 appears less complex and is a good candidate for understanding the minimal requirements for membrane fusion. The fusion proteins of enveloped viruses and intracellular fusion machinery have evolved efficient mechanisms for regulation, specificity and rapidity that could then be distinguished from the minimal fusion determinants through comparison to p10.

Membrane fusion is an essential process for the survival of cells and enveloped viruses. Biological membranes are, however, designed to act as effective barriers between compartments, cells, and other lipid-enclosed particles. The specificity and regulation of membrane fusion events is ensured by the controlled activity of proteinaceous fusion machinery required to drive the merger of lipid bilayers. Both cellular and viral fusion proteins have evolved to induce rapid, efficient, leakage-proof fusion at the right time and with the correct target membrane.

Intracellular vesicle transport mediated by SNARE proteins must be kept under strict regulation to ensure cellular integrity. Vesicles must fuse with the correct target membrane to ensure that contents are transported to their respective locations. Intracellular vesicle fusion ensures that vesicles are targeted and fused to the correct compartmental membrane by regulating the specific pairing of vesicle and target membrane bound proteins. Although SNARE proteins are broadly distributed (Hay et al., 1998; Holthuis et al., 1998; Yang et al., 1999) some studies show that SNARE pairing between vesicle- and target- SNARE proteins (v- and t- SNARE, respectively) is specific (Weber et al., 1998). Other defined receptor-ligand interactions between correct target and donor membranes, called tethering proteins, permit docking of the vesicle and promote subsequent regulatory steps. In *in vitro* experiments, correct SNARE pairing appears sufficient for membrane fusion, but biological conditions require additional regulatory components.

The complexity of the methods used for temporal regulation of intracellular fusion can be depicted by describing the sequence of events leading to endosome fusion within yeast. Rab GTPases must first be recruited to the vesicle membrane and stabilized by nucleotide exchange proteins. In the GTP bound state, Rab proteins can recruit tethering proteins which themselves are regulated by phosphorylation. The oligomerization of tethering proteins may be necessary for recruitment of sufficient regulatory components or for efficient docking to target membranes (McBride et al., 1999). Following the docking of donor and

target membranes, SNARE proteins are locally activated by the activities of soluble proteins NSF and α -SNAP. When SNARE proteins are in their inactive conformation, NSF and α -SNAP are recruited and their activity results in the refolding of SNARE proteins that permits v-SNARE-to-t-SNARE interactions. In summary, specificity, regulation, efficiency and rapidity of intracellular fusion is mediated by conformational changes between active and inactive states, specific protein-protein interactions, formation of oligomeric structures and tethering platforms concentrated with fusion machinery. Clearly, the cellular fusion machinery has evolved elaborate strategies to coordinate the fusion function with a propensity to be controlled.

The complexity of intracellular fusion has made enveloped virus mediated fusion proteins appear more amicable for study. Viruses cannot afford to carry multiple proteins and factors for activity, as they must reduce their genome size and antigenicity. Yet, enveloped viruses must ensure that they enter specific cells at the correct time. Specificity of fusion between enveloped viruses and target cells is conferred by the presence of cellular receptor binding domains within, or adjacent to the viral fusion proteins. Receptor binding determines specificity, brings target and donor membranes into closer proximity, and in some viruses, provides the trigger for fusion protein activity. For other viruses, low pH encountered following receptor-mediated endocytosis acts as the trigger for fusion. In both cases, the correct trigger provokes changes in protein conformation and/or oligomeric structure of the fusion proteins that activate their

function. The fusion proteins of enveloped viruses must be sufficiently large and multimeric to permit burying of the membrane-seeking and destabilizing fusion peptides prior to activation, yet must be capable of exposing the fusion peptides for insertion into the target membrane and subsequent induction of fusion.

The fusion reaction between enveloped viruses and cells must also occur in a highly efficient manner. Rapid fusion prevents leakage and disruption to the cellular membrane. Efficient fusion would ensure that virus entry into correct cells, an important stage in the host-pathogen battle, is rapid. Rapid and efficient fusion is promoted by the accumulation of fusion proteins at the site of membrane merger through oligomerization, concentration within envelopes, and further aggregation during the fusion process. To facilitate rapid membrane fusion, enveloped virus fusion proteins are produced with native structures that are primed to undergo rapid and energetically favorable conformational changes. Because enveloped viruses have evolved to fuse rapidly, erroneously activated proteins can be eliminated by conversion to inactive conformations if fusion does not proceed promptly. Only if sufficient specific triggering is provided is the activity of the fusion proteins synchronized to result in fusion rather than inactivation.

In summation, specificity, regulation, efficiency and rapidity of enveloped virus entry by membrane fusion is mediated by conformational changes between active and inactive states, specific protein-protein interactions, formation of

oligomeric structures and concentration of the fusion machinery. Enveloped viruses have reduced the complexity and number of regulatory components as compared with the intracellular fusion machinery, but have evolved equally elaborate strategies to ensure regulated fusion events. Although, at first glance, the enveloped virus fusion proteins seem to be better candidates for understanding membrane fusion because the fusion machinery involves fewer components, they actually present equally difficult problems in deciphering the events essential for membrane merger. With enveloped virus fusion proteins it is difficult to distinguish those events that have evolved because they are necessary for membrane merger from those that are involved in regulation, specificity, efficiency or rapidity.

The nonenveloped avian and Nelson Bay reoviruses (ARV and NBV, respectively) encode fusion proteins (p10) that have evolved under different limitations as compared to the fusion machinery of intracellular transport and enveloped viruses. Characteristics of the virus life cycle and of the fusion protein of these nonenveloped viruses strongly suggest that regulation, specificity, efficiency and rapidity are not essential for p10 function. A trigger for p10-induced fusion is not apparent, as the protein functions under physiological conditions. Furthermore, p10 does not appear to bind cellular receptors. P10 has evolved as an accessory protein with the minimal fusion determinants for the induction of unregulated membrane fusion. By comparison with other known

fusion proteins, p10 will permit dissociation of those events necessary for membrane merger from those involved in other aspects of the fusion reaction.

2.C. RESULTS

2.C.1. Characteristics of the ARV life cycle and fusion protein support the absence of regulation of the fusion process.

Processes that require membrane fusion are not essential for the life cycle of nonenveloped viruses. Nonenveloped viruses do not acquire lipid envelopes or membrane-bound glycoproteins during exit via cell lysis (Figure 2.1). Evidently, in the absence of lipid bilayers, entry of nonenveloped viruses into target cells cannot be accomplished through membrane fusion. This is in contrast to enveloped viruses that exit by budding from cells and thereby acquire envelopes composed of cellular lipid bilayers that are used for subsequent entry through viral envelope-to-cell membrane fusion.

Several investigations confirm that the membrane fusion protein (p10) of ARV is not involved during entry. First, p10 of ARV is a nonstructural protein (i.e. not present within extracellular virions) and therefore cannot be used during entry (chapter 1). Second, previous characterization of ARV strains with variable membrane fusion activity suggested that p10 is an accessory protein, and is not essential for the life cycle of ARV (Duncan and Sullivan, 1998). Lastly, the prototypic member of the orthoreovirus genus (*Reoviridae* family), mammalian reovirus (MRV) does not encode a fusion protein yet successfully undergoes replication using similar strategies as ARV. That p10 plays an accessory role in the virus replication cycle and is not involved in entry of ARV into cells suggests

that ARV faced different evolutionary pressures in the development of p10 than those faced by enveloped viruses during evolution of their fusion proteins.

Although the role of p10 is proposed to be accessory, it is of course beneficial to the life cycle and has therefore been maintained within fusogenic reoviruses (Duncan and Sullivan, 1998). Potential roles for p10 include; enhanced cell lysis for more rapid exit, expansion of intracellular resources through cell-cell fusion, and enhanced evasion from the immune system through extended intracellular movement. The accessory nature of p10 suggests that its evolution was not restricted by an absolute essentiality for its function. Based on the accessory role of p10 in the ARV life cycle, p10 is proposed to have freely evolved to the minimal determinants necessary for membrane fusion.

The life cycle of ARV suggests that the fusogenic activity of p10 is nonspecific and post-translationally unregulated. If only ARV-infected cells generate p10, and every infected cell undergoes p10-mediated fusion with neighboring membranes, then specificity of donor and target membranes is inherent in the infection-dependent expression of p10. In effect, there is no benefit to determinants of specificity since p10 can only function within infected cells, which can only fuse to neighboring cells. With respect to regulation of fusogenic activity, the extent and timing of p10-mediated fusion could be easily controlled by variable expression rather than elaborate conformational changes or protein-protein interactions. Overall, the accessory nature of p10 and membrane fusion

in the life cycle of ARV suggests that p10 has evolved the fundamentals to induce membrane fusion in the absence of specificity and regulation.

That the function of p10 is nonspecific and controlled by timely expression rather than protein structure and conformational changes is supported by the characteristics of the p10 protein itself (Figure 2.2). The most striking difference between p10 and the fusion proteins of enveloped viruses is that p10 is significantly smaller. The ectodomains of enveloped virus fusion proteins that undergo changes during activation and regulated fusion range from as small as approximately 160 amino acid residues (*filoviridae*) up to 400 (*paramyxoviridae*), 500 (*togaviridae*, *flaviviridae* and *rhabdoviridae*) and 600 (*coronaviridae*) residues in length. In comparison, p10 is at least four fold smaller, with only 40 residues in the ectodomain (Figure 2.2). The large size of enveloped virus fusion protein ectodomains permits conformational rearrangements that transform the inactive state with buried fusion peptide to a fusion peptide exposed, active conformation. The small size of p10 ectodomain suggests that complex conformational changes are not likely. The twenty N-terminal residues in p10 have been shown to function as a fusion peptide and evidence suggests that they form a disulphide bond stabilized loop (Figure 2.2)(chapter 3). The conserved domain adjacent to the membrane is predicted to have little secondary structure and function as a flexible linker between the fusion peptide and membrane anchor (Figure 2.2)(chapter 3). In view of the small size and simple structure of the p10 ectodomain, it is difficult to reconcile the existence of two conformations, one that

successfully buries the looped internal fusion peptide, and another that exposes it.

In addition of the small size of p10, the absence of heptad repeats and multimerization support that conformational changes involved in regulation of membrane fusion activity are improbable. P10 does not have N-terminal and C-terminal heptad repeats that form coiled-coil structures and mediate the transition from inactive to fusion active states for the fusion proteins of coronaviruses, filoviruses, paramyxoviruses, arenaviruses, orthomyxoviruses and retroviruses. The togaviruses and flaviviruses are unique from the remaining enveloped virus families because their fusion protein activities are regulated by changes in protein structure mediated through conversion between different oligomeric states. The fusion proteins of togaviruses and flaviviruses initiate in trimeric form and undergo conformational change to dissociate into monomers and re-associate into homotrimeric structures. Chapter 5 described various experimental strategies that suggest a monomeric structure for p10, supporting that p10 is not regulated through multimer dissociation. The coiled-coil interactions and multimerization in enveloped virus fusion proteins is essential for creating 'cavities' for fusion peptide burying. Based on the amino acid sequence, predicted secondary structure, monomeric structure and small size, it is unlikely that p10 has the capacity to form a hydrophobic cavity that would shield the fusion peptide in the inactive state yet undergo significant conformational changes to expose the fusion peptide under regulated activation conditions.

Thus, the two most common methods of structural rearrangements and fusion peptide concealment used by enveloped viruses are not consistent with the mechanism of p10. Either p10 is not regulated, as we are presently suggesting, or it has evolved a very condensed yet effective novel method of activation.

The life cycle of ARV and the structure of p10 also support the hypothesis that efficiency and rate of membrane fusion are not critical for p10-mediated fusion. Membrane fusion has evolved to occur late during the ARV life cycle. Despite potential for more rapid fusion by increased expression of p10, ARV has evolved to slowly accumulate p10 at the surface of cells and within localized areas with sufficient total activity to favor membrane merging. P10 may not require characteristics that ensure rapid activity such as multimerization and aggregation. Rather, given the life cycle of ARV and the simple domain organization and small size of p10, we predict that p10 has only evolved and maintained the minimal requirements necessary for the ability to cause changes to lipid bilayers that culminate in membrane fusion.

2.C.2. P10-mediated cell-cell fusion does not require cleavage, low pH or receptor binding.

In addition to characteristics of the life cycle and fusion protein of ARV, the ability of p10 to function in the absence of activation triggers would suggest that it does

not undergo regulated activity. Enveloped viruses use two different mechanisms for activation of fusion protein activity. For togaviruses, flaviviruses, rhabdoviruses, arenaviruses and orthomyxoviruses, low pH encountered within endosomes following specific interactions between viral glycoproteins and cellular receptors triggers fusogenic activity. For the remaining viruses, entry can be accomplished directly at the cell surface because cell receptor binding is sufficient to induce the conformational changes in the fusion protein that activate fusion activity. Since p10 fuses cellular membranes at physiological temperature and neutral pH and does not undergo proteolysis (data not shown) we focused on cell receptor binding as the potential triggering mechanism. Conclusive demonstration that the fusion peptide of p10 inserts into the target membrane in the absence of other interactions between p10 and target membrane components would support that p10 fusogenic activity is neither specific or regulated once integrated within membranes.

Strategies used in attempt to detect multimerization of p10 including cross-linking reagents and co-immunoprecipitation analysis failed to detect p10 binding between p10 to itself or other cellular proteins (chapter 5). Cross-linking is commonly used to identify receptor-ligand interactions (Laburth et al., 19984; Wood and O'Dorisio, 1985). The inability of cross-linking to detect p10-cellular receptor interactions could, however, be a consequence of the low surface expression of p10 (chapter 3) or the weakness of p10-receptor interactions.

The small size of the p10 ectodomain makes it unlikely that receptor-binding domains are present. The ectodomain of p10 consists of the 20 residue fusion peptide sequence and a conserved domain adjacent to the transmembrane anchor (Figure 2.2). We speculate that the fusion peptide does not participate in specific and avid binding reactions, as they would interfere with its insertion into target membranes. Note that although the fusion peptide interactions with lipids in the bilayer can be interpreted as binding, they would not confer specificity or regulation since the fusion peptide would already be en route to inducing membrane fusion. P10 should therefore be distinguished from the glycoprotein of vesicular stomatitis virus that binds phosphatidylserine and phosphatidylethanolamine specifically (Pozzi et al., 1993; Conti et al., 1991; Yamada and Ohnishi, 1986) prior to fusion peptide exposure and insertion into target membranes. It is also unlikely that the conserved region binds target membrane components based on comparison to the enveloped virus proteins fusion- and other proteins whose binding regions are discrete domains found furthest away from the transmembrane anchor. To determine whether the conserved region binds cellular membrane components, peptides corresponding to the conserved region could be assessed for association with target membranes. Such experiments have been undertaken using the entire ectodomain of p10.

When the ectodomain of p10 was expressed as a soluble secreted protein in cells, it bound to cellular membranes. The binding of the ectodomain could have

resulted from fusion peptide-membrane association, receptor-p10 interactions, or both. To determine whether cellular receptors were essential for the ectodomain-membrane interactions, we transcribed and translated the p10 ectodomain *in vitro* using rabbit reticulocyte lysates in the presence of canine microsomal membranes. We assessed whether removal of membrane-associated proteins by trypsin treatment effected the association of p10 ectodomain with microsomal membranes. Cellular receptors tend to be large and would be expected to have multiple accessible lysine or arginine residues susceptible to cleavage. An N-terminal signal peptide was added to the ectodomain of p10 to provide a similar treatment and folding environment as the ectodomain in the context of the entire p10 protein. Because transport of p10 across microsomal membranes and signal peptide cleavage required membrane-associated proteins, trypsin treatment was done after translation was complete. Microsomes with associated p10 ectodomains were opened up by passage through a 30-gauge syringe to permit access of the luminal proteins to trypsin. Figure 2.3 demonstrates that the p10 ectodomain is associated with membranes in the absence of membrane bound proteins. The ectodomain of p10 associated with membranous pellets and not soluble supernatants (Figure 2.3, lanes 1 and 2) and was resistant to treatment with salt supporting nonionic binding interactions (Figure 2.3, lanes 3 and 4). When p10-ectodomain-membranes were treated with trypsin, the ectodomain remained associated with membranous pellets suggesting an interaction with lipids rather than proteins (Figure 2.3, lanes 7 and 8). The inability to immunoprecipitate truncated species of N-terminally HA-tagged p10

with HA-epitope specific antibodies following similar trypsin digestion confirmed that trypsin did effectively reach the intra-microsomal compartment and remove the HA-epitopes (data not shown). The membrane association of p10-ectodomains is predicted to result from fusion peptide insertion into the lipid bilayer (chapter 3). Whether p10-cellular protein interactions occurred in addition to fusion peptide insertion could not be addressed in this experiment.

Previous analysis demonstrated that inhibition of host transcription with actinomycin D did not affect syncytium formation induced by ARV (Ni and Ramig, 1993). This experiment suggests that if cellular receptors are necessary for p10 activity, they are stable and incessantly on the surface of cells. Furthermore, p10 is capable of inducing membrane fusion with target membranes of a wide variety of cell types. A cell-cell fusion assay was designed using two populations of cells, each pre-loaded with different intracellular fluorescence markers (cell tracker green and blue). Fusion between the two populations, only one of which expressed p10 by transfection, was monitored by cofluorescence. The fusogenic activity of p10 on target membranes of quail fibroblasts (Figure 2.4A), human epithelial cells (Figure 2.4B) and human primary dendritic cells (Figure 2.4C) was clearly visible using this technique. A list of cell types capable of acting as target cells for p10-mediated fusion is provided in figure 2.5. The ability of p10 to function on monkey, human, quail, chicken, hamster, mouse and canine cells of kidney, dendritic, muscle, macrophage, and other tissue origins suggests that either p10 does not require binding to cellular surface proteins for function, or

that the cell receptor for p10 is ubiquitous and highly conserved across species and cell types. Given our current understanding, cellular surface proteins that are constitutively expressed, highly conserved, and ubiquitous are unlikely.

One possibility is that p10 uses a carbohydrate rather than a protein receptor. Influenza, as an example, binds to sialic acid moieties, which are in fact constitutive, conserved and ubiquitous across species and cell types. Previous analysis, however, showed that inhibitors of glycosylation had no effect on the fusion mediated by p10 (Duncan et al., 1996). This study suggested that p10 does not require N-linked glycosylation on target cells.

Although we can easily eliminate the need for cleavage or low pH as triggers for p10 fusogenic function, we have yet to conclusively demonstrate that p10 does not bind a cellular protein for activation. Nevertheless, the inability to detect p10-receptor interactions by coprecipitation and cross-linking experiments, the small size of the p10 ectodomain supporting the absence of receptor binding domains, the ability of p10 ectodomains to associate with membranes following trypsin treatment, the indiscriminate use of target membranes, and the ability to fuse in the absence of protein glycosylation suggest that p10 does not require binding to cellular proteins or carbohydrates for function. Overall, these results and rationales suggest that p10-mediated fusion is not specified by binding or regulated post-translationally by activation triggers such as those common to other membrane fusion inducing proteins.

2.C.3. P10 activity is controlled by expression and surface localization.

In the absence of visible triggers for fusion, we have hypothesized that p10 is made in an active conformation and awaits transport to the cell surface for activity. Thus, we predict that syncytium formation is correlated with sufficient accumulation of p10 at the surface of cells. Previous analysis demonstrated that brefeldin A (BFA), an inhibitor of vesicular transport to the cell surface (Pelham, 1991; Chatterjee and Sarkar, 1992; Eggers et al., 1992) was capable of preventing syncytium formation in ARV infected cells (Duncan et al., 1996). Removal of BFA restored surface transport and cell-cell fusion within 1-2 hours (data not shown). Similarly, low temperatures that prevent protein localization to the cell surface successfully blocked fusion until raised above 20°C (data not shown). These experiments suggest that surface localization and accumulation of p10 may be a bottleneck for p10-mediated fusion.

The expression of p10 by virus-infected cells was monitored to determine whether ARV temporally regulates the production of p10 (Figure 2.6). Expression of p10 followed the same pattern as other ARV proteins. P10 production started at three hours post-infection rose gradually until nine hours post infection and drastically increased by twelve hours post infection. This pattern of expression matches the two-phase life cycle of ARV where progeny subviral particles made during the first round of replication are used for further rounds of mRNA transcription, release into the cytoplasm, and translation.

Release of newly formed ARV between 12 and 24 hours post infection corresponds to the time of maximum protein production and rise in syncytium formation. All ARV encoded proteins, structural and nonstructural, increase progressively during the infection.

Although the timing of protein production appears similar for all ARV encoded proteins, figure 2.6 demonstrates that production of sigma C and p10 is lower than the other viral proteins. Of all structural proteins, sigma C is required in the lowest quantities because it is present only within the vertices of virus particles. The open reading frames for both sigma C and p10 are present within the only polycistronic segment of ARV (10 segments of dsRNA total). Furthermore, the p10 open reading frame, although first on the mRNA, has a poor translational start sequence. P10 also undergoes rapid degradation (chapter 3), which together with the low translation, may account for the low p10 production relative to structural genes. Sigma C, on the other hand, is the third of three sequential overlapping reading frames and may also have reduced translation as a result of the mRNA organization. Thus, both the nonstructural p10 and the structural protein that is required in the least amount have reduced production. Altogether, it is possible that the S1 segment of ARV has evolved to ensure low, but sufficient, production of p10 and sigma C. The low production of p10 may be the method by which ARV synchronizes syncytium formation with the life cycle.

We wanted to confirm that sufficient expression and surface localization of p10 results in syncytium formation. The rapid degradation of p10 (chapter 3) and consequential low surface expression relative to intracellular production suggests that large amounts of p10 must be produced prior to accumulation at the surface of cells. The enlarged syncytium formation in ARV infected cells closely correlated with the increased expression of p10 (Figure 2.7). P10 was first detected at 11 hours post-infection by both immunoprecipitation and immunohistochemical staining. The amounts of p10 were, however, low in view of the rapid degradation (>90%) that occurs within the first 45 minutes following p10 production. By 15 hours, significant p10 expression and syncytium were seen. Between 15 and 34 hours post-infection, only a small rise in p10 amounts was seen, most likely due to constant rates of production and degradation. On the other hand, syncytium formation continued to increase reflecting the continuous supply of active p10 proteins on the surface.

The correlation between p10 expression and extent of fusion is maintained in cells transfected with p10. The p10 protein naturally has a weak translational start sequence. When the authentic p10 construct was transfected into quail fibroblasts, expression of p10 fell below detectable limits (Figure 2.8). Small syncytium were clearly evident, however, suggesting that authentic p10 expressed in low amounts relative to the virus-infection scenario was sufficient for low fusogenic activity. Optimization of the translation start sequence of p10 resulted in increased expression of p10 (Figure 2.8) and subsequent syncytium

formation. We can therefore clearly state that the fusion activity of p10 is controlled by infection/transfection-dependent-expression. The production-based control of p10-mediated membrane fusion also supports that triggers, conformational changes, multimerization changes and protein-protein interactions would not be necessary for regulation of the cell-cell fusion activity of p10.

2.C.4. P10 may depend on cell-cell protein interactions.

The life cycle of enveloped viruses imparts an additional constraint on the roles fulfilled by their fusion proteins that is absent in ARV p10. As mentioned in the introduction, membrane fusion is presumed to involve the approach of opposite membranes and their destabilization. Because enveloped viruses are in suspension, adherence to cellular membranes is a stipulation for membrane fusion. Since p10 only functions within infected cells, it could hypothetically rely on intercellular interactions that maintain adherence and cell-cell communication to correctly arrange opposing membranes.

The implications of using cell-cell interactions that are independent of ARV infection and p10 production for apposing donor and target membranes during p10-mediated fusion are two fold. First, the reliance on cell-cell interactions for membrane apposition in p10-mediated fusion would support the absence of a need for domains within p10 that are involved in cellular receptor binding. Secondly, it would suggest that the refolding of enveloped virus fusion proteins to

conformations that appose donor and target membranes is necessary for intimate intermembrane contact, but not necessarily for release of energy coupled to fusion. Discovery that p10-mediated fusion necessitates cell-cell contact mediated through cellular surface protein interactions would suggest that membrane binding and apposition could be separated from the fusion protein complex.

Several attempts to fuse nonadherent cells failed and suggest that cell-cell contacts or forced proximity may be necessary for the activity of p10. P10 failed to induce fusion between p10-transfected cells and red blood cells (RBC) (data not shown). The RBC fusion assay has been used extensively for the study of hemagglutinating enveloped virus fusion proteins and involves adding RBCs prelabeled with membrane-localized or intracellular fluorescent probes to cells expressing the fusion protein. The fusion protein is activated by attachment or low pH and functions to fuse RBCs with the adherent cells. Hemifusion, the fusion intermediate where outer but not inner membrane leaflets are fused between opposing membranes, can be monitored by the transfer of dyes from the RBC to the cellular lipid bilayer. Content mixing results from complete fusion and the transfer of intracellular fluorescent probe between RBCs and adherent cells. The standard use of the RBC fusion assay to study the activity of enveloped virus fusion proteins prompted our attempt to apply this assay to studies on p10. The RBC fusion assay is used routinely in the laboratory of Dr. Judith White at University of Virginia. Even with the expert advice of Dr. White,

and using her RBC fusion assay system during a visitation in her laboratory, we were unable to show complete or hemifusion between p10-transfected cells and RBCs.

The inability of p10 to function in the RBC fusion assay may be a consequence of its slow fusion rate, the absence of regulation, or the absence of cell-cell contact. The RBCs only maintain the fluorescent probes for a limited time period before leakage or lipid probe transfer is seen. Because the RBC fusion assay is only functional for approximately 30-60 minutes, the inability of p10 to function in this assay may simply reflect a difference in the rate and efficiency of p10-mediated fusion. As mentioned previously, enveloped virus fusion proteins have evolved to fuse rapidly, and may therefore be easily monitored for activity. Furthermore, because enveloped virus fusion proteins are regulated and do not fuse until correct conditions are established, fusion between adherent cells and RBC can be assessed immediately upon activation of the fusion protein. P10 on the other hand, may have evolved to function slowly and without regulation, making assays that rely on rapid and controlled fusion impossible. Alternatively, p10-mediated fusion may depend on pre-existing cell-cell contact that would be absent between adherent cells and RBCs. Our attempt to include nonfusogenic influenza hemagglutinin proteins with p10 during transfection to permit RBC-adherent cell contact failed to permit p10-mediated fusion. Although on first inspection this would suggest that cell-cell contact is not sufficient for p10-mediated fusion of RBC to adherent cells, one cannot assume that HA and p10 localize together.

The finding that HA pulls RBC to the ends of cells (Armstrong, communication) may reflect a localized binding between RBC and adherent cells where p10 is absent. The inability of p10 to induce fusion of RBCs could, therefore, reflect a need for cell-cell interactions during p10-mediate fusion.

Support for the requirement of cell-cell contact mediated by cellular surface proteins was provided by the inability of p10 to fuse transfected cells in suspension. Transfection of human monocyte lymphoma (U937), insect ovary epithelial (SF21), human lymphoblast (Jurkat), and mouse blood macrophage (J774) suspension cell lines with p10 did not induce cell-cell fusion (data not shown). Syncytium formation rather than fluorescent probe transfer is a better indicator of p10-mediate fusion as it permits sufficient time for the activity of p10 to reach significant levels. In our assay for p10-mediated fusion of suspension cells, however, we did not account for the difference in transfection efficiency found between suspension and adherent cells. A more telling experiment would be to transfect a monolayer of cells (i.e. QM5 or Vero) with p10. Addition of live suspension cells prelabeled with intracellular cell tracker green dye (taken up by live cells and maintained within cells for up to 72 hours) could be added to p10-transfected suspension cells. Following immunohistochemical staining for p10 with texas red, microscopy could be used to quantify red-green cofluorescence indicative of syncytium formation. This proposed experiment would provide more accurate controls for transfection efficiency, expression, stability, and surface localization of p10. Conclusive evidence showing that p10 cannot fuse cells that

are not in direct contact would strongly suggest a requirement for intercellular interactions during p10-induced membrane fusion. A cell line that can switch from a suspension to an adherent growth phenotype under varying conditions would be optimal for this endeavor, if it exists.

2.D. DISCUSSION

This chapter provides preliminary data that suggests the fusion proteins (p10) of nonenveloped avian and Nelson Bay reovirus have evolved as minimal machinery for catalysis of biological lipid bilayer fusion devoid of complexities necessary for specificity, regulation, efficiency or rapidity of membrane fusion. The accessory role of p10 in the life cycle of ARV and the involvement of p10 in fusing infected cells rather than in entry suggest that p10 evolved under different limitations than the fusion proteins of enveloped viruses. Notably, the life cycle of ARV provides the rationale for why p10 would have been free to evolve without pressures to function rapidly, efficiently, specifically and under strict regulation. Characteristics of p10 such as its unusually small size, monomeric structure, and lack of heptad repeats support the hypothesis that p10 is produced in a fusion-active state and that p10 activity is regulated by slow protein accumulation rather than activation, conformational and multimeric changes, and protein-protein interactions.

In agreement with the proposition that p10 is synthesized in the fusion active state, no trigger for activation of p10 activity has been identified. P10 fuses at physiological conditions and probably does not bind to specific cellular proteins or carbohydrates. Instead, syncytium formation correlates with p10 expression and accumulation within ARV- infected and p10-transfected cells. That syncytium formation is most significant late during the ARV life cycle may have evolved to ensure that cell damage does not precede virion production. Although

p10 is produced throughout the life cycle of ARV like other viral proteins, the weak translational start sequence, the polycistronic mRNA organization and the rapid degradation of p10 likely contribute to the relatively slow accumulation of p10 within infected cells.

If p10 does in fact initiate in a fusion active conformation, it would be the only biological fusion protein to do so, and would be useful in dissecting determinants for fusion from those involved in regulation, specificity, and kinetics. Significant evidence for a fusion competent conformation of p10 directly upon synthesis is provided in chapter 3 where fusion peptide exposure is presumed to occur within the preliminary p10 structure, which directly accounts for rapid degradation of p10. Because this hypothesis is so distinct from the present paradigm of membrane fusion, numerous strategies will be essential to convince the scientific community.

The binding of the p10 ectodomain to membranes pretreated with trypsin could be used to discern whether p10 initiates in a fusion-active state or whether it requires binding to membrane proteins for activation. Unlike the experiment described in the results section, the ectodomain of p10 would have to be constructed without an N-terminal signal sequence to eliminate the need for membrane-associated proteins involved in translocation and signal peptide cleavage. This would allow membranes to be pre-treated with trypsin without the risk of deleterious effects on p10 ectodomain processing. The association

between the fusion peptides within p10 ectodomains and trypsin-pretreated membranes, in itself, would suggest that p10 is produced in a fusion active state and does not require activation for function. We predict that the structure of p10 ectodomains expressed alone would resemble the ectodomain structures produced in the presence of the entire protein. This prediction is primarily founded by principles of membrane protein folding that suggest an independent folding process for extracellular and transmembrane domains (Heijne, 1996; Popot and Engelman, 1990, 2000; Baldwin and Rose, 1999a, 1999b; White and Wimley, 1999). The ability of the ectodomain to bind to trypsin-treated membranes through the fusion peptide would suggest that the fusion peptide was exposed in the primary conformation of p10 and did not require additional triggers such as low pH or receptor binding for activity.

Other methods to determine whether several states are involved in p10-mediated membrane fusion are contingent on finding conditions that would favor activation of p10, if present. If changes in p10 conformation or multimerization are necessary for fusion, antigenicity of p10 would be altered. Monoclonal antibodies could be generated to a panel of linear ectodomain epitopes and assessed for their binding to p10 prior to, and following exposure to target membranes. Biophysical analysis of p10 structure in the presence or absence of lipids could be undertaken. Cross-linking reagents could assist in 'locking' pre- and post-fusion states. These additional confirmatory experiments would substantiate the absence of regulation of p10. In combination with the rationale and data

provided in this chapter, we may succeed in demonstrating that p10 function is not specific, regulated, rapid or efficient and has evolved as minimal fusion machinery.

In addition to addressing the characteristics of p10 that contribute to the fusion of cellular membranes, this chapter suggests that additional cellular components could participate in p10-mediated fusion. Specifically, we propose that cell-cell interactions may be necessary to bring donor and target membranes into close apposition. Whether p10 does or does not necessitate membrane-membrane interactions for fusion will assist the understanding of protein-mediated fusion as a whole. With the exception of some paramyxovirus strains, all enveloped virus fusion proteins necessitate membrane binding for attachment to the donor membrane and for consequential conformational changes that activate the protein. With enveloped virus fusion proteins that have receptor binding and fusion domains on separate proteins, interactions between these proteins are speculated to occur. Our findings would suggest that these interactions between binding and fusion components are not necessary for fusion per se, but may be involved in the regulation of enveloped virus fusion proteins that have evolved a method to link binding with fusion activity.

An interesting consideration in understanding protein-mediated membrane fusion is the minimum distance required between membranes and whether this distance is so close that it requires energetic mechanisms to pull donor and target cells

towards one another. The enveloped virus fusion proteins are proposed to bind target cells in an extended conformation through the binding subunits, to insert the fusion peptide into the target membrane, and to undergo further conformational change that reduce the distance between fusion peptide and transmembrane domains. The six helix bundle formed by paramyxoviruses, orthomyxoviruses and other virus families would bring donor and target membrane to a distance similar to that predicted for p10. If membrane fusion requires active forces for close membrane apposition, what fulfils this role for p10? Are these forces necessary, or is this distance easily achievable? Do surface protein interactions between cells sometimes bring membranes into such close proximity? For p10 to interact with target membranes, the close apposition is assumed to occur, but how? Studies using p10-containing liposomes in the presence or absence of biological membrane components would provide answers to these important questions. Answering these questions will not only help us understand p10-mediated fusion, but will help us understand the minimal requirements for membrane fusion mediated by proteins in general.

2.E. CHAPTER 2 FIGURES

Figure 2.1. The replication of ARV and NBV. Nonenveloped viruses enter the cytoplasm following receptor mediated endocytosis and exit from the endosomes through mechanisms poorly understood. In the absence of lipid envelopes, entry of ARV and NBV does not involve membrane fusion. Partial uncoating stimulates the RNA polymerase activity and positive sense RNAs are made within the particles and released into the cytoplasm where they function as templates for translation by host machinery. Orthoreoviruses have ten segments of RNA that must undergo this process. Structural proteins assemble into new virus particles that package the sense RNA. Within the particles, sense RNA is copied to produce a dsRNA genome. Like all nonenveloped viruses, ARV and NBV exit infected cells through lysis and do not acquire cellular membrane or membrane bound proteins. Unique to the fusogenic orthoreoviruses, production of the integral membrane p10 protein, which is transported to the cell surface, results in cell to cell fusion. P10 is not integrated into newly formed virus particles and is not necessary for the entry, replication, and exit of ARV and NBV.

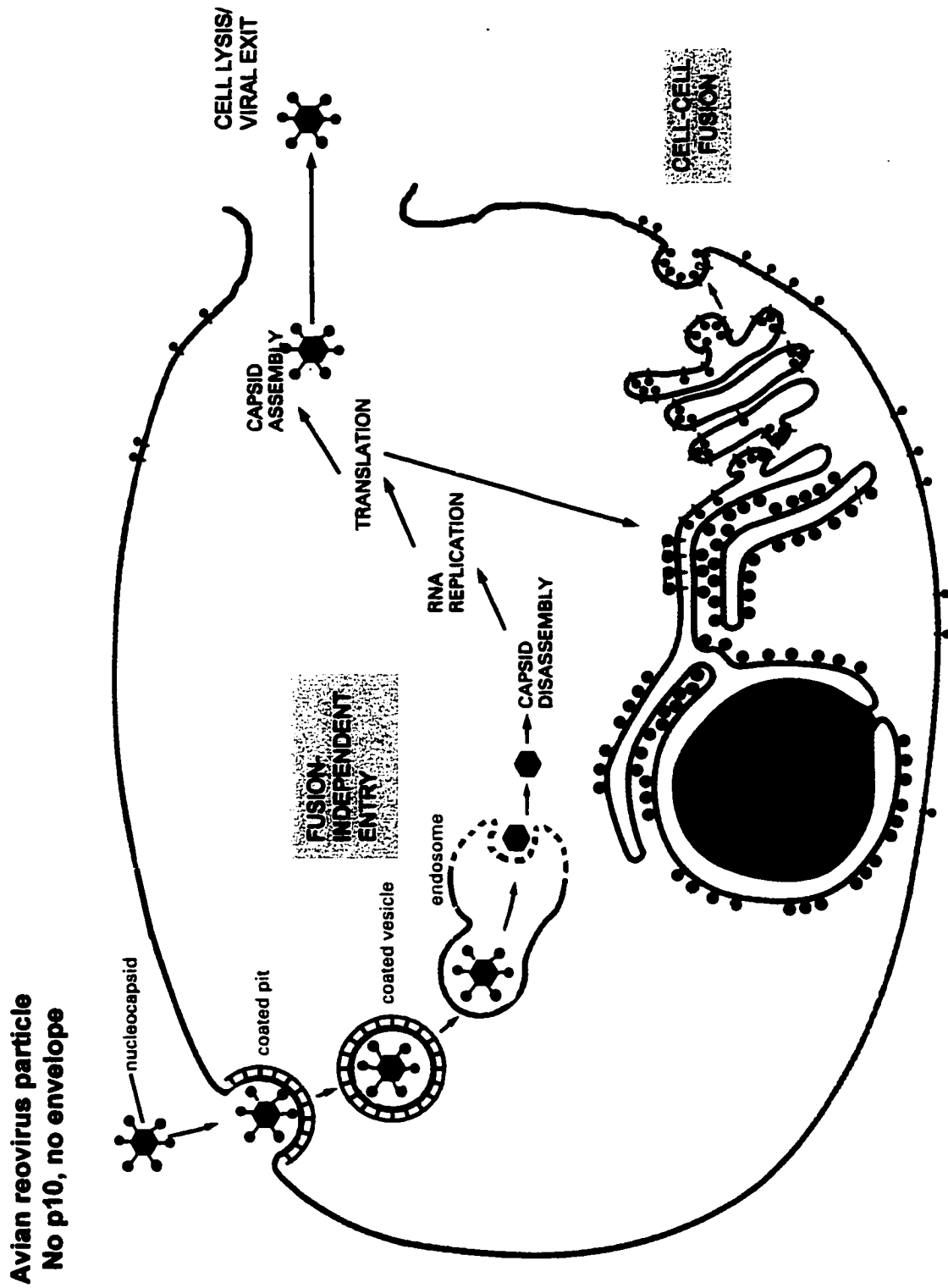


Figure 2.1

Figure 2.2. Characteristics of p10. The cartoon depicts the domain organization of p10. At the N-terminus is the hydrophobic patch (HP) that functions as the fusion peptide (FP) flanked by two cysteines, which is predicted to fold into a disulphide-bonded loop structure. Between the HP (yellow) and transmembrane domain (red) is the conserved region (CR, green) present in both ARV and NBV p10 proteins. The intracellular motifs of p10 include the non-conserved region (NON-CR), the basic region (blue), and two cysteines that are palmitoylated (pink moon). The amino acid sequence of the ecto- and endo-domains is provided, as are the positions of the first and last residues.

Figure 2.3. Ectodomain-membrane association is maintained following cellular protein cleavage with trypsin. *In vitro* transcribed and translated p10 ectodomains, in the presence of canine microsomal membranes, were subjected to high speed centrifugation to separate membranous (P) from soluble (S) components. The p10 ectodomain associated strongly with membranes. Membrane disruption in the presence of NaCl, or trypsin followed by repeated centrifugation was used to assess the continued association of p10 ectodomain under these conditions. Membrane dissolution with detergent (RIPA) resulted in complete solubilization of p10 ectodomain. Methods are described in chapter 3.

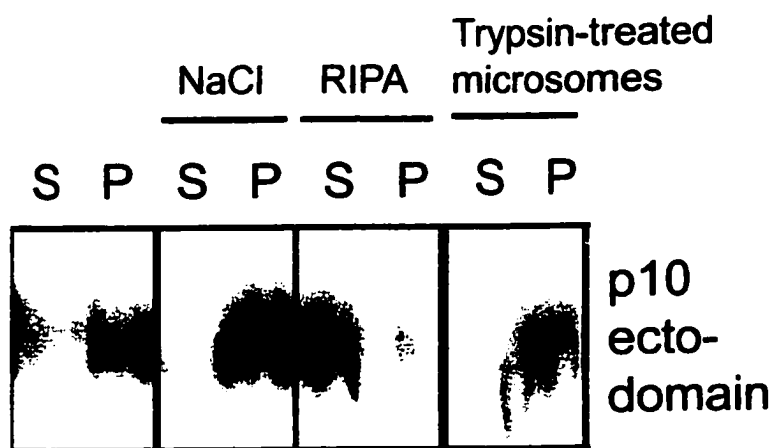


Figure 2.3

Figure 2.4. P10 functions with diverse target membrane origin. Quail cells (A), human epithelial cells (B) and primary human dendritic cells (C) were incubated with cell tracker green (green cells) and added to quail cells transfected with p10 and pre-incubated with cell tracker blue (blue cells). Twenty four hours following the mixing of cell populations, methanol fixation and immunohistochemical staining for p10 using texas red-conjugated secondary antibodies identified p10-expression and multinucleated syncytia (red). An overlay of blue, green, and red fluorescence showed clearly that all cells types were successfully used as target membranes for membrane fusion. Methods provided in chapter 3.

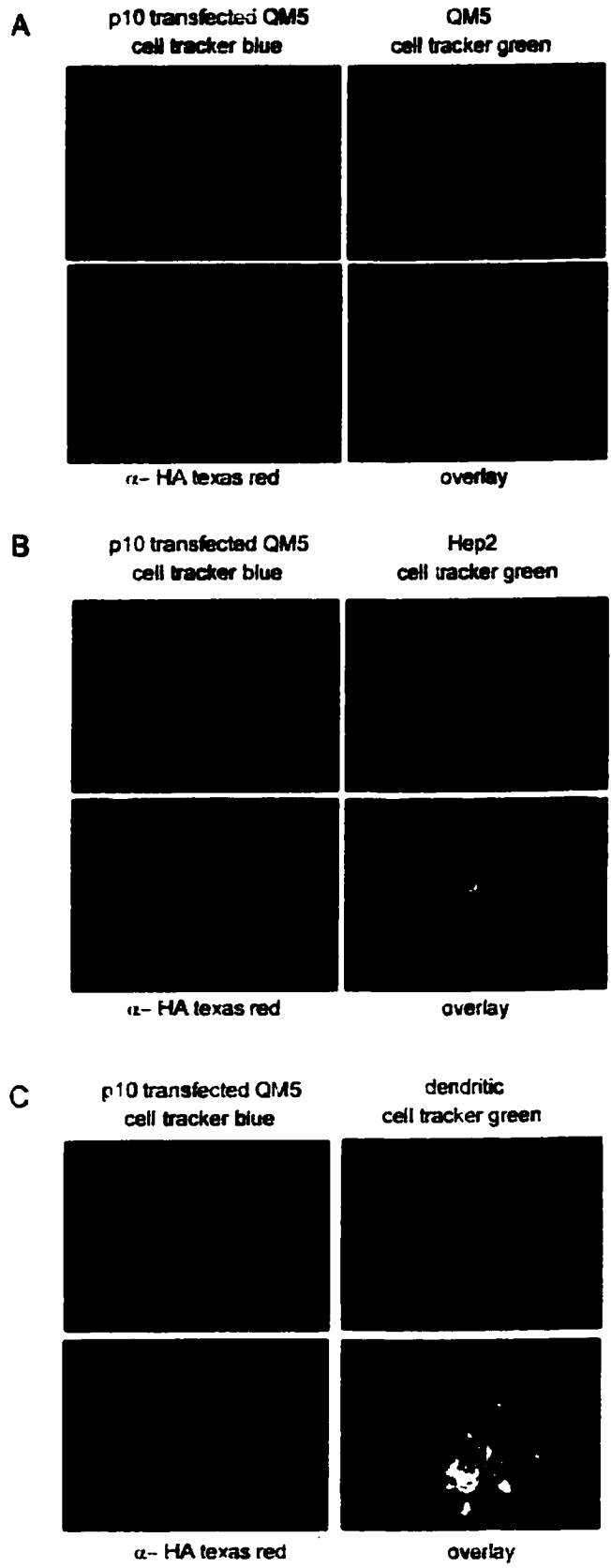


Figure 2.4

Figure 2.5. Cell lines susceptible to p10-mediated fusion. A summary table is provided of all cell lines shown to function as targets for p10-induced cell-cell fusion. The common abbreviated name of each cell type, the animal and tissue of origin, and the tissue type are listed. All cells are adherent when propagated in cell culture.

CELL TYPE	ANIMAL ORIGIN	TISSUE ORIGIN	ADHERENT?
VERO	African green monkey	Kidney fibroblast	Adherent
COS-1	African green monkey	Kidney fibroblast	Adherent
CV-1	African green monkey	Kidney fibroblast	Adherent
Dendritic	Human	Primary dendritic fibroblast	Adherent
QM5	Quail	Muscle fibroblast	Adherent
HD-11	Chicken	Macrophage-derived	Adherent
HT-29	Human	Colon adenocarcinoma epitheloid	Adherent
BHK-21	Syrian hamster	Kidney fibroblast	Adherent
3T3	Swiss albino mouse	Embryonic fibroblast	Adherent
Hep2	Human	Laryngeal carcinoma epithelial	Adherent
MDCK	Canine	Kidney epithelial	Adherent

Figure 2.5

Figure 2.6. Expression profile of p10 and structural proteins during the life cycle of ARV. Cells infected with ARV at a MOI of 10 were labeled at 3, 6, 9, and 12 hours post-infection for 30 minutes with [³⁵S]-methionine (75uCi/ml), lysed and immunoprecipitated with either p10-specific antibodies (anti-p10) or polyclonal serum raised against ARV (anti-ARV). Locations of bands corresponding to structural lambda, mu, and sigma proteins are indicated. Locations of sigma C, and p10 bands are also indicated. Methods are identical to those described in chapter 1.

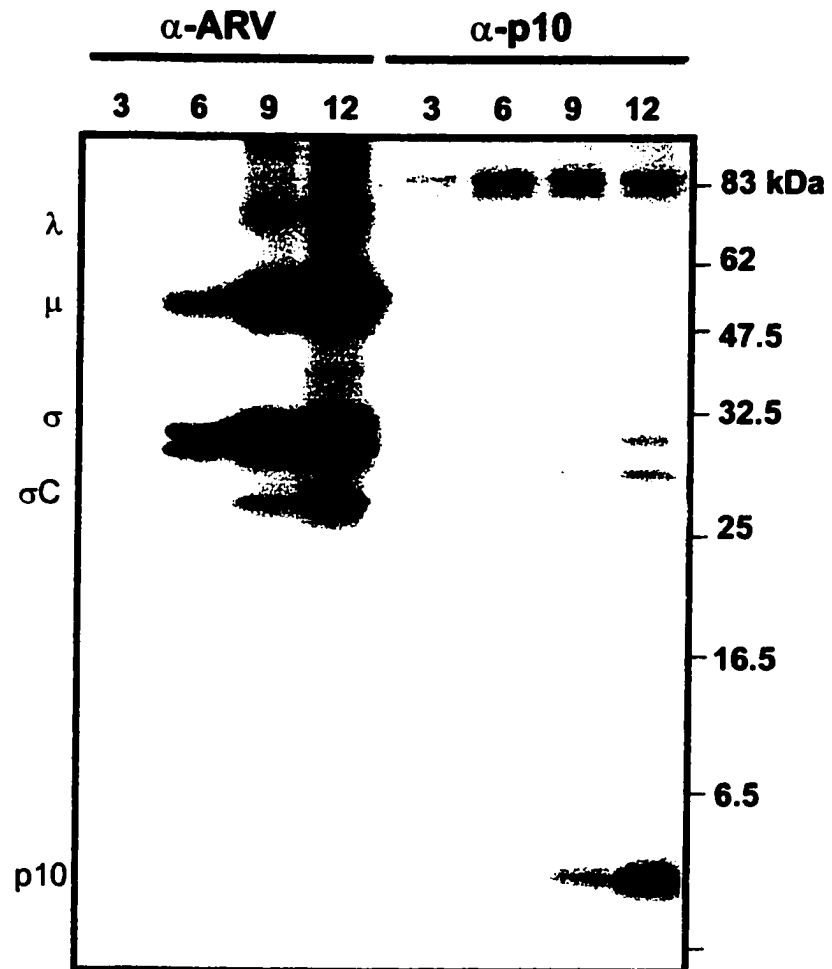


Figure 2.6

Figure 2.7. Expression of p10 in infected cells correlates with the extent of syncytia formation. Cells infected with ARV at a MOI of 0.1 were labeled with [³⁵S]-methionine (75uCi/ml), lysed and immunoprecipitated with p10 specific antibodies to monitor the expression levels of p10 at 6, 11, 15, 18, 23 and 34 hours post-infection. At each time point, another well of infected cells was fixed with methanol and immunostained with p10-specific antibodies to visualize p10-expressing syncytium (arrows). Methods are identical to those described in chapter 1.

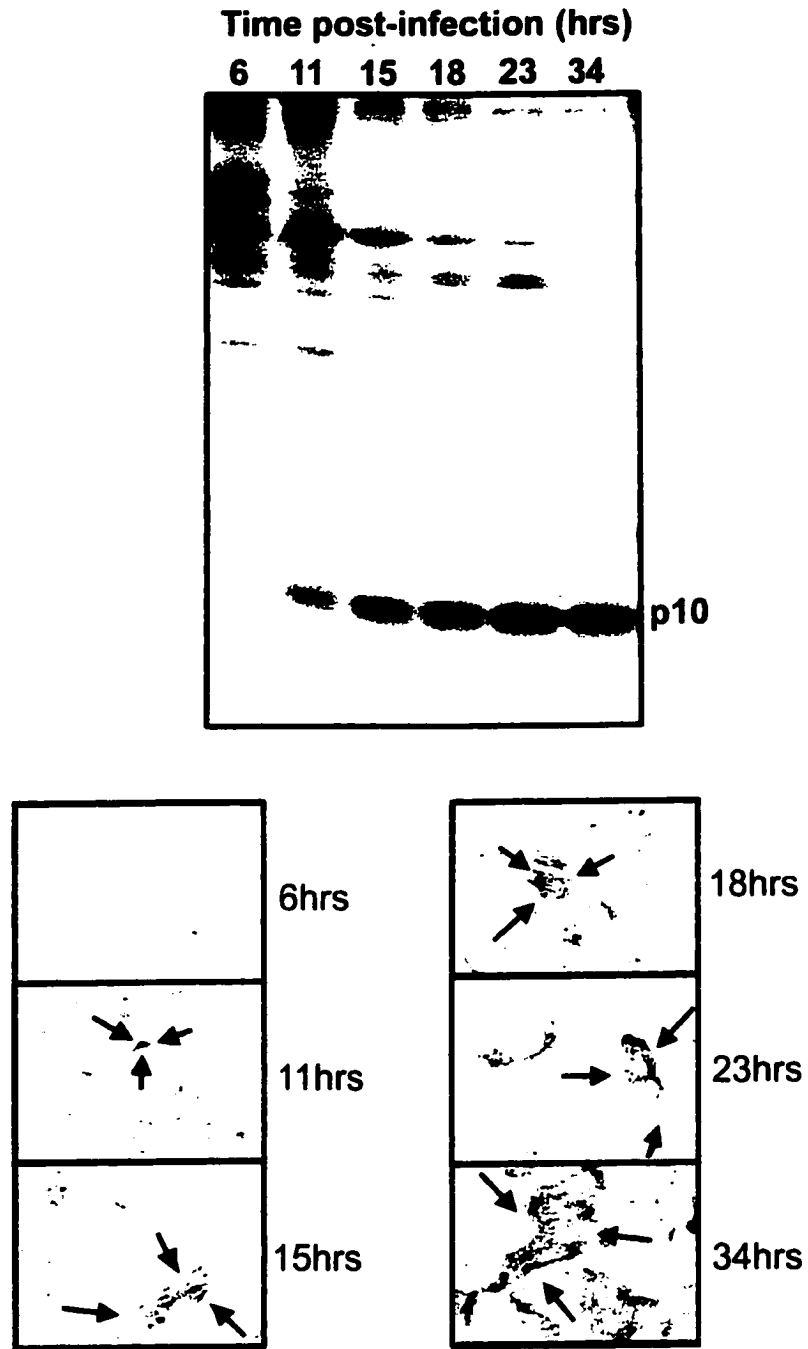


Figure 2.7

Figure 2.8. Expression of p10 in transfected cells correlates with the extent of syncytia formation. Cells were transfected with the authentic p10 construct (auth) or the p10 construct in which the translational start sequence was optimized (opt). To ensure that transfection conditions did not result in p10 saturation, cells were also infected with ARV at an MOI of 0.1 (ARV). Cells were labeled with [³⁵S]-methionine (75uCi/ml), lysed and immunoprecipitated with p10 specific antibodies (anti-p10) to monitor the expression levels of p10 at 24 hours post-transfection/infection. For cells transfected with authentic or optimized p10 constructs, another well of cells was fixed with methanol and immunostained with p10-specific antibodies to visualize p10-expressing syncytium (arrows). Methods are identical to those described in chapter 1.

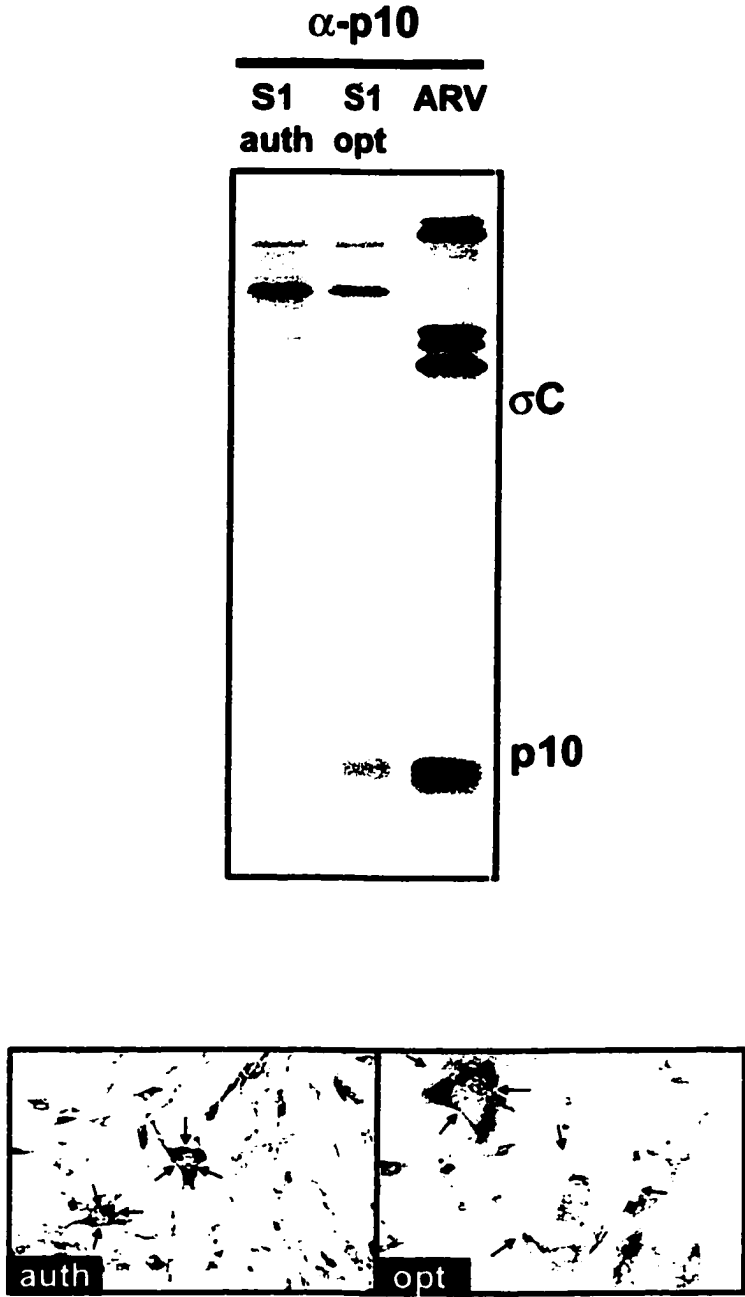


Figure 2.8

CHAPTER 3. An exposed internal fusion peptide leads to rapid protein degradation and suggests the avian reovirus p10 protein naturally exists in a fusion competent conformation.

3.A. ABSTRACT

The smallest biological proteins capable of inducing cell-cell fusion encoded by the nonenveloped avian and Nelson Bay reoviruses (ARV and NBV, respectively) are providing a better understanding of the minimal machinery necessary for membrane fusion. Our present analysis showed that a hydrophobic patch near the N-terminus of these proteins (called p10) shares the following characteristics with the fusion peptides of enveloped virus fusion proteins: an abundance of hydrophobic, glycine and alanine residues, the ability to be modeled as an amphipathic secondary structure, association with membranes when translated *in vitro* and in cell culture, membrane-seeking characteristics that correspond to the degree of hydrophobicity and the ability to fuse liposomes. The hydrophobic patch of p10 is therefore predicted to provide a similar function in the mechanism of membrane fusion as the fusion peptides of enveloped virus fusion peptides, the association with and destabilization of opposing lipid bilayers. Insertions at the N-terminus had minimal effects on the activity of p10 demonstrating that the p10 fusion peptide is internal. The p10 fusion peptide is predicted to have a loop structure based on the conservation and absolute requirement of cysteine residues that flank the hydrophobic patch, and to be composed of two antiparallel beta sheets as suggested by sequence-based structural predictions and CD spectra analysis. Unexpectedly, the fusion peptide of p10 resulted in rapid proteasome-dependant protein degradation during transport from the endoplasmic reticulum to the cell surface. The correlation between degradation and the hydrophobicity of the p10 fusion peptide suggests that the peptide is

exposed to the degradation machinery previous to surface localization. P10 therefore differs from the fusion proteins of enveloped virus fusion proteins, which require specific triggers prior to fusion peptide exposure and consequential fusogenic activation. As the non-structural p10 is not essential for entry of ARV and NBV, folding to bury and inactivate the fusion peptide may not be necessary. P10 may represent the first fusion protein that is fully loaded and poised for induction of membrane fusion.

3.B. INTRODUCTION

Our understanding of the minimal membrane fusion machinery remains confounded by the inherent need for enveloped virus- and intracellular fusion proteins to be highly specific and under strict regulation. It is, therefore, difficult to separate the events that are involved in membrane fusion, from those involved in regulating the fusion event. Thus, in concert with studies on the complex enveloped virus and vesicle fusion protein systems, identification of a fusion-inducing protein that is devoid of the requirement for specificity and regulation would play a key role in understanding the rudimentary fusion machinery required for fusion of biological membranes.

In the previous chapter (chapter 2), we provided the rationale and preliminary data to suggest that p10 has evolved under less stringent restrictions. Like all non-enveloped viruses, ARV and NBV do not require membrane fusion for entry into cells (Duncan and Sullivan, 1998) and therefore maintain p10 as a non-structural protein, expressed only following viral entry and productive replication within cells. The accessory role of p10 during viral infection suggests that p10 may have been free to evolve to the minimal determinants necessary for promoting membrane fusion. There is no evidence for the activation of p10 by a trigger. Rather, p10 appears to fuse cellular membranes of many cell types (kidney and muscle fibroblast, kidney epithelial, dendritic, macrophage, from monkey, mouse, hamster, human or quail origin) non-discriminatorily, regulated

merely by its infection-dependent expression. For these reasons, p10 is a likely candidate for identifying the minimal fusion machinery.

We are interested in defining the characteristics of p10 that are involved in fusion activity. In this chapter, our objective was to determine whether p10 shares similarities with SNARE proteins involved in intracellular vesicle fusion or enveloped virus fusion proteins. Our findings showed that p10 in donor and target membranes do not undergo pair-wise interactions such as those necessary for SNARE proteins. Similar to the enveloped virus fusion proteins, however, the short ectodomain of p10 does contain a fusion peptide. Structural and biophysical analysis suggest that the fusion peptide of p10 is an internal loop composed of beta sheet and is attached to the transmembrane domain by a 20 residue flexible linker.

The interactions between fusion peptides of enveloped virus fusion proteins and target membranes have long been speculated to play a pivotal role in the fusion process. With enveloped viruses, structural changes (described in the section I) induced by specific triggers are required for the exposure of initially buried fusion peptides. For many enveloped viruses, coiled-coil interactions between heptad repeats drive these refolding events. Proposed models for enveloped virus fusion proteins suggest that in addition to protein-lipid interactions, hypothetical energy released during refolding of the metastable fusion proteins may help overcome the energy barriers to membrane fusion (Bollough et al., 1994; Bron et

al., 1993; Carr et al., 1997; Carr and Kim, 1993; Weissenhorn et al., 1997; Kozlov et al., 1998; Bentz, 2000; Baker et al., 1999; Bentz and Mittal, 2000).

The absence of multiple heptad repeats within the 40 residue ectodomain of p10 suggests that p10 does not undergo conformational changes to form coiled-coil structures. Furthermore, since the internal fusion peptide occupies one half of the p10 ectodomain, it is difficult to envision complex refolding events similar to those shared by enveloped virus fusion proteins. In this chapter, we demonstrate that the fusion peptide of p10 is not buried in the initial conformation. The fusion peptide targets p10 for rapid degradation of p10 on route from the ER to the cell surface. The degradation of p10 is suspected to involve the intracellular quality control machinery. The rate of degradation correlates with fusion peptide hydrophobicity suggesting that the fusion peptide in functional p10 is solvent exposed rather than buried within the short ectodomain of p10. The evidence suggests that unlike all other fusion proteins that are large, complex, metastable structures that require regulated refolding for activation, p10 may exist in a fusion competent conformation directly upon synthesis. The results presented in this chapter question whether structural changes, in addition to fusion peptide-bilayer interactions, are necessary to overcome the energy barriers to biological membrane fusion.

3.C. RESULTS

3.C.1. Cell-cell fusion only requires p10 in the donor membrane.

The unusual biological and structural properties of the reovirus p10 fusion-inducing proteins precludes assurances that p10 functions similar to the fusion proteins of enveloped viruses. The p10 protein more closely resembles the SNARE proteins in size. Furthermore, hydrophobic residues found in the 'a' and 'd' positions of the hydrophobic patch near the N-terminus of p10 (see figure 3.2A) could permit the association of p10 proteins in two apposing membranes, although less avidly than the longer coiled coil interactions found between donor and acceptor membrane-bound SNARE proteins. We therefore determined whether p10 was necessary in both donor and acceptor cell membranes.

Using cell-tracker dyes, we were able to distinguish between two populations of cells, only one of which was transfected with a p10-expressing plasmid. Hep2 human epithelial cells (Figure 3.1, green fluorescence) were mixed with the p10-transfected QM5 quail fibroblasts (Fig 1, blue fluorescence). Expression of p10 was confirmed using immunofluorescent staining with antibodies specific to the HA epitope tags inserted at the N-terminus of p10 (Figure 3.1, red fluorescence). The p10-induced syncytia present within the mixed population contained nuclei from both p10-expressing and non-expressing cells (Figure 3.1, overlay). The ability of nontransfected cells to function as the acceptor membrane during cell-cell fusion indicates that p10 is only necessary in the donor membrane.

3.C.2. Mutational analysis of the ectodomain of p10.

If p10 functions similar to the fusion proteins of enveloped viruses, the ectodomain of p10 should be essential for interactions with target membranes. Previous studies have demonstrated that the ectodomain of p10 is critical for p10-mediated fusion (Shmulevitz and Duncan, 2000). HA-specific antibodies inhibited membrane fusion induced by p10 containing HA epitopes at the N-terminus. Furthermore, partial deletion of the ectodomain completely abrogated p10-mediated fusion. To identify the characteristics of the ectodomain essential for p10 function, a thorough mutagenesis study was conducted.

Two HA epitopes were added to the N-terminus of all p10 constructs for ease and consistency of immunoprecipitation and surface immunostaining protocols. Experiments demonstrated that HA-tagging the N-terminus of p10 did not inhibit fusion but merely delayed the kinetics of syncytium formation (see Figure 3.5A). Syncytium formation for all of the 2HAN constructs was therefore followed for 4 days, the endpoint for maximal syncytium formation, before concluding a particular mutant was incapable of inducing fusion. All mutants were transfected into quail fibroblasts and assessed for their expression levels, membrane association, surface expression, and fusogenic ability by visualization of multi-nucleated syncytium formation.

Sequence-based protein predictions and comparative analysis of the NBV and ARV p10 proteins identified two domains with distinct characteristics within the

ectodomain. The conserved domain is a stretch of 10 residues that are absolutely conserved between NBV and ARV despite an overall identity of only 33% between the two p10 proteins (Figure 3.2A). Three mutations were introduced within the conserved region (G29A, D31A, and L32A). In all three mutants, although expression and membrane integration was identical to the authentic 2HAN construct, surface localization was diminished (Figure 3.2). The absence of surface expression is most likely not a result of reverse orientation within the membrane as the distribution of positively charged residues adjacent to the transmembrane domain, which remained identical for all mutants, is the primary determinant of protein topology (Whitley et al., 1993, Matlack et al., 1998). Reduced surface expression suggests that the conserved domain is involved in the traffic to, or retrieval from the surface of cells. Since the extent of surface localization likely impacts the fusogenic potential of p10, these results did not address whether the conserved domain is also involved in the fusion reaction.

Near the N-terminus of p10 is a hydrophobic patch of 16 residues flanked by conserved cysteines (Figure 3.2A). Mutating valines at positions 15 or 19 to methionine ablated p10-mediated fusion without effecting p10 expression or membrane association (Figure 3.2B). Abrogation of the fusion activity for V15M and V19M could not be explained by decreased surface expression since surface localization was actually enhanced for these mutants (Figure 3.2D). In contrast, the polar threonine within the hydrophobic patch withstood mutation to

methionine without effecting p10-induced cell-cell fusion. Thus, the ectodomain appears to have two essential domains. The conserved region within p10 influences surface expression while the hydrophobic patch is directly implicated in the fusion process, a function dependent on the conservation of hydrophobicity in this region.

In addition to the hydrophobicity, the conserved cysteines flanking the hydrophobic patch and the secondary structure appear to be essential for p10-mediated fusion (Figure 3.2B). Substitution of each cysteine with alanine or serine completely abrogated membrane fusion. Although serine more closely resembles cysteine in polarity and molecular shape, substitution to alanine was also performed to ensure that reduction in hydrophobicity did not account for the inactivation of the ectodomain. The necessity of cysteines suggests that disulphide bonds may be involved in the folding of p10. In addition, the insertion of an alanine within the hydrophobic patch, which would have minimal effects on overall hydrophobicity but would distort the residue arrangement within all secondary structures, eliminated the functionality of p10 (Figure 3.2). These results suggest that in addition to the hydrophobicity, secondary structure may be an essential characteristic of the hydrophobic patch.

3.C.3. The hydrophobic patch within the ectodomain of p10 accounts for rapid protein degradation.

The stability of p10 and p10 mutants within transfected cells was assessed to ensure that increased protein turnover was not responsible for reduced fusion competency of V15M, V19M, G29A, D31A and L32A. Surprisingly, pulse-chase analysis consistently demonstrated a rapid rate of degradation of authentic HA-tagged p10 (Figure 3.3A). Sigma C, a structural protein of ARV, remained stable under identical conditions (Figure 3.3A). To exclude the possibility that the short half-life of p10 reflected the formation of insoluble p10 aggregates, harsh extraction conditions (denaturation and ionic detergent) were used to solubilise p10 (Cuesta et al., 2000). The levels of 2HAN over time showed a similar pattern under both mild and harsh lysis indicating that degradation, and not the formation of insoluble p10 aggregates, was responsible for the short half-life of p10 in transfected cells (Figure 3.3B). The observations that 2HAN is functional, that C-terminally-tagged p10 displayed the same rapid degradation profile (data not shown), and that specific mutations in p10 prolonged p10 half-life (see below), implies the short half-life is an inherent feature of p10 and is not the consequence of the N-terminal HA epitope tags. In spite of this rapid degradation, p10 is still found on the cell surface (Figure 3.2D). Prolonged exposures of the autoradiograms shown in Figure 3.3 indicated a low steady-state level of p10 (data not shown) suggesting that a population of p10 that

escapes the rapid degradation pathway is maintained in a relatively stable state in cells, possibly at the cell surface (see below).

Further studies demonstrated that the characteristics of the ectodomain were responsible for the rapid degradation of p10. Cloning and expression of the HA-tagged p10 ectodomain containing the cleavable signal sequence of the influenza hemagglutinin (2HANE), for translocation into the endoplasmic reticulum, showed that the ectodomain alone is degraded at a similar rate as the full-length 2HAN protein (Figure 3.3A). To determine the characteristic(s) of the ectodomain responsible for the rapid degradation of 2HANE and 2HAN, the stability of different p10 mutants was evaluated. As expected, deleting the ectodomain from p10 prevented rapid degradation (Figure 3.3C). Furthermore, mutations to the hydrophobic residues and conserved cysteines in the hydrophobic patch eliminated rapid protein turnover (Figure 3.3C). In comparison, mutations to residues found outside of the hydrophobic patch (D31A and K67M) had no effect on the degradation rate. A more detailed time course analysis clearly demonstrated the correlation between hydrophobicity of the hydrophobic patch and the rate of p10 degradation (Figure 3.3D). The half-life of p10 was approximately 5 min. with almost undetectable amounts following a 30 min chase. Substitution of the polar threonine residue with the slightly more hydrophobic methionine residue (T13M) resulted in an increased rate of degradation, with a protein half life of less than 5 minutes and near undetectable levels by 15 min. In contrast, substitution of the hydrophobic valine residue to the

less hydrophobic methionine residue (V15M) lead to a prolonged p10 half-life of approximately 30 min. with detectable levels of p10 persisting for up to 1-2 hrs (Figure 3.3D). These results suggested that the rapid degradation of p10 is a consequence of the hydrophobicity found within the ectodomain. The increased stability of V15M, V19M, C9A/S and C21A/S most likely accounts for their increased surface expression described previously (Figure 3.2B).

3.C.4. The hydrophobic patch in the ectodomain of p10 has membrane-seeking qualities.

The preservation of the hydrophobic patch despite the severe consequence of rapid degradation suggested that the hydrophobicity of this region is critical for p10 function. Furthermore, the hydrophobicity profiles of both ARV and NBV p10, calculated using the Kyte-Doolittle hydrophathy index (Kyte and Doolittle, 1982) and the Wimley-White interfacial hydrophobicity scale (Wimley and White, 1996), demonstrated a conservation of the hydrophobic characteristic despite significant divergence in primary amino acid sequence (data not shown). The plots suggested that the hydrophobic patch of p10 might partition at the bilayer interface, a property common to the fusion peptides of enveloped viruses (Nieva and Suarez, 2000). However, the overall hydrophobicity of the p10 hydrophobic patch, calculated using the Eisenberg Consensus Index (Eisenberg, 1984), is significantly less than those of other fusion peptides (Shmulevitz and Duncan, 2000; White, 1992).

We confirmed that the ectodomain of p10 possesses overall hydrophobic and membrane-seeking qualities using Triton X-114 phase-partitioning analysis (Bordier,1981). As expected, the cytoplasmic reovirus sigma C protein associated almost exclusively with the aqueous fraction (Fig 4A). In contrast, the transmembrane domain-bearing 2HAN preferentially associated with the detergent fraction. Interestingly, the ectodomain of p10 (2HANe) showed characteristics of both soluble and integral membrane proteins, partitioning approximately equally to both the aqueous and detergent phases. Modifications to the primary sequence of the conserved domain as well as small changes to the hydrophobicity of the hydrophobic patch did not translate into noticeable effects on the preferential distribution between aqueous and detergent fractions, as T13Me, A14/15e, V15Me, V19Me, C21Ae, G29Ae, and L32Ae all behaved similar to 2HANe. We assume that the slight changes to the hydrophobicity of these mutants do not sufficiently alter their partitioning properties in spite of the profound effects of these mutations on the fusogenic activity of p10.

The Triton X-114 partitioning analysis confirmed sequence-based predictions and demonstrated that the ectodomain of p10 does possess hydrophobic characteristics. The membrane interaction potential of p10 and of the ectodomain was confirmed by cell fractionation (Figure 3.4B). Analysis of subcellular protein localization revealed that the soluble sigma C protein was found almost exclusively in the soluble (S) fraction, while full length 2HAN and the 2HANe ectodomain construct were entirely present within the membranous pellet (P)

fraction of cells. The association of 2HANE with cell membranes was resistant to extraction with high salt and NaCO_3 at high pH conditions, supporting that membrane association was avid and dependent on forces other than ionic interactions (Figure 3.4B). Mutations that slightly reduced the hydrophobic characteristics of the hydrophobic patch (V15Me, V19Me) decreased, but did not eliminate, membrane association while the slightly more hydrophobic T13Me mutant displayed no such change in its membrane association (Figure 3.4B). The differences in the membrane interaction potentials of these mutants, which were not evident in the Triton X-114 partitioning analysis, coincided with the relative fusion activity of the mutants. Substitution of the conserved cysteine with alanine (C9Ae) had only a minimal effect on the association of the ectodomain with cellular membranes suggesting that if a disulphide bond does exist in p10, the ectodomain may fold sufficiently well in the absence of such a bond to associate with membranes. The insertion of alanine within the hydrophobic patch had a slightly more noticeable effect on membrane association (Figure 3.4B), again supporting that the positioning of the residues within the patch is important. These results did not address the possibility that modifications to the hydrophobic patch have more drastic effects on the characteristics of membrane interactions such as depth and angle of insertion, which may reflect the loss of fusion activity with these mutants.

The membrane fraction from disrupted cells was further subjected to sucrose gradient centrifugation in order to establish that the p10 present in the membrane

pellet reflected membrane association and not the presence of insoluble protein aggregates. In all cases, the 2HANE proteins present in the cellular membrane pellet floated at the interphase between the 50% and 5% sucrose layers (fraction 2), with little protein evident in the insoluble protein pellet (fraction 4). These results confirmed that p10 and the p10 ectodomain are present in the membrane pellet from disrupted cells due to association with membranes, a property shared with the fusion peptides of enveloped viruses.

3.C.5. The p10 hydrophobic patch induces liposome fusion.

Several characteristics of the hydrophobic patch are reminiscent of the fusion peptides of enveloped viruses. In addition to the hydrophobic and membrane association qualities, the hydrophobic patch of p10 is rich in alanine and glycine residues, is located on a transmembrane anchored protein directly involved in membrane fusion, and is located near the N-terminus (White, 1990; White 1992). Studies supporting the direct role of fusion peptide insertion and destabilization of lipid bilayers in promotion of membrane fusion have accumulated for over a decade (Duzgunes and Shavnin, 1992; Durell et al., 1997; Martin et al., 1999; Pecheur et al., 1999). Amongst the most compelling evidence is the ability of synthetic peptides generated from the fusion peptide sequences of many enveloped viruses to induce liposome fusion (Wharton et al., 1988; Duzgunes and Shavnin, 1992; Martin et al., 1994; Nieva et al., 1994). Although biological membranes are expected to place additional constraints on the fusion process and the remaining fusion protein domains would be necessary for biological

membrane fusion, liposome fusion serves as a functional marker for membrane destabilizing fusion peptide activity. Consequently, it was interesting to find that synthetic peptides corresponding to the ectodomain and the hydrophobic patch of p10 were able to induce low, but detectable, fusion of liposomes.

In collaboration with Dr. Richard Epand (McMaster University) the ability of synthetic peptides to promote membrane fusion of large unilamellar vesicles (LUV) composed of dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), and cholesterol at a molar ratio of 1:1:1 was assessed by monitoring lipid mixing using the resonance energy transfer assay (Struck et al., 1981). No lipid mixing was found between vesicles in the absence of peptide (Figure 3.5A, DMSO control). A 10 μ M concentration of the peptide corresponding to the hydrophobic patch of p10 flanked by the two conserved cysteines (p10hp) promoted approximately 7% lipid mixing. A similar concentration of the longer p10 ectodomain peptide (p10ed) that encompasses both the hydrophobic patch and adjacent conserved region resulted in approximately 15% lipid mixing. By comparison, the moderately fusogenic SIV fusion peptide at a concentration of 5 μ M resulted in approximately 20% lipid mixing (Figure 3.5A). Although moderate, these results indicated that the p10 ectodomain and its hydrophobic patch are capable of inducing lipid bilayer mixing. Together, the sequence-based and mutational analyses, the strong hydrophobic and membrane-seeking qualities, and the ability to induce fusion of liposomes, strongly suggest that the hydrophobic patch of p10 functions as a

fusion peptide similar to, although less efficiently than, those found in enveloped virus fusion proteins.

3.C.6. Structural characteristics of the p10 fusion peptide.

The fusion peptides of enveloped viruses have been classified as either N-terminal or internal depending on their location within the fusion protein. The internal fusion peptides of enveloped viral fusion peptides are commonly 16-20 residues and are bound by charged residues. In comparison, N-terminal fusion peptides are generally longer (20-36 residues). The p10 fusion peptide bears resemblance to the internal fusion peptides of enveloped viruses with an estimated length of 16-19 residues flanked by charged residues. Mutagenesis studies confirmed that the fusion peptide of p10 is internal, as extensions made to the N-terminus of p10 did not abolish p10-induced fusion (Figure 3.5B). Progressive addition of HA epitopes to the N-terminus increased the duration of time preceding cell-cell fusion as a consequence of delayed membrane association (data not shown). The addition of an HA epitope adjacent to the transmembrane domain, however, abrogated fusion despite continued expression, membrane integration and surface localization (Figure 3.5B). This result suggested that, in addition to a role in influencing p10 surface localization (Figure 3.2D), the linker region between the transmembrane domain and the p10 fusion peptide is involved in the ability of p10 to induce fusion of biological membranes. The linker region could impact the biological function of p10 by modifying the length, folding or flexibility of the ectodomain.

Structural studies of synthetic fusion peptides revealed that, with few exceptions (Epand et al., 1992; Rodriguez-Crespo et al., 1996, 2000), an alpha helical structure is most commonly associated with the membrane-bound state of the peptides (Martin et al., 1993; Rapaport and Shai, 1994; Lunenberg et al., 1995; Kliger et al., 1997). Penetration of amino acids into the hydrocarbon core is favorable only if the peptide bonds are hydrogen bonded (White and Wimley, 1999), thus favoring either helical conformations or beta-strands that are arranged in antiparallel or parallel sheets. A beta strand of identical residue composition as an alpha helix would be more than twice the length, a characteristic essential for determining the depth of fusion peptide penetration. We therefore analyzed the secondary structure of the p10 ectodomain and hydrophobic patch.

Computer based structural analysis suggested that the fusion peptide of p10 has a beta strand structure (Chou and Fastman, 1978). Furthermore, the Walsh-Crofts structural index, which take into account that buried structures differ in composition than surface structures, suggested that a buried beta strand is the most favorable structure (Crofts,1994). A hydrophobic face characteristic of fusion peptides is more obvious when the p10 fusion peptide is depicted as a beta strand as compared to an alpha helix (Figure 3.6A). Note that the hydrogen bonding of cysteine, serine, and threonine residues makes them more hydrophobic in membrane environments (Popot and Engelman, 2000). The

secondary structure of the synthetic fusogenic peptides p10ed and p10hp was also directly evaluated by circular dichroism (CD) (Richard Epand, McMaster University). The spectra of both peptides suggested the absence of alpha helix secondary structure as reflected by the absence of minima at 208 and 222nm (Figure 3.6B). Bilayer induction of helical secondary structure was not significant as secondary structure preference was similar in the presence and absence of lysophosphatidylcholine (LPC) (Figure 3.6C). Even in the helix-promoting solvent TFE, both peptides existed preferentially as a beta sheet structure. The elevated helical content of the p10hp peptide in the presence of 66-100% TFE (Figure 3.6B, top panel) is not significant. For both peptides, beta strand accounts for the bulk of the secondary structure (Figure 3.6C). Both secondary structure predictions and CD data support that the internal fusion peptide of p10 adopts a predominantly beta sheet structure. Furthermore, the entire ectodomain of p10 appears to have no helical content, consistent with secondary structure predictions suggesting that the fusion peptide and transmembrane domain are joined through a flexible linker.

As demonstrated by evolutionary conservation and mutagenesis (see Figure 3.2B), the cysteines flanking the internal fusion peptide of p10 are essential for fusion activity. Electrophoretic analysis of p10 under reducing and non-reducing conditions indicated identical gel mobilities demonstrating that the cysteines are not involved in intermolecular disulphide bonds (Figure 3.6D). This did not preclude the possible participation of cysteines in intramolecular disulphide bonds as small loops rarely affect the mobility of proteins sufficiently for detection

by electrophoresis (Gruarin et al., 1997; Glombik et al. 1999). The conserved and essential role of the cysteines in the absence of any role in intermolecular p10 interactions suggested that the internal p10 fusion peptide might adopt a beta sheet-loop-beta sheet structure stabilized by an intramolecular disulphide bond (Figure 3.6B). Support for this conjecture was obtained by assaying the ability of the synthetic peptides to induce lipid mixing under reducing versus non-reducing conditions. As previously demonstrated (Figure 3.5A), the larger ectodomain peptide p10ed induced a greater degree of lipid mixing than the shorter peptide, p10hp, representing the hydrophobic patch. This was true under both reducing and non-reducing conditions, and the extent of lipid mixing was dependent on the peptide concentration (Figure 3.6E). For both peptides, a reducing environment decreased the extent of lipid mixing, an effect most noticeable for the p10hp peptide whose activity was decreased by 50%. Secondary structure assessment by CD spectra analysis under reducing and non-reducing conditions demonstrated a preservation of the predominantly beta strand structure for both p10ed and p10hp peptides under reducing and non-reducing conditions (Figure 3.6C). Overall, studies using synthetic peptides corresponding to the fusion peptide of p10 suggest that disulphide bonds between cysteines may impact, but are not essential, for secondary structure and function.

3.C.7. Failed attempts to show disulphide bonded loop formation and its essential role in p10-induced membrane fusion

The inability of p10 containing substitutions of the ectoplasmic cysteines to induce membrane fusion is predicted to be a consequence of the inability to form the disulphide bonded loop structured fusion peptide. In hopes to show that disulphide loop formation in the ectodomain of p10 is essential for fusion peptide and p10 function, we assessed the effect of several impermeable sulhydryl-reducing or permeable sulfhydryl-reactive reagents on syncytia formation (Figure 3.7A). While reducing agents were expected to disengage disulfide bonds of cell surface localized proteins, the sulfhydryl-reactive agents were used to covalently bind free cysteine residues and prevent disulphide bond formation. Unfortunately, all reagents were toxic to cells by two hours at various concentrations. Even the impermeant reagents were toxic, most likely a consequence of internalization through endocytosis. Reduction of disulfide bond formation of cellular proteins cannot be tolerated for the time period necessary to detect significant differences in syncytia formation. This approach would be feasible with p10 constructed to function more rapidly. Cloning of p10 with N-terminal cleavable added sequence that would permit accumulation of p10 at the cell surface but prevent activity until the correct protease was added is an example of a method by which assays that require rapid fusion could be used to study p10-induced membrane fusion.

Two biochemical approaches were attempted to confirm that the fusion peptide of p10 forms a disulfide bonded loop. For the purpose of these approaches, western blot detection of 2HAN was developed. Optimization of the procedure showed that the limiting factors for detection are the concentration of the 2HAN protein within transfected cells and the antibody concentrations used to detect it. More specifically, it was found that the equivalence of a 25cm² flask of QM5 cells transfected with 2HAN or 2HANe per lane and a 1:100 dilution of mouse monoclonal anti-HA antibodies was required (Figure 3.7B). The use of [³⁵S]-methionine labeled proteins was able to demonstrate that only one half of 2HAN and 2HANe successfully transferred from the gel onto the PDVF membrane, although transfer for 60 minutes was better than 30 minutes (Figure 3.7B). Two alternative transfer buffers were used to determine whether a change in the charge of 2HAN and 2HANe would effect the transfer efficiency of these proteins. Addition of 0.1% SDS to the transfer buffer or the use of a transfer buffer with a pH of 9.0 (more basic than the p10) did not improve the detection of these proteins (data not shown). Although the detection of 2HAN was possible, the requirement for large lysate quantities makes this method of detection inadequate for most applications, especially because in this experiment, pre-immunoprecipitation of the lysate with anti-HA prior to western blot analysis would have removed the high lysate background that would otherwise appear if such large protein loads were used. Recommendations include improving the transfer conditions such that more protein is transferred or increasing the stability

and therefore concentration of 2HAN in cells prior to lysis using of proteasome inhibitors.

The ability to detect immunoprecipitated p10 by western blot analysis permitted biochemical assays for the presence of disulfide bonded loops. The first approach was to cleave p10 at the methionine residues inserted within the predicted loop (T13M, V15M, V19M) with cyanogens bromide and analyze the fragment lengths by SDS-page in the presence, and absence, of reducing agents. Following complete cleavage, a mobility shift would be expected following reduction of p10 if a disulfide bonded loop was present near the N-terminus. This technique required many laborious manipulations and resulted in undetectable yields of p10. As an alternative approach, the six-residue factor Xa cleavage sequence was inserted into the loop region in several places. P10 containing the cleavage sequence within, or at the ends of the fusion peptide was not functional. Nonetheless, these constructs could be used to identify disulfide bonded loops in p10. We have not yet optimized the protocol for factor Xa cleavage and therefore loop formation in p10 remains to be confirmed.

3.C.8. Degradation of p10 is intracellular and involves the proteasome.

The endoplasmic reticulum-associated degradation (ERAD) pathway is an intracellular quality control mechanism that assures misfolded proteins are retained and targeted for proteolysis (reviewed in Ellgaard et al., 1999; Wickner et al., 1999). The signals for degradation include a hydrophobic surface normally

buried in protein-protein interactions or in the core regions of correctly folded proteins (Sadis et al., 1995; Gilon et al., 1998; Johnson et al., 1998). The rapid degradation of p10, shown to correlate with the hydrophobicity of the fusion peptide, suggests that the p10 fusion peptide is exposed to the degradation machinery rather than buried like the fusion peptides of enveloped virus fusion proteins. To strengthen this prediction, we sought to determine whether the intracellular quality control machinery was responsible for the degradation of p10.

Analysis of the degradation rate of p10 proteins that had already been translocated to the membrane fraction of cells (Figure 3.8A) revealed the same rapid rate of protein turnover previously noted for total p10 (see Figure 3.3). Immunofluorescence of single and multinucleated cells expressing 2HAN showed high concentrations of 2HAN predominantly within the cell and surrounding the nuclei (Figure 3.8B). Furthermore, co-immunofluorescence of transfected cells expressing 2HAN with antiserum to HA and to Concalavin A demonstrated an abundance of p10 within the ER at various times post-transfection (Figure 3.8C). Inhibitors of lysosomal acidification (chloroquine and MA) or lysosomal proteases (E64C) were unable to reverse the rapid degradation of 2HAN suggesting that p10 is not transported to the lysosome for removal (Figure 3.8D). Conversely, the proteasome inhibitor MG-132 (Cbz-LLL-OH, Lee and Goldberg, 1998) successfully reduced the rate of p10 degradation. These results are in agreement with a model that predicts the hydrophobic p10

fusion peptide is exposed, thereby contributing to rapid p10 degradation via the ERAD pathway.

Although degradation by the proteasome is usually accomplished before proteins leave the ER, proteasome-dependent proteolysis at post-Golgi compartments including the plasma membrane has been demonstrated (Benharouga et al., 2001). The turnover rate of p10 (i.e. less than 30 min.) was consistent with ER-associated degradation which was previously demonstrated to confer a half life of approximately 30 minutes for the misfolded cystic fibrosis transmembrane conductance regulator. We used two strategies to confirm that degradation of p10 was not a consequence of retrieval from the cell surface. The first strategy was to approximate the time required for surface expression of p10 by following secretion of 2HANE. The degradation of 2HAN occurred well before the time required for 2HANE secretion, which began at 60 minutes and peaked at 2 hours post synthesis (data not shown), suggesting that degradation precedes surface expression. The second strategy was to monitor the stability of surface localized 2HAN. HA-specific antiserum was added to live 2HAN-transfected cells which were then incubated for various times under optimal cell growth conditions prior to fixation and detection by fluorescence. Quantities of surface localized antibody-bound 2HAN remained relatively constant for up to 90 minutes (Figure 3.8E) suggesting that p10 is relatively stable once expressed at the cell surface. The stability of surface localized 2HAN was comparable to V19M (Figure 3.8E)

despite the substantial difference in overall protein turnover of these two p10 constructs (see Figure 3.3).

Reagents that slow cytoskeletal-dependent processes such as endocytosis did not prevent rapid degradation of 2HAN supporting that p10 degradation did not depend on removal from the cell surface for degradation (Figure 3.8F). Inhibitors of proteases expected to function in the endoplasmic reticulum also failed to prevent p10 degradation (Figure 3.8F). Interestingly, inhibition of intracellular transport by reagents (brefeldin A and monensin) or by temperature greatly increased the stability of p10 (Figure 3.8F). Since temperature changes, brefeldin A and monensin have multiple effects on cells, it is difficult to predict the cause of this inhibitory effect on p10 degradation. As all conditions were present at least an hour prior to the pulse-chase analysis of p10, one prediction for the increase in p10 stability is that prevention of intracellular transport resulted in cellular protein degradation that saturated the ubiquitination and proteasomal machinery. Results in figure 3.8F are difficult to interpret and open to criticism but are added in this chapter in hopes that future investigations on p10 may elucidate these curious findings.

The data suggests that the fusion peptide of p10 is exposed prior to surface expression in contrast to enveloped fusion peptides that become exposed at the cell surface after activation-induced conformational changes. The lack of

complete fidelity of the quality control machinery supports that some p10 could escape and accumulate at the cell surface to induce cell-cell fusion.

3.D. DISCUSSION

Our results have demonstrated that the p10 protein of the nonenveloped ARV shares certain similarities with the enveloped viral fusion proteins. Unlike the SNARE proteins but similar to the enveloped virus fusion proteins, the p10 fusion protein is required only in the donor membrane to promote membrane fusion (Figure 3.1). Interactions between fusion peptides and target membranes have been shown for many enveloped viruses (Harter et al., 1989; Stegmann et al., 1989, 1991, 1995; Guy et al., 1992; Tsurudom et al., 1992; Brunner and Tsurudome, 1993; Pak et al., 1994; Weber et al., 1994; Hughson, 1995; Durrer et al., 1995, 1996; Durell et al., 1996; Doms and Peiper, 1997), and are essential for membrane merging (Bosch et al., 1989; Freed et al., 1992; Delahunty et al., 1996; Durell et al., 1997; Printske et al., 1999; Pecheur et al., 1999). We have identified a hydrophobic patch in the ectodomain of p10 that shares numerous similarities to the fusion peptides of enveloped virus fusion proteins: (1) this N-proximal hydrophobic patch is rich in glycine and alanine residues; (2) it has inherent and membrane interaction potential and appears to be capable of inserting into membranes (i.e. it resists extraction with high salt or high pH); (3) it has the potential to form an amphipathic secondary structure that may exist in a loop conformation with two anti-parallel beta sheets; (4) it is essential for the fusogenic activity of p10 and moderate changes to the hydrophobicity of this peptide abrogate p10-induced membrane fusion; and (5) it displays a low, but significant, ability to promote lipid bilayer mixing. These results provide compelling evidence that the hydrophobic patch in the ectodomain of p10

represents an internal fusion peptide that serves to destabilize target cell membranes.

Whether p10 functions alone to mediate the fusion reaction or in concert with undefined host proteins is still not clear. As previously discussed (Shmulevitz and Duncan, 2000), the probability of p10 interacting with a cellular fusion protein in either the donor or target membranes seems unlikely. Such a host fusion protein would need to be ubiquitously distributed in view of the promiscuous fusogenic nature of p10. Moreover, the ability of anti-HA monoclonal antibodies to inhibit fusion induced by the 2HAN construct (Shmulevitz and Duncan, 2000) suggests the direct involvement of p10 in the fusion reaction. Our present demonstration that the ectodomain of p10 can avidly associate with membranes (Figure 3.4) and promote lipid bilayer mixing (Figure 3.5) provides additional data in support of p10 functioning directly as a fusion protein. Such an assertion does not preclude the possible involvement of cellular proteins to mediate the cell-cell interactions required for promoting close apposition of adjacent cell membranes. The p10 protein may differ in this regard from most enveloped virus fusion proteins that mediate both receptor binding and membrane fusion (White, 1990).

In addition to the hydrophobic patch, our current results also suggest the presence of a second distinct region in the ectodomain of p10. A stretch of 10 residues that lie adjacent to the hydrophobic patch are completely conserved in the p10 proteins of ARV and NBV (Figure 3.2A). The involvement of the

conserved domain in protein transport to the surface was predicted from the reduced surface expression of p10 constructs bearing mutations within the conserved domain (Figure 3.2D) despite continued membrane association and similar protein stability to the authentic 2HAN construct. Computational sequence-dependent structural predictions suggested that the conserved domain does not form stable secondary structures but functions as a flexible linker between the hydrophobic patch and the transmembrane domain. Insertion of a nine amino acid HA epitope tag between the conserved and the transmembrane domains resulted in a loss of fusion supporting a need for conservation within the linker region (Figure 3.5B). The length and/or sequence specificity of the membrane proximal region has been shown to be critical for membrane fusion induced by several enveloped virus fusion proteins (Fass et al., 1996; Zhou et al, 1997; Salzwedel et al, 1999; Tong et al., 2001).

The similarities between the p10 hydrophobic patch and the fusion peptides of enveloped viruses outlined above strongly support that they play a similar functional role in the mechanism of membrane fusion. The CD spectral analysis and computational secondary structure predictions both agree with a high beta sheet content within the p10 fusion peptide. The conservation and essential role of the cysteines that flank the fusion peptide suggests the presence of a loop structure, a supposition supported by the reduced fusogenic activity displayed by the p10hp peptide under reducing conditions (Figure 3.6E). Since a reducing environment did not eliminate lipid mixing induced by the synthetic peptides, we

predict that the p10 fusion peptide loop is stabilized by additional hydrophobic and hydrogen bonding interactions between two antiparallel beta strands and may autonomously fold in solution to induce liposome fusion. The essential role of the conserved cysteines for p10-induced syncytium formation may reflect a greater need for the stabilizing effects of an intramolecular disulphide bond in the context of the entire p10 protein and for fusion of biological membranes as opposed to pure lipid bilayers. We therefore propose that the fusion peptide of p10 exists as cysteine stabilized loop containing two anti-parallel beta sheets.

The fusion peptide of p10 is reminiscent of a recently proposed structure common to many internal fusion peptides consisting of a loop formed by two antiparallel amphipathic secondary structures. The crystal structure of the tick-borne encephalitis virus (TBE) fusion protein ectodomain reveals a beta strand-turn-beta strand structure for the fusion peptide (Rey et al., 1995). A similar structure has been proposed for the internal fusion peptide of avian sarcoma/leukosis virus (ASLV) (Delos et al., 2000). The proposition that a loop structure interacts with lipid bilayers is not limited to the internal fusion peptides of enveloped viruses. Lipases such as hepatic lipase, pancreatic and lipoprotein lipase, and cholesterol oxidase have a flexible amphipathic loop that is postulated to create a hydrophobic surface that interacts with the lipid phase (Dugi et al., 1992; van Tilbeurgh et al., 1994; Egloff et al., 1995; Carriere et al., 1997; Sampson et al., 1998). The hydrophobic residues within the loop region of the prothrombin Gla domain were shown to partition into the phospholipid

membrane (Falls et al, 2001). Furthermore, several antimicrobial peptides that lyse lipid membranes, including dodecapeptide, protegrins, NK lysine and granulysin, have a sheet-loop-sheet or helix-loop-helix structure (Aumelas et al., 1996; Andreu et al., 1999; Chen et al., 2000; Raj et al., 2000;). It may be that differences in the depth and/or angle of insertion of these various loop structures into the lipid bilayer may dictate the outcome (i.e. lysis, fusion, or no effect) of the membrane interaction (Brasseur et al., 1997). Interestingly, disulfide bonds commonly stabilize the loops of internal fusion peptides, lipases, and antibacterial peptides, and elimination of the disulphide bonds through mutagenesis of participating cysteines has been shown to reduce the activity of lipoprotein lipase, protegrins, and the internal fusion peptide of ASLV (Hill et al., 1991; Iwanaga et al., 1994; Harwig et a., 1996; Henderson et al., 1996; Delos and White, 2000). There is, therefore, ample precedent for the involvement of a disulphide stabilized loop structure in membrane interacting proteins, similar to our proposed model for the fusion peptide of p10.

In spite of several similarities between the fusion peptide of p10 and those of enveloped viral fusion proteins, significant differences exist. The fusion proteins of enveloped viruses share in common a transition to the active state through conformational changes within the large ectodomain that are induced by specific triggers (Carr and Kim, 1993; Chan and Kim, 1998; Weissenhorn et al., 1997, 1999; Furuta et al., 1998, Gray and Tamm 1998; Berger et al., 1999; Domico et al., 1998; Skehel and Wiley, 2000; Eckert and Kim, 2001). The final antiparallel

coiled coil (trimer-of-hairpins) conformation forces close membrane apposition (Chen et al., 1995; Chan et al., 1997; Malashkevich et al., 1998, 1999; Sutton et al., 1998; Baker et al., 1999; Zhao et al., 2000). Aside from the fusion peptide, only 20 residues remain within the ectodomain of p10 suggesting that p10 be incapable of similar complex rearrangements. In support for the absence of gross refolding events within p10, we have provided evidence for the exposure of the p10 fusion peptide prior to surface localization. The fusion peptide is directly responsible for rapid degradation of p10 by the intracellular proteasome-dependant quality control machinery that recognizes characteristics of misfolded proteins including exposure of hydrophobic/amphipathic domains. As the degradation of p10 correlated with the hydrophobicity of the fusion peptide, it appears that the fusion peptide is exposed to the degradation machinery. The difference in size between the ectodomains of p10 and other fusion proteins may therefore reflect the requirement of other fusion proteins to have conformations that bury or expose the fusion peptide under the regulation of specific triggers. Burying the fusion peptide may be required for fusion proteins on the membrane of enveloped viruses to prevent premature insertion into incorrect bilayers, protein aggregation, instability, or degradation prior to availability of the correct target cell membrane. The final coiled coil structure could be essential for promoting close apposition of donor and target membranes, a role that could be assumed by cellular proteins involved in cell-cell contact in the context of p10-induced syncytium formation. The accessory role of p10 in the replication cycle of ARV suggests that p10 may have freely evolved to the minimal components

necessary to promote fusion of closely apposed membranes, devoid of regulatory components that would unnecessarily increase the size of the viral genome.

The role of p10 in the life cycle of the nonenveloped ARV provides an explanation for the evolution of a fusion protein with a naturally exposed fusion peptide. Enveloped viruses require fusion for entry and must therefore induce rapid and efficient fusion using very hydrophobic fusion peptides which, of necessity, are buried within the final tertiary structure of the protein. In contrast, ARV does not use p10 for entry and only expresses p10 within infected cells. The reovirus replication cycle may not necessitate rapid and efficient cell-cell fusion, and it would not require regulatory components if fusion of every infected cell was beneficial to promote rapid virus egress. Rather, p10-mediated fusion would commence later in the virus replication cycle once sufficient levels of 'poised' p10 accumulate on the surface of infected cells. In agreement with this proposal, translation of p10 in ARV-infected cells is driven by a sub-optimal translation initiation site which, in conjunction with the short half-life of p10, likely contributes to delayed fusion in infected cells (Shmulevitz and Duncan, 2000). The consequence of producing a fully poised fusion protein would be degradation. The hydrophobicity of the p10 fusion peptide would then have evolved to provide a delicate balance between the degradation and fusion pathways (Figure 3.9). This model serves to explain the reduced fusogenic activity of the p10 fusion peptide in liposome fusion assays, a property that correlates with the decreased

hydrophobicity of the fusion peptide as compared to other viral fusion peptides. Increased hydrophobicity of the p10 fusion peptide, which could contribute to more efficient membrane fusion, would also likely target the protein for unacceptably high levels of recognition by the ERAD pathway. The fact that very subtle decreases in the hydrophobicity of the p10 fusion peptide eliminate the fusogenic activity of the protein suggests that the fusion peptide is on the cusp of the transition from the minimal hydrophobicity required to promote membrane fusion and the maximal hydrophobicity that is tolerated before the extent of degradation exceeds tolerable levels.

An additional explanation for the relatively low level of fusogenic activity displayed by the p10 versus the SIV fusion peptides is the absence of transmembrane anchors. The essential role of specific interactions between the transmembrane domains of fusion proteins and the viral membranes has recently emerged (Freed et al., 1992; Pecheur et al., 1997; Cleverley and Lenard, 1998; Armstrong et al., 2000; Langosch et al., 2001;). If fusion peptide interactions with target membranes and transmembrane domain interactions with donor membranes conspire together to favor membrane fusion, the transmembrane domain of p10 may play a more essential role in the fusion process in order to compensate for the weakly fusogenic activity of the p10 fusion peptide. We have previously shown that slight changes to the transmembrane domain of p10 eliminate the fusogenic activity of the protein without effecting membrane association or surface expression (Shmulevitz and Duncan, 2000) clearly

implicating the transmembrane domain of p10 in the fusion reaction.

Interestingly, electron microscopy failed to reveal any changes to intracellular membranes (Stoltz and Duncan, communications) and suggested that intracellular p10-induced membrane fusion is not a consequence of p10 expression. If p10 is expressed with an exposed fusion peptide, why does it not interact with and fuse intracellular membranes during its transport to the cell surface? Furthermore, if the fusion peptide is exposed, why does it not associate with donor membranes? We can speculate on three scenarios consistent with the poised hypothesis of p10-mediated membrane fusion. (1) The p10 fusion peptide does insert into donor membranes and this is the mechanism by which p10 induces membrane fusion. This possibility would agree with the 'dimple hypothesis', which suggests that fusion peptide insertion into donor membranes results in bulging of the donor membrane towards the target membrane and induces sufficient stress on the donor membrane to favor membrane merger (Kozlov and Chernomordik et al., 1998). If this were the case, however, p10 would be expected to fuse intracellular membranes. (2) The linker region between the p10 fusion peptide and the transmembrane anchor is highly conserved to ensure insufficient flexibility and length to permit fusion peptide association with the donor membrane. In this case, the fusion peptide could only insert into target membranes when they are sufficiently close. To decipher whether the p10 fusion peptide inserts into membranes of donor or target cells, or both, photolabelling experiments using lipid probes imbedded into donor or

target membranes could be completed. (3) Insertion into the target membrane requires cellular receptor-ligand interactions to appose donor and target membranes (see chapter 2) and, therefore, does not occur within the cell where such interactions are likely prevented to ensure that the compartments are functional and do not collapse. The latter two speculations, together, suggest that interactions between p10 and the intracellular compartmental membranes are not possible and provide a hypothesis for why p10 only functions to induce membrane fusion at the cellular surface.

Our poised model of p10-mediated membrane fusion may have several implications on the present understanding of the mechanism of membrane fusion. This model suggests that p10 resembles the final fusion competent conformation of enveloped virus fusion proteins, with two membranes held in close apposition through the fusion peptide and the transmembrane domain separated by a short linker. The model therefore questions whether energy released during conformational changes and coiled coil formation is essential for membrane merging. The recent demonstration that a polypeptide corresponding to the ectodomain of the influenza HA fusion protein is in its lowest energy (fusion competent) conformation can induce hemifusion between biological cell membranes suggests that energy released during the 'spring-loaded' conformational changes is not required for the initial steps of HA-mediated membrane fusion (Leikina et al., 2001). The ARV and NBV p10 fusion proteins

offer an additional model system to explore the minimal determinants required for protein-mediated fusion of biological membranes.

Future studies could aim at confirming that p10 is expressed in the fusion-active conformation. X-ray crystallography is the present method by which enveloped virus fusion protein structure has been confirmed. The conformation of enveloped virus fusion proteins are assessed before and after triggering with low pH. Since triggers for p10-induced membrane fusion have not been identified, results of crystallography would provide the structure of p10, but would fail to confirm that this structure is maintained throughout the 'life' of p10 molecules. If p10 molecules used in crystallographic analysis are also shown to fuse biological or liposomal membranes, then this analysis would confirm that the conformation is relevant to the active state of p10. A more feasible method to determine whether p10 is expressed with an exposed fusion peptide would be to generate antibodies specific to the disulphide-bonded hydrophobic patch of p10. If the p10 fusion peptide is exposed, then these antibodies would inhibit p10-induced membrane fusion. Furthermore, positive immunohistochemical staining of live and fixed/permeabilized p10-expressing cells with fusion peptide-specific antibodies would suggest that the fusion peptide is exposed upon p10 expression and surface localization. The use of photoreactivatable lipid probes to label protein insertion into the bilayer could then be used to confirm that the fusion peptide of p10 inserts into the membrane similar to the fusion peptides of enveloped virus fusion proteins.

3.E. CHAPTER 3 FIGURES

Figure 3.1. p10 is only necessary within the donor membrane. Heterotypic cell-cell fusion was visualized in monolayers containing a 3:2 ratio of 2HAN-transfected QM5 cells (CellTracker™ blue) and Hep2 cells (CellTracker™ green). In the overlay, the multinucleated cell positive for expression of p1-2HAN (red fluorescence) contained nuclei from both QM5 (blue) and Hep2 (green) origin.

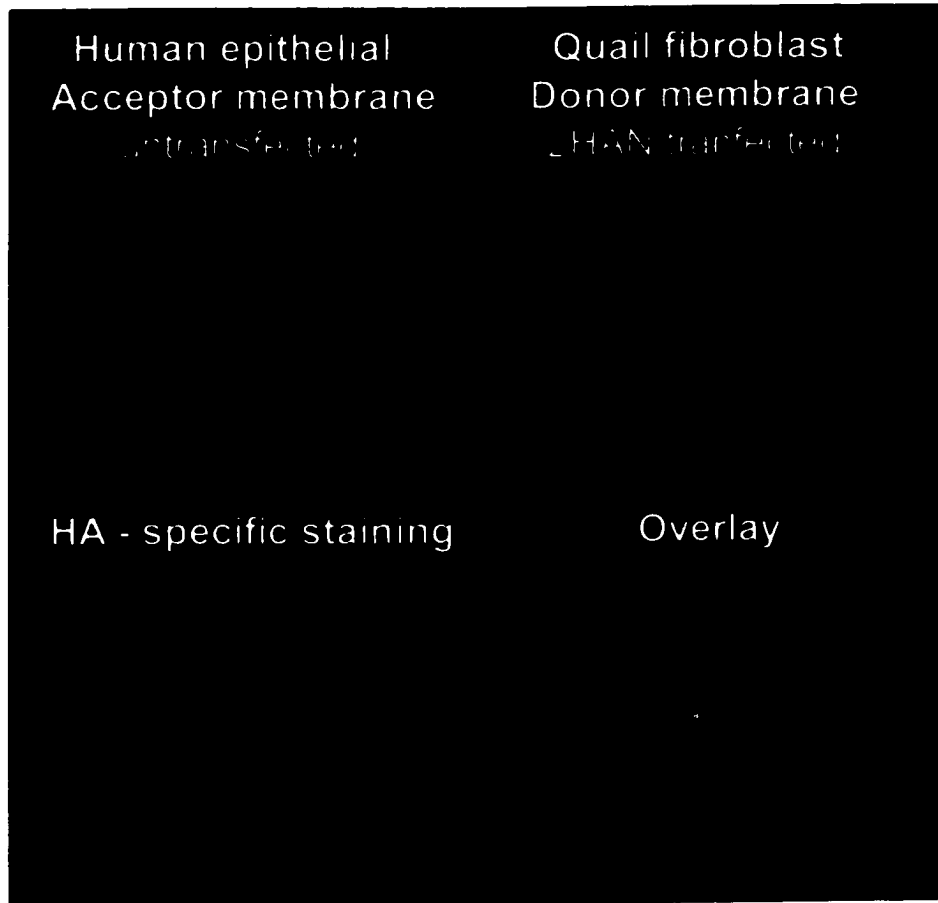


Figure 3.1

Figure 3.2. The hydrophobic patch and conserved domain of p10 are necessary for fusion and surface localization, respectively. A. A schematic representation of the p10 ectodomain including the sequence and structural conservation between ARV strains 138 and 176, and NBV. **B.** Complete deletion of the ectodomain and various site-specific substitutions were created in the ARV 2HAN construct and assessed for their expression, membrane association, fusogenic activity and surface localization in transfected cells. Constructs are named using the single letter amino acid code to indicate the identity of the authentic amino acid, its position, and the identity of the substituted residue. **C.** To assess membrane insertion, membranes were purified from transfected cells and immunoprecipitated, [³⁵S]-methionine labeled proteins were resolved by SDS-PAGE and detected by fluorography. **D.** Immunohistochemical staining identified foci of transfected protein expressing cells, and the degree of cell-cell fusion was scored visually according to the abundance of nuclei within the foci (top panel). Surface immunofluorescence was used to detect the relative levels of surface localized protein on transfected cells. Cells were photographed under identical parameters for comparison by intensity of fluorescence (bottom panel).

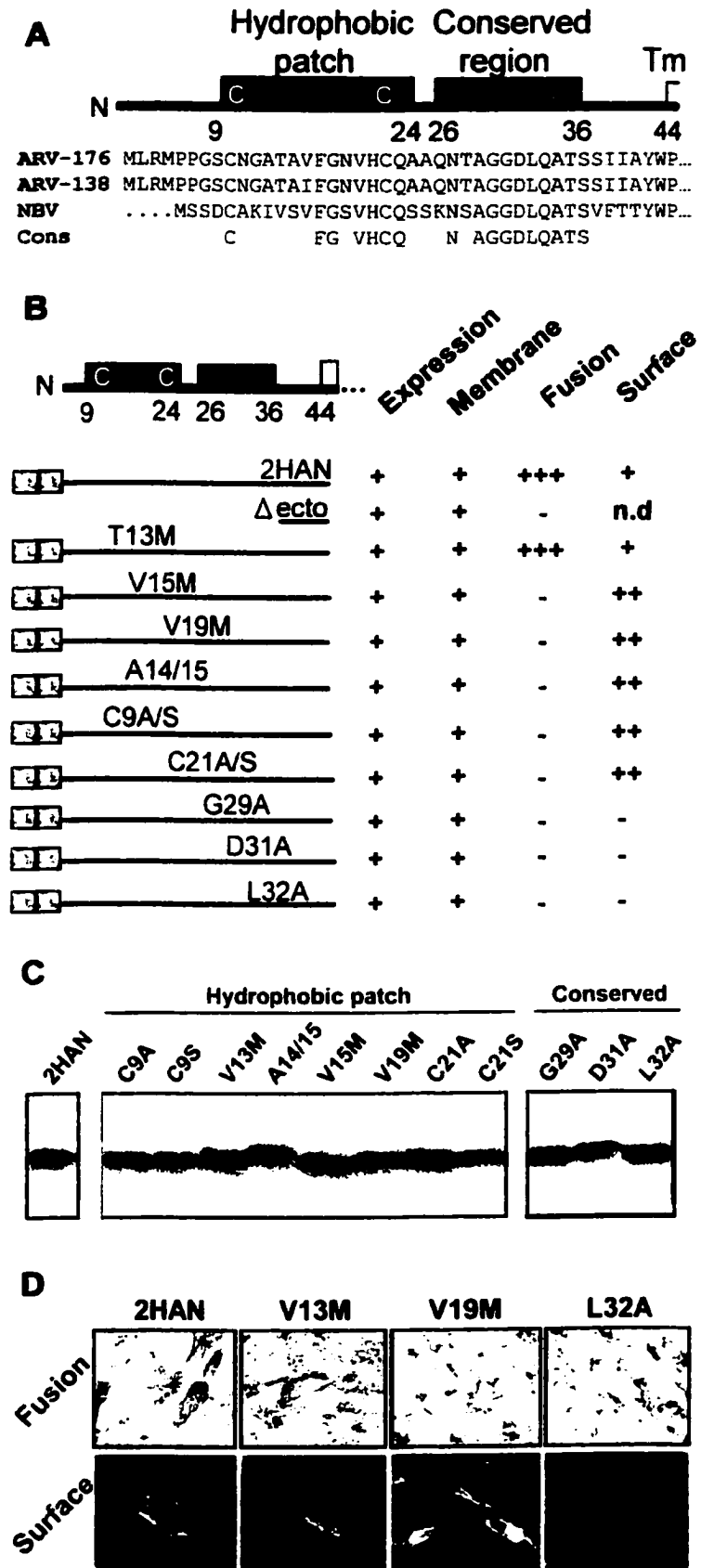


Figure 3.2

Figure 3.3. The hydrophobic patch accounts for the rapid degradation of p10. **A.** Transfected cells expressing the full length 2HAN construct (2HAN), the 2HAN ectodomain (2HANE), or the soluble sigma C protein of ARV were pulse-labeled with [³⁵S]-methionine for 10 minutes and chased for the indicated periods in minutes. Radioactive proteins were immunoprecipitated, resolved by SDS-PAGE, and visualized by fluorography. **B.** The full length 2HAN construct was examined as in panel A except cells were disrupted under harsh lysis conditions to ensure complete solubilisation of any aggregated p10 protein complexes. **C.** The degradation rates of 2HAN constructs containing single amino acid substitutions in different domains were assessed as described in panel A. See Figure 3.2 for the location of the mutations and the mutant nomenclature scheme. **D.** A time course analysis of protein turnover was performed as described in panel A using more precise time points to compare authentic 2HAN (2HAN) to two p10 mutants containing single residue substitutions (T13M and V15M) that altered the hydrophobicity of the hydrophobic patch.

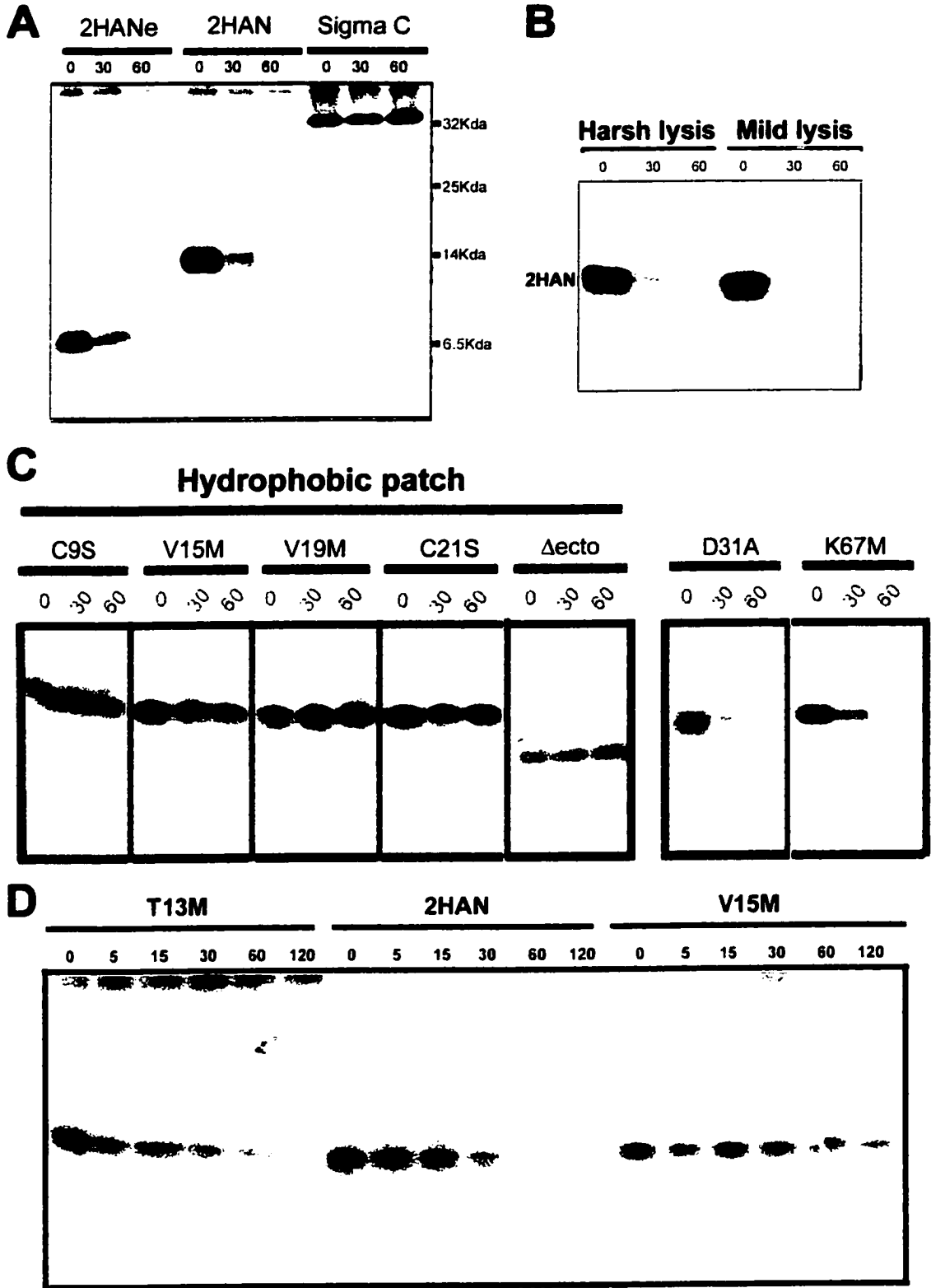


Figure 3.3

Figure 3.4. The hydrophobic patch has membrane association properties.

A. Triton X-114 partitioning analysis was performed on transfected cells to determine the preference of 2HAN, sigma C, or the ectodomain of authentic (2HANE) or mutated (T13Me and A14/15e) HA-tagged p10 for the aqueous (aq) versus detergent (det) phases. **B.** Transfected cells were separated into the membranous pellet (P) or supernatant (S) fractions to determine the preferred location of the indicated radiolabeled protein. The membrane pellet fraction was then mixed with sucrose to a final concentration of 66% and overlaid with equal volumes of 50% and 5% sucrose. Following centrifugation, the gradients were fractionated to assess the distribution of the indicated proteins in the pelleted protein aggregate fraction (4), in the soluble protein fractions containing 66% or 50% sucrose (3 and 2), and in the membrane-associated fraction at the 5%-50% sucrose interphase (1). In some experiments, the membrane fraction from cells transfected with the 2HANE construct were further extracted using NaCO_3 , pH 11.3, 500 mM NaCl, or PBS and the membrane and soluble fractions were re-isolated by centrifugation.

Figure 3.5. The hydrophobic patch of p10 induces liposome fusion. A. Resonance energy transfer was used to follow the time course of lipid mixing induced by 10 μ M concentrations of the hydrophobic patch peptide (p10hp) or the ectodomain peptide (p10ed) of p10 using 50 nM LUVs composed of DOPC:DOPE:Cholesterol (1:1:1). A DMSO control was included in the assays.

B. The fusion-inducing ability of p10 constructs containing sequential N-terminal HA epitope additions was qualitatively assessed at the indicated time points post-transfection. Similar analysis was performed on p10 containing an HA epitope insertion between the transmembrane and ectodomains. The extent of syncytium formation was monitored by immunostaining transfected cells with a p10-specific polyclonal antiserum.

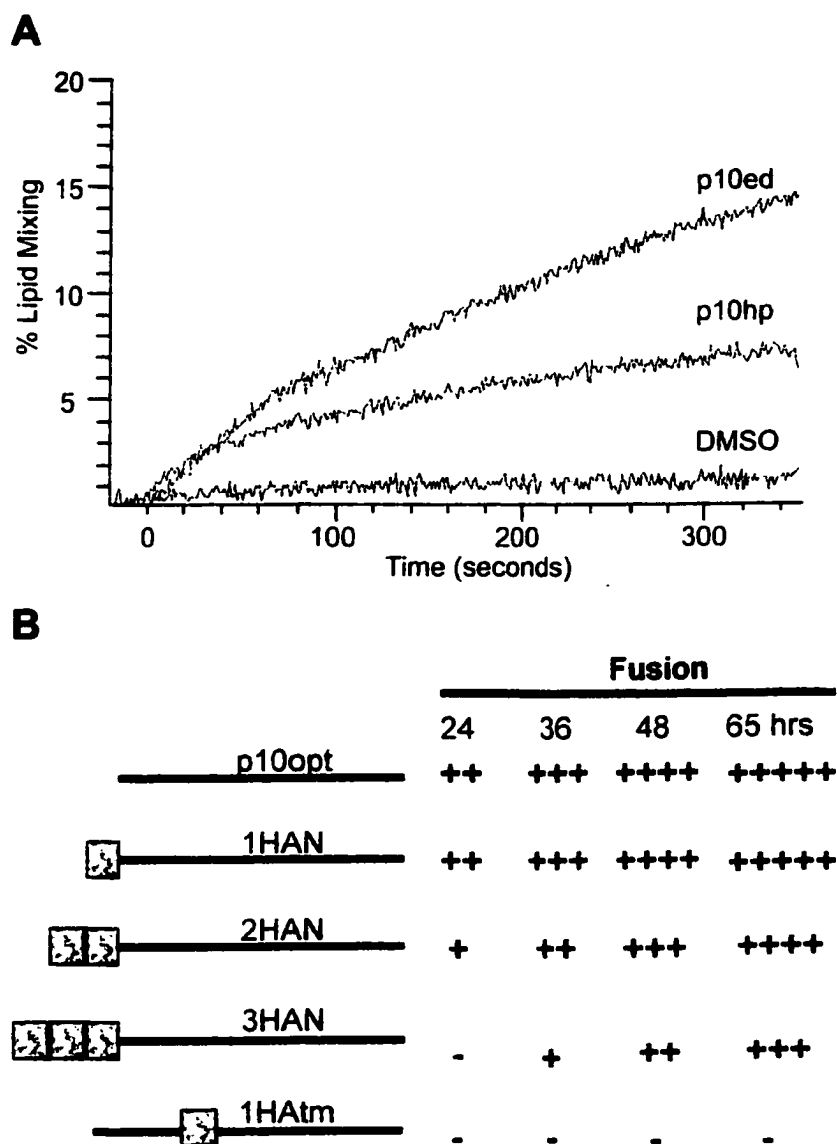


Figure 3.5

Figure 3.6. Characteristics of the p10 fusion peptide. A. Three diagrams depict the amino acid arrangement within the p10 hydrophobic patch when depicted as an α -helix, a β -sheet or a cysteine-bonded loop composed of anti-parallel β -sheets. Residues most commonly in contact with bilayer lipids (V, I, L, F, A and G) are bold and circled, residues that can form hydrogen or covalent bonds to favor their presence within membranes (T, S, and C) are circled, while residues that do not prefer direct contact with lipids (Q, N, H, D, E, R and K) are neither bold or circled. **B.** CD data expressed as the mean residue ellipticity was obtained for the p10hp (top panel) and p10ed (bottom panel) peptides. The top CD spectra was obtained at 25°C using 100 μ M of the p10hp peptide (p10hp) in the following environments: in LPC at a lipid to peptide molar ratio of 10 (∇), in 33% TFE (π), in 66% TFE (\bullet), or in 100% TFE (\blacksquare). The lower CD spectra was obtained using 100 μ M p10 ectodomain peptide (p10ed) in the following environments: in LPC at a lipid to peptide molar ratio of 10 (∇), in 50% TFE (\bullet), or in 100% TFE (\blacksquare). Spectra under oxidized and reduced conditions were superimposable. **C.** The secondary structure was estimated with the program Selcon3 for p10ed and p10hp peptides under the indicated conditions. **D.** The gel mobility of p10 under reducing and non-reducing conditions was assessed by SDS-PAGE analysis and fluorography of immunoprecipitated [35 S]-methionine labeled 2HAN in the presence or absence of 2% β -mercaptoethanol. **E.** The percent lipid mixing induced by DMSO, or the hydrophobic patch (p10hp) or ectodomain (p10ed) peptides was quantified at 350 seconds using 50 μ M LUVs composed of DOPC:DOPE:Cholesterol (1:1:1) under oxidizing or reducing

(buffer in the presence of 4 mM DTT) conditions. The following conditions apply to each bar grouping; 1st bar - oxidizing conditions with 5 μ M peptide; 2nd bar - oxidizing conditions with 10 μ M peptide; 3rd bar - reducing conditions with 5 μ M peptide; 4th bar - reducing conditions with 10 μ M peptide.

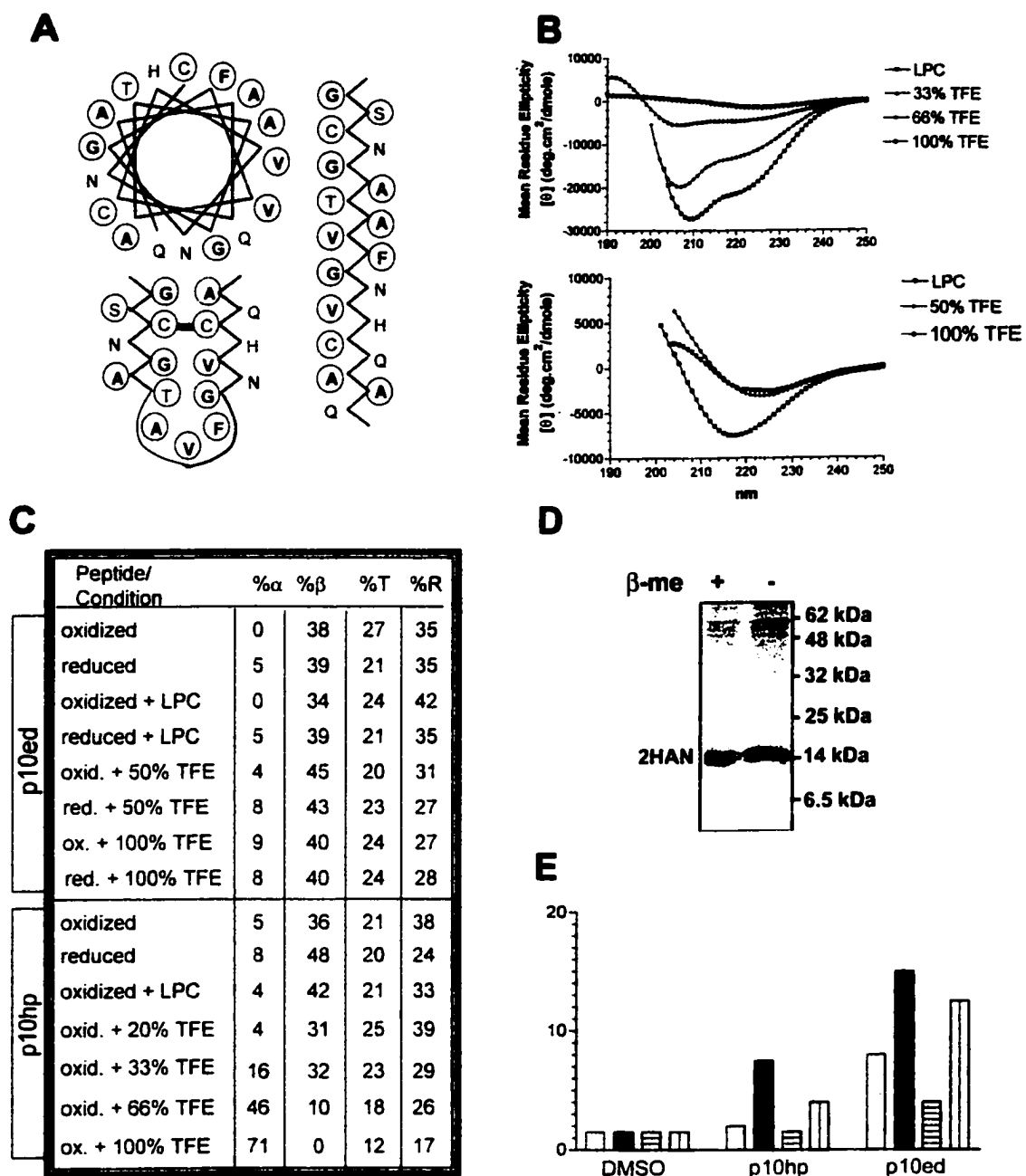


Figure 3.6

Figure 3.7. Attempts to demonstrate that intramolecular disulfide bonds are essential for p10-induced membrane fusion. A. Characteristics of the sulfhydryl-reactive and reducing reagents used in cell culture to test the effect of disruptions to intramolecular disulfide bonds in p10 on syncytia formation are summarized. Reagents were added to 2HAN transfected or ARV infected (MOI 0.1) at the onset of syncytia formation and cells were visualized for changes in membrane fusion activity. All reagents proved toxic by two hours and were useless in distinguishing significant changes in syncytia formation. **B.** Cells transfected with 2HAN, 2HANE, or pcDNA and labeled with [³⁵S]-methionine were immunoprecipitated, run on SDS-PAGE gels, and subjected to western blot analysis under differing conditions described in materials and methods. PDVF membranes (PDVF) or the SDS-PAGE gel following transfer (gel) were immunostained (stained) or exposed to X-ray film ([³⁵S]-methionine) for detection of p10.

A

In cell culture inhibition of disulphide bond formation			
Blocker	mechanism	permeability	concentrations used
2-Aminoethyl-2'-aminoethanethiosulfonate	sulfhydryl-reactive	impermeant	5mM, 2mM, 100uM
β -(4-Hydroxyphenyl)ethyl iodoacetamide	sulfhydryl-reactive	permeant	5mM, 2mM, 100uM
N-Ethylmaleimide (NEM)	sulfhydryl-reactive	permeant	5mM, 2mM, 100uM
Dithiothreitol (DTT)	sulfhydryl-reducing	impermeant	5mM, 2mM, 100uM
β -mercaptoethane sulfonic acid (MESNA)	sulfhydryl-reactive	impermeant	25mM, 5mM, 1mM, 100uM
Glutathione, reduced form (GSH)	sulfhydryl-reducing	impermeant	25mM, 5mM, 1mM, 100uM
β -mercaptoethanol	sulfhydryl-reducing	impermeant	25mM, 5mM, 1mM, 100uM

NOTE: prolonged incubation of live cells with impermeant chemicals may still be toxic due to internalization through endocytosis and intracellular accumulation.

B

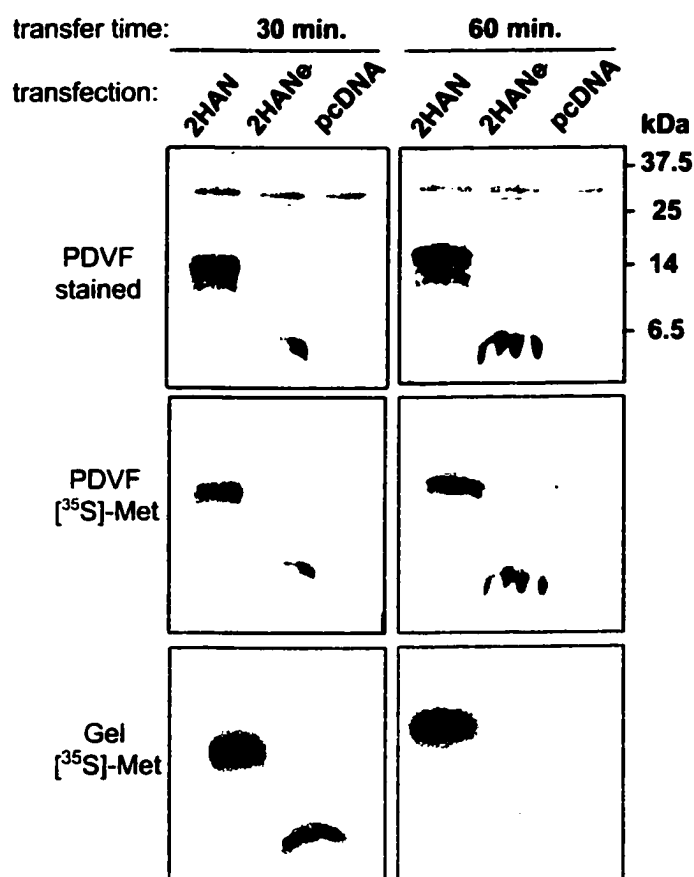


Figure 3.7

Figure 3.8. p10 is degraded intracellularly by the proteasome. A. Pulse-chase analysis for the indicated times was performed on p10 obtained from the membrane fraction of transfected cells. **B.** Immunofluorescent staining of 2HAN shows strong perinuclear staining in single and multinucleated syncytia despite low surface staining. **C.** The intracellular localization of 2HAN (red fluorescence, middle panels) corresponded with the Con A stained ER (green fluorescence, left hand panels) as visible in the overlay (yellow fluorescence, right hand panels). **D.** A 10 minute pulse labeling with [³⁵S]-methionine followed by indicated chase times demonstrated that although lysosomal degradation inhibitors (E64C, chloroquine and MA) did not prevent rapid degradation of 2HAN, the proteasome inhibitor Cbz-LLL-OH reduced the rate of p10 turnover. **E.** To determine the stability of surface localized 2HAN, antibody-2HAN complexes that remained on the surface following incubation at 37°C for the indicated durations were visualized with secondary FITC-labeled antibodies. **F.** A 10 minute pulse labeling with [³⁵S]-methionine followed by indicated chase times demonstrated that cytoskeletal growth and endocytosis inhibitors (cytochalasin B, monodansyl cadaverine and nocodazole) and endoplasmic reticulum localized proteases (pepstatin, aprotinin, and leupeptin) did not prevent rapid degradation of 2HAN, while inhibitors of intracellular traffic (brefeldin A and monensin) and low temperatures reduced the rate of p10 turnover.

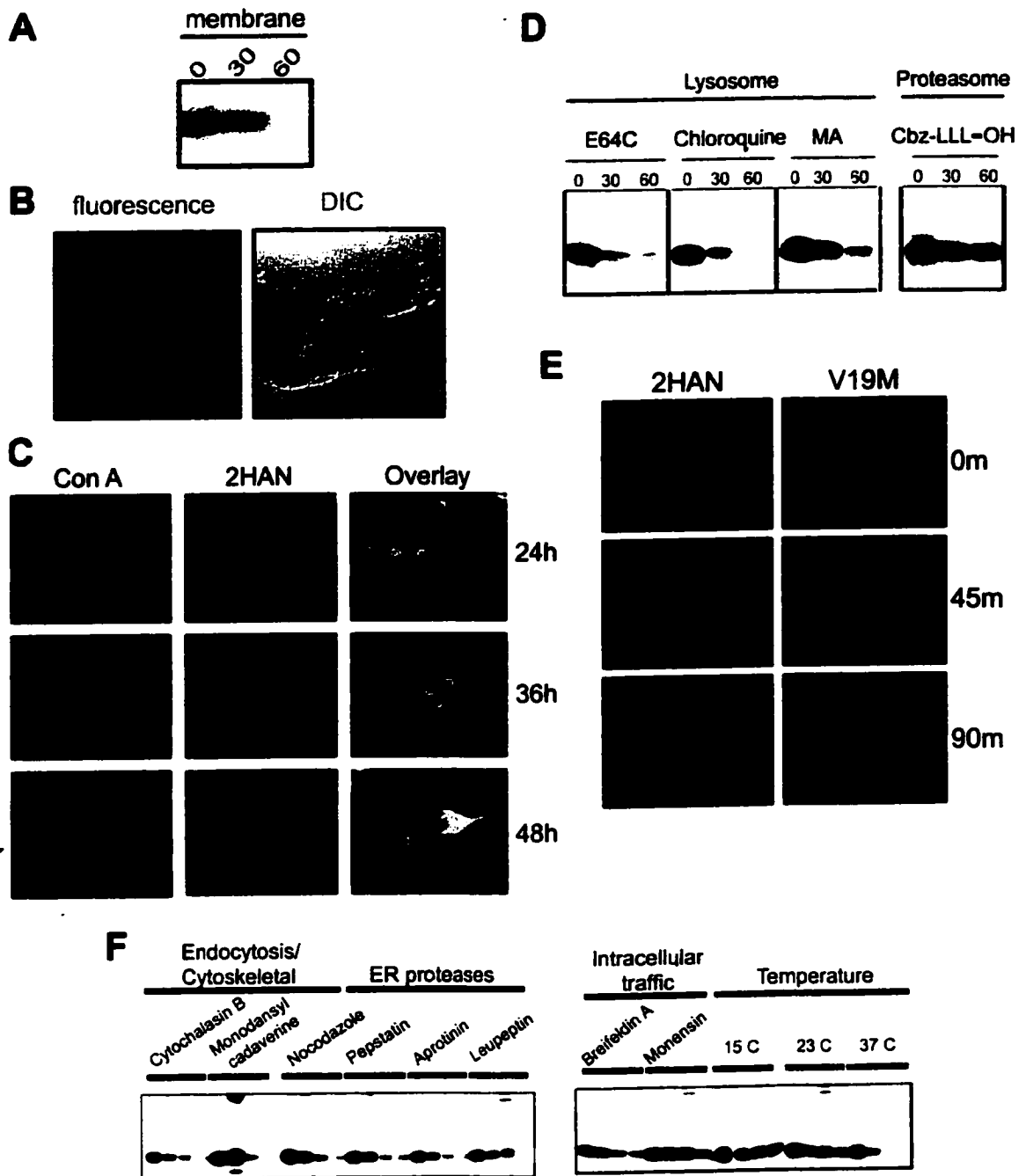


Figure 3.8

Figure 3.9. The exposed hydrophobic patch of p10 results in fusion or degradation. The model depicts the proposed balance that exists between the two alternate fates of p10. Having a fusion peptide exposed directly upon protein synthesis would signal proteasome-dependent degradation. P10 capable of escaping the degradation machinery would localize to the cell surface and once present in sufficient quantities would trigger cell-cell fusion by using the internal fusion peptide to associate with and destabilize the opposing membrane similar to the fusion peptides of enveloped virus fusion proteins.

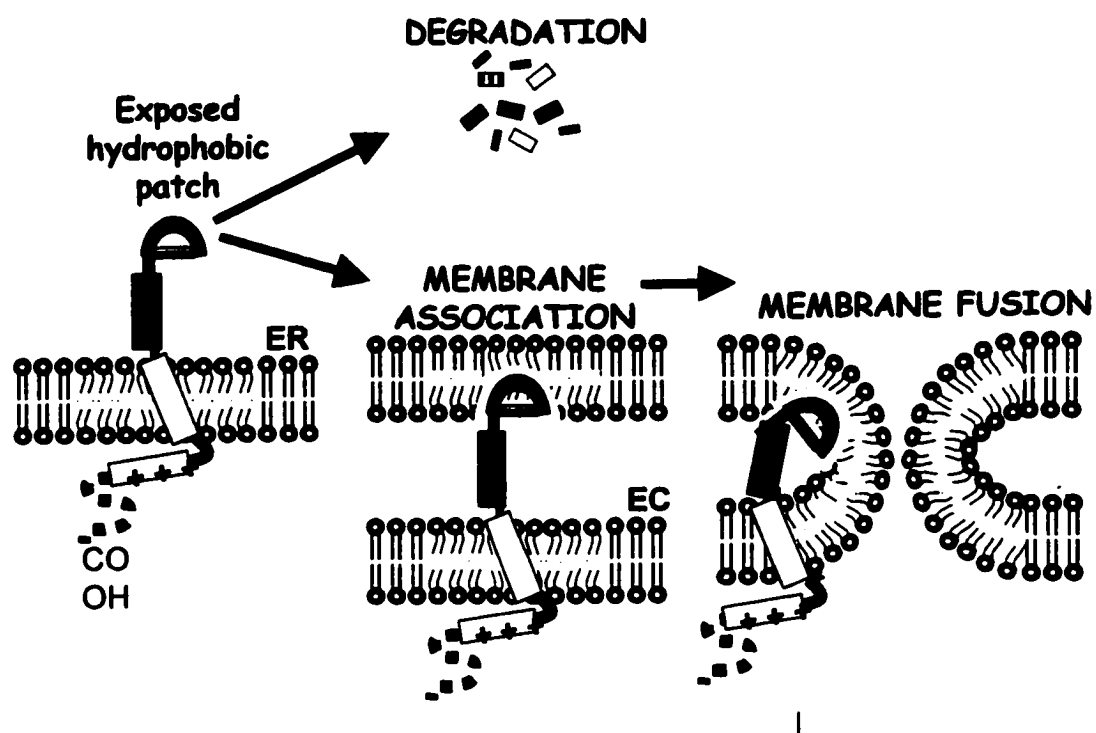


Figure 3.9

3.F. MATERIALS AND METHODS

Abbreviations used

DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; LPC, lysophosphatidylcholine; N-Rh-PE, N-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; N-NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine; LUV, large unilamellar vesicle; DMSO, dimethylsulfoxide; HBSS, Hanks balanced salts solution; SIV, Simian Immunodeficiency Virus.

Plasmids and cells

The continuous quail cell line, QM5 (Duncan and Sullivan, 1998) was maintained in growth medium consisting of medium 199 supplemented with 10% fetal bovine serum (FBS), and penicillin/streptomycin (50U/ml and 50 μ g/ml, respectively). The continuous Hep2 human epithelial cell line used for heterotypic cell-cell fusion assays was maintained under similar conditions using 5% FBS. The 12CA5 anti-HA mouse hybridoma cells were grown in RPMI 1640 containing 10% FBS and penicillin/streptomycin as above. The eukaryotic expression vector pcDNA3 (Invitrogen) was used for expression of p10 and its derivatives.

Materials

All lipids, including the fluorescently labeled lipids, were purchased from Avanti Polar Lipids (Alabaster, AL). The p10 hydrophobic patch peptide (p10hp) was

synthesized by Biosource (Hopkinton, MA) and the p10 ectodomain peptide (p10ed) by Waterloo Peptide Synthesis (Waterloo, ON); both were purified to >95% purity by HPLC. The 12CA5 anti-HA (IgG2b, kappa) antibodies were produced in house from a mouse hybridoma. Cells secreting the monoclonal antibodies were maintained in serum-free media containing penicillin/streptomycin for 10-15 days until cells were dead. The media, clarified from cells by centrifugation, was used directly for surface staining and was estimated to contain 0.12 μ g/ μ l of anti-HA antibodies. Purification of the IgG by precipitation with 35% ammonium sulfate and extensive dialysis against PBS produced a concentrated stock (approximately 1.6 μ g/ μ l) of anti-Ha used for immunoprecipitation and intracellular staining. Alkaline phosphatase-, Texas Red-, and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibodies and protein G-agarose were obtained from Life Technologies Inc. while FITC-labeled concanavalin A (Con A) was purchased from Sigma-Aldrich (Sigma Chemical Company, St. Louis, Mo., USA). CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) and CellTracker Blue CMAC (7-amino-4-chloromethylcoumarin) were purchased from Molecular Probes (Oregon USA). Lysosome and proteasome inhibitors including E64C ((2S, 3S)-trans-epoxysuccinyl-L-Leucylamido-3-methyl-butane), methylamine (MA), Chloroquine, and N-Cbz-leu-leu-leu-OH were purchased from Sigma-Aldrich. The pSAAM protein sequence analysis and modeling program was used for secondary structure predictions (Crofts, 1994).

Cloning and transfection

Construction of the HA-tagged p10 (2HAN) expression vector was previously described (Shmulevitz and Duncan, 2000). Two HA epitopes were introduced at the N-terminus of p10 with an optimal translation start sequence. All mutations made within 2HAN were created by 3-primer PCR using a forward primer corresponding to the N-terminal sequence of 2HAN (see Shmulevitz and Duncan, 2000) containing a *HindIII* restriction site, a reverse primer corresponding to the C-terminal sequence of 2HAN containing the *EcoRV* restriction site, and a middle primer corresponding to the site of mutation and containing the correct nucleotide substitutions to generate the desired amino acid mutation. First round PCR was performed using the forward primer and the middle primer followed by product purification using the Qiagen PCR purification kit according to manufacturer's instruction and used in the second round PCR along with the reverse primer. All primer sequences can be obtained by request of the corresponding author. The final PCR reaction consisted of 1x Vent polymerase buffer, 2mM MgSO₄, 0.2 mM of each dNTP, 0.05 pmol of 2HAN template, 40 pmol of each primer and 0.5 µl of Vent polymerase (Life Technologies Inc.) in a final volume of 100 µl. After denaturation of samples at 94°C for 4 minutes, 5 rounds of denaturation (94°C, 1 min), annealing (56°C, 54 °C, 52 °C, 50 °C, 48 °C or 46 °C, 30 sec) and elongation (72 °C, 45 sec) were performed for each annealing temperature. Reaction products were gel purified using Qiax II gel purification kit (Qiagen) according to manufacturer's instructions. Products and vector were digested with *HindIII* and *EcoRV*, gel purified, ligated,

and transformed by electroporation into Top-10 cells according to standard protocols. The ectodomains of 2HAN and 2HAN containing mutations were amplified using a single touch-down PCR reaction using the same forward and reverse primers but different templates created above. The forward primer corresponded to the N-terminus of 2HAN and contained, in addition to the restriction site, the signal peptide of influenza HA with a signal peptidase cleavage site (MLTIIALSYIFCLALG). The reverse primer corresponded to the most C-terminal residues of the ectodomain depicted in Figure 3.2A. All constructs were sequenced in house using the ³³P-sequencing kit according to manufacturer's instructions.

Transfection was performed using the Lipofectamine reagent (Life Technologies Inc). For every twelve-well equivalence containing QM5 cells at 70% confluence, 1 µg of DNA and 3 µl of Lipofectamine reagent were used in the procedure for transient transfection of adherent cells recommended by the manufacturer. Medium was replaced with serum-containing medium following incubations of 16-18 hours with the DNA-lipofectamine complexes.

Intracellular staining, co-immunofluorescence with Concanavalin A (Con A), and surface immunofluorescence

HA-tagged p10 was detected within transfected cell monolayers by immunocytochemical staining using purified anti-HA antibodies diluted 1:800 in phosphate-buffered saline (PBS) containing 2 % bovine serum albumin (BSA).

Following extensive washes, foci were visualized using goat anti-mouse IgG conjugated with alkaline phosphatase in PBS/2% BSA at a 1:400 dilution according to standard protocols (Harlow and Lane, 1988). Stained cells were visualized/photographed on a Nikon Diaphot inverted microscope at 100 X magnification.

Con A was used as a marker for ER membranes (Virtanen et al., 1980). For double labeling of 2HAN-transfected cells with anti-HA antibodies and Con A, cells were fixed at 24, 36 and 48 hours post-transfection with methanol on ice for 2 minutes, incubated with purified anti-HA antiserum (1:200) for 1 hour at RT then with FITC-Con A (1:100) and goat anti-mouse texas red antiserum (1:100) for 1 hour at RT with extensive washes following each incubation.

Surface immunoprecipitation was performed on live cells. Twenty-six hours post-transfection, cells were incubated with a 2/3 dilution of anti-HA antibody supernatants (0.12 $\mu\text{g}/\mu\text{l}$) in HBSS/10% FBS for 45 min at 30°C. Following extensive cold washes, cells were fixed with ice cold methanol for 2 min. FITC-labeled goat anti-mouse antibody was used at 1:25 dilution in PBS/2% BSA and incubated for 45 min at RT.

Immunoprecipitation

Prior to immunoprecipitation, all samples were brought to a 1ml volume in 1x final lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl , 1% Nonidet P40, 0.5%

sodium deoxycholate, and 1 $\mu\text{g/ml}$ each of aprotinin, leupeptin and pepstatin). For immunoprecipitation of samples under harsh lysis conditions, a 2x final lysis buffer solution was used containing 0.1% SDS. For every 2×10^6 cells, 4 μl of purified anti-HA antibodies were pre-incubated with 15 μl of protein G-agarose for 1 hour with shaking at RT. Antibody-protein G-agarose complexes were washed once with lysis buffer and incubated with samples for 1 hour at RT followed by two stringent washes with each of the following: lysis buffer, high salt buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% Nonidet P40, 0.05% sodium deoxycholate, and 1 $\mu\text{g/ml}$ each of aprotinin, leupeptin and pepstatin) and low salt buffer (50 mM Tris-HCl pH 7.5, 0.1% Nonidet P40, 0.05% sodium deoxycholate, and 1 $\mu\text{g/ml}$ each of aprotinin, leupeptin and pepstatin) to completely remove non-specifically bound proteins. Immune complexes were released by boiling in SDS protein sample buffer (0.5% SDS, 50 mM Tris-HCl pH 8.0, 2% 14.4M β -mercaptoethanol) before SDS-PAGE using 15% acrylamide gels.

Heterotypic cell fusion assay

Sixteen hours following transfection with the 2HAN constructs, QM5 cells were incubated with HBSS containing 10 μM final CMAC for 45 min at 37°C. The membrane permeable probe, once inside the cell, was converted into a cell-impermeant reaction product while the excess probe was washed away through several quick HBSS rinses before and after an additional incubation for 1 hour in media 199/10% FBS. Similarly, nontransfected Hep2 cells were labeled with

5 μ M final CMFDA. QM5 cells and Hep2 cells were suspended by incubation in PBS in the absence of calcium and magnesium salts for 20 min at 37°C and reseeded at a ratio of 3:2 (QM5:HepG2). Forty-eight hours following the original transfection, cells were immunofluorescently labeled using purified HA monoclonal antibody (1:200) and goat anti-mouse IgG conjugated with Texas Red (1:25) as previously described (Shmulevitz and Duncan, 2000).

Protein stability assessment (pulse-chase)

At 30 hours post-transfection, cells were incubated at 37°C for 20 min with methionine-free DMEM followed by a 10 min incubation with 50 μ Ci/ml [³⁵S]-methionine in methionine-free DMEM. To ensure removal of unincorporated [³⁵S]-methionine, cells were washed 8 times with HBSS. Cells were incubated for different time periods with media 199 at 37°C prior to lysis with 1 X lysis buffer. When the effects of proteasome or lysosome degradation inhibitors were assessed, E64C, chloroquine, MA, or cbz-LLL-OH were added at 200 μ M, 0.5 mM, 15 mM, or 100 μ M final concentrations, respectively, to all incubations described above excluding the washes. Stock solutions of degradation inhibitors were always 1000X to ensure that DMSO solubilized reagents (E64C and cbz-LLL-OH) were non-toxic. When the stability of membrane localized 2HAN was assessed, membrane purification was performed as described instead of direct lysis with 1 X lysis buffer.

Stability of surface localized protein assessment

Transfected cells were incubated on ice for 30 min to slow cellular activity at 36 hours post-transfection. Cells were incubated for 30 min on ice with purified anti-HA antibodies in HBSS containing 10% FBS in a 1:20 ratio followed by 8 washes with cold HBSS to remove non-specifically bound antibodies. Medium 199 with 10% FBS was added to cells which were then incubated for various times (0, 45 or 90 minutes) at 37 °C before addition of cold secondary FITC-labeled anti-mouse antibodies in HBSS/10%FBS and incubation on ice for 30 minutes on ice to detect the anti-HA antibody bound surface localized 2HAN that remained on the surface of cells. Cells were washed as before and fixed with 4% paraformaldehyde prior to visualization and photography with appropriate filter sets.

Triton X-114 partitioning assay

Twenty-four hours post-transfection, cells were incubated for 30 min with methionine-free DMEM followed by a 30 min incubation with 50 μ Ci/ml [³⁵S]-methionine in methionine-free DMEM. Cells were incubated for 10 min on ice, washed with cold PBS, and incubated on ice with a cold solution of 1% Triton X-114, 10 mM Tris-HCl, pH 7.4 and 150 mM NaCl for 15 min rocking. Solubilized cells were centrifuged at 2500 g for 5 min at 4°C to remove debris and nuclei followed by centrifugation at 15,000 g for 15 min at 4 °C to remove other insolubles. The supernatant was then incubated at 31 °C for 10 min and the aqueous and detergent phases were separated by centrifugation at 300 g for 3 min at room temperature (RT). Both fractions were brought up to a 1 ml volume

with 5x lysis solution and water to produce a final 1x lysis solution and immunoprecipitated as described above.

Membrane fractionation of transfected cells and sucrose gradient centrifugation

Membranes of [³⁵S]-methionine labeled transfected cells were purified exactly as previously described (Shmulevitz and Duncan, 2000) using a 30 gauge syringe to disrupt cells and following removal of insoluble debris, high speed centrifugation was used to pellet the membrane fraction. To remove peripheral membrane-associated proteins, the membranous pellet was re-syringed into PBS and treated with 100 mM NaCO₃, pH 11.3, or 500 mM NaCl for 30 minutes on ice prior to recovery of membranes by centrifugation at 100,000 g for 25 min. Sucrose gradient centrifugation was used for separation of membrane material from protein aggregates that also pellet at high speeds. Membrane fractions were syringed 10 times through a 30 gauge syringe in a small volume of PBS and mixed with 80% sucrose/PBS to produce a 66% sucrose solution that was overlaid with equal volumes of 50% and 5% sucrose/PBS solutions. Centrifugation at 200,000 g for 2 hours separated the lipid fraction that floated above the 50% sucrose from protein aggregates that formed tight pellets. Each fraction was resuspended in a volume of 1 ml 1x final lysis buffer for immunoprecipitation.

Circular dichroism (CD)

The CD spectra were recorded using an AVIV model 61 DS CD instrument (AVIV Associates, Lakewood, NJ). The sample was contained in a 1 mm pathlength cell that was maintained at 25 °C in a thermostated cell holder. The CD data are expressed as the mean residue ellipticity. The secondary structure was estimated with the program Selcon3 (Sreerama et al.,1999). All CD runs were made with protein dissolved in 10 mM sodium phosphate buffer containing 0.15 M NaF and 1 mM EDTA at pH 7.4. When CD runs were made under reducing conditions, the buffer also contained 4 mM DTT

Lipid mixing assay for membrane fusion

The resonance energy transfer assay of Struck *et al.* (1981) was used to monitor membrane fusion. LUVs were prepared containing DOPC:DOPE:Cholesterol at a molar ratio of 1:1:1. Two populations of LUVs were prepared, one unlabeled and one labeled with 2 mol % each of N-Rh-PE and N-NBD-PE. A 9:1 molar ratio of unlabeled to labeled liposomes were used in the assay. Fluorescence was recorded at excitation and emission wavelengths of 465 nm and 530 nm, respectively; using a 490 nm cut-off filter placed between the cuvette and the emission monochromator, with 4 nm bandwidths, using an SLM Aminco Bowman AB-2 spectrofluorimeter. Siliconized glass cuvettes (1 cm²) were used with continuous stirring in a thermostated cuvette holder. Measurements were carried out using a buffer containing 5 mM HEPES, 5 mM MES, 5 mM citric acid, 0.15 M NaCl and 1 mM EDTA, pH 7.4. When working under reducing conditions the buffer contained, in addition, 4mM DTT. LUVs at a final lipid concentration of 50

μM were added to 2 mL of buffer in the cuvette at 37° C and then the peptide was injected from a solution of DMSO. Fluorescence was recorded for several minutes and then 20 μL of 10% Triton X-100 was added (final concentration 0.1 %). The initial residual fluorescence intensity, prior to acidification, F_o , was taken as zero. The maximum fluorescence intensity, F_{max} , was obtained by dilution of the labeled lipids with 20 μL of 10% Triton X-100. Percent lipid mixing at time t is given by: $[(F_t - F_o)/(F_{\text{max}} - F_o)]100$. All runs were done in duplicate and were found to be in close agreement. Controls were done using comparable volumes of DMSO in the absence of peptide. The intensity of maximal fluorescence with Triton was found to be close to that obtained when the mol fraction of labeled lipids was reduced by 10-fold with unlabeled lipid and was taken as the fluorescence corresponding to 100% fusion.

Western blot of 2HAN

One 25cm² flask of QM5 cells transfected with pcDNA, 2HAN or 2HANe was labeled with [³⁵S]-methionine and lysed at 30 hours post-transfection with lysis buffer followed by immune-precipitation as previously described (Shmulevitz et al., submitted). The entire sample was loaded on a 15% SDS-Page gel.

Transfer to PDVF membrane using the semi-dry transfer apparatus (BioRad) was done according to manufacturer's instructions with exception to the following; gel, methanol-wetted PDVF membrane, and Wattman papers were soaked in transfer buffer (25mM Tris pH 7.4, 192mM glycine pH 8.3, 1:10 methanol) for 15 minutes prior to assembly, four Wattman papers were placed beneath and on top of the

gel-membrane and transfer was performed at 25 volts for 30 or 60 minutes. Alternatively, a transfer buffer containing 0.1% SDS or Tris pH 9.0 were used to assess the effects of altered protein charge on transfer efficiency. The gels were dried and exposed to film to assess the amount of protein that did not transfer successfully to the membrane. The membrane was incubated at 4°C for over 4 hours in PBS/ 2% milk followed by incubation for 1 hour in mouse monoclonal anti-HA supernatant (1:100) and an hour incubation in alkaline phosphatase conjugated goat anti-mouse antiserum (1:5000). Antibodies were diluted in PBS/2% milk and extensive washes with PBS/ 0.1% Tween 20 followed each antibody incubation.

CHAPTER 4: Evidence for the involvement of fusion protein domains associated with the donor bilayer in protein induced membrane fusion:

The conserved transmembrane tri-glycine motif, two palmitoylated cysteines and intracellular membrane-proximal basic region are directly involved in membrane fusion induced by the unique p10 fusion protein.

4.A. ABSTRACT

Mutational analysis showed that the transmembrane and cytoplasmic domains are involved in p10-mediated fusion and not in the processing of p10. Specifically, we identified a tri-glycine motif that is conserved within the transmembrane domains of ARV and NBV p10 proteins, which can not withstand semi-conservative substitution without loss of p10 fusion activity, supporting a role for the unique features of glycines in the fusion process. Mutational analysis also demonstrated that two conserved cysteines within or adjacent to the transmembrane domain are palmitoylated and are essential for fusion. P10 was found localized to detergent-insoluble, glycolipid rich membrane domains following solubilization of cells with Triton X-100 at 4°C, but raft association was independent of palmitoylation. Finally, a membrane-proximal domain characterized by a large proportion of basic residues was found to be involved in the fusion process. These motifs within the transmembrane and cytoplasmic domains of p10 are predicted to have perturbation effects on the donor membranes. The donor membrane perturbation by p10 may be more substantial than by enveloped virus fusion proteins because the latter would jeopardize the viral envelope stability. The relatively weak fusion peptide present within p10 suggests that unlike the fusion proteins of enveloped viruses, interactions between p10 and the donor membrane may play a more significant role in membrane fusion. These studies expand the present understanding of the minimal requirements for fusion by providing evidence for sequence-dependent determinants within donor membrane localized protein domains.

4.B. INTRODUCTION

The final fusion competent conformation predicted for enveloped virus fusion proteins consists of a membrane anchor and fusion peptide embedded in the donor and target membranes, respectively. One model suggests that the intervening sequence is flexible and allows rotational mobility of both transmembrane anchor and fusion peptide within their respective membranes that destabilize the lipid bilayers and favor membrane merging (Weissenhorn et al., 1997). A second model proposes that a rigid intervening sequence couples the ectodomain and transmembrane domain, each exerting their own local perturbation forces on their respective membranes, again favoring lipid bilayer fusion (Melikyan et al., 1995). In either case, the models are consistent with the involvement of both fusion peptide and transmembrane anchor in membrane changes that are necessary for membrane fusion. In addition to the transmembrane domain, acyl-chain additions commonly found in the fusion proteins of enveloped viruses and other features of the cytoplasmic tail may contribute to the forces that act on the donor membrane. Despite the predicted involvement of both transmembrane domain and fusion peptide association with the membranes in the fusion process, most studies have focused on the role of the fusion peptide.

The fusion proteins of avian (ARV) and Nelson Bay reoviruses (NBV) (p10, figure 4.1A) differ from the fusion proteins of enveloped viruses in that they are significantly smaller and appear to be primed for induction of membrane fusion

upon synthesis (see previous chapters). Because p10 proteins are nonessential for the virus life cycle, they may have freely evolved the minimal fusion machinery, devoid of the complexities involved in the regulation and specificity of the fusion reaction. If p10 represents the minimal fusion machinery, then analysis of p10 proteins would permit the identification of those domains involved directly in promoting membrane merger. The absence of coiled-coil structures within the p10 ectodomain, as an example, questions whether the conformational changes and the coiled-coil motif common to various enveloped virus fusion proteins are essential for their fusogenic activity or for their regulation. The finding that p10 and fusion proteins of enveloped viruses share in common the presence of a fusion peptide, however, suggests that direct interactions between the fusion peptides and donor and/or target membranes is a universal requirement for protein-mediated fusion. Analysis of p10 transmembrane and intracellular domains may therefore be useful in understanding the minimal donor membrane interactions necessary for fusion.

The nonstructural nature of p10 also makes it a good candidate for studies on the effects of fusion proteins on donor membranes. For enveloped viruses, the structural envelope glycoproteins may not have strong destabilizing effects on the donor membrane, since doing so would compromise the stability of the virus. Conversely, because p10 is not included in the virus structure, there is no need to limit or repress domains that destabilize the donor membrane. If membrane fusion requires the perturbation of both donor and target membranes, then p10

could afford more freedom in its activity on donor membranes and may, therefore, provide more strong donor-membrane destabilizing activity that is active upon synthesis.

The domain organization of p10 is intriguing since the ecto- and endo-domains are of equal length (approximately 40 residues each), perhaps indicating that they both contain important information for protein processing and/or fusion activity (Figure 4.1A). Previous analysis demonstrated that the nonconserved domain within the cytoplasmic tail of the p10 was involved in protein processing in a sequence-independent manner (chapter 1). We proposed that the non-conserved region allows the signal recognition particle, which is responsible for insertion of membrane proteins into the endoplasmic reticulum bilayer, to co-translationally bind the transmembrane anchor of p10. Continued fusogenic activity of constructs devoid of the nonconserved domain but containing an N-terminal cleavable signal sequence to direct p10 insertion into the membrane in an N-terminus out orientation would help confirm our hypothesis.

Our present mutagenesis analysis of the transmembrane anchor and membrane proximal residues within the ARV p10 protein identified motifs that are involved in the fusion process. Specifically, a conserved tri-glycine motif in the transmembrane domain, two palmitoylated cysteines, and a membrane-proximal intracellular basic domain are necessary for the fusogenic activity of ARV p10. Conservation of these motifs between ARV and NBV further suggests that they

are essential for p10 processing or function. Furthermore, isolation of detergent-resistant membranes demonstrated that p10 is associated with rafts, suggesting that the lipid composition of donor membranes may be important for fusion. The potential roles of each motif in the fusion process are discussed.

4.C. RESULTS AND DISCUSSION

4.C.1. Direct involvement of the p10 transmembrane domain in fusion

GPI-anchored p10 is fusion deficient

The participation of fusion protein transmembrane domains in membrane fusion was first suggested by studies using a glycosylphosphatidylinositol (GPI)-linked influenza HA fusion protein ectodomain. Several strategies demonstrated that although GPI-HA was capable of inducing the mixing of outer membrane leaflets, complete fusion was blocked at the hemifusion state (Kemble et al., 1994; Nussler et al., 1997). More recent analysis showed that the GPI-HA was deficient in promoting expansion of the fusion pore (Razinkov et al., 1999; Markosyan et al., 2000). The GPI-HA retained the ability to multimerize, bind red blood cells, and undergo conformational changes under conditions that normally trigger HA-mediated fusion (Kemble et al., 1993). From these studies, it is presumed that the transmembrane anchor of HA is mechanistically coupled to the ectodomain and participates in the reorganization of the donor membrane lipids during membrane fusion.

The fusion proteins of HIV (gp160) and vesicular stomatitis virus (VSV-G), when anchored to the membrane through a GPI-anchor, were also unable to induce complete membrane merger (Salzwedel et al., 1993; Odell et al., 1997). Interestingly, the SNARE proteins involved in intracellular vesicle transport were also non-functional when anchored to the membrane through a lipid-anchor

(McNew et al., 2000). These studies support that the anchor of fusion proteins has specific requirements.

When the transmembrane of p10 was replaced with the signals for addition of a GPI-anchor and transfected into quail cells, p10 did not induce cell-cell fusion (data not shown). Expression of GPI-p10 was evident by positive immunohistochemical staining (data not shown), however, surface localization was not examined. Because the transmembrane domain, cytoplasmic domain and palmitoylation of p10 would presumably affect the inner but not outer leaflets, it would be interesting to test whether GPI-p10 still succeeds in inducing the mixing of the outer leaflet. Nonetheless, the inability of GPI-p10 to induce complete fusion does lend credence to the possible involvement of the transmembrane domain in fusion.

The tri-glycine motif within the transmembrane anchor of p10 is essential for membrane fusion.

Two mechanisms by which fusion protein transmembrane domains could participate in the fusion reaction have been proposed. One hypothesis suggests that there is a sequence-independent but length-dependent requirement for the anchor of fusion proteins. This hypothesis is supported by studies on fusion proteins with truncated or substituted transmembrane domains. Armstrong et al (2000) showed that HA could withstand deletions of up to 10 residues within the transmembrane domain without changes to the membrane fusion capability of

HA. The SNARE proteins also showed a length requirement of their membrane anchor, requiring sufficient length to span one, or both membranes for t- and v-SNAREs, respectively (McNew et al., 2000). Furthermore, when the transmembrane of HA was substituted with the transmembrane domain of other viral and non-viral proteins, fusion was not affected (Roth et al., 1986; Dong et al., 1992; Melikyan et al., 1999). The fusion proteins of Sendai virus (F), VSV, and HIV also withstood substitution without loss of fusogenic activity (Schroth-Diez et al., 1998; Kozerski et al., 2000; Odell et al., 1997; Wilk et al., 1996). These reports support a length-dependent but sequence-independent requirement for the transmembrane domain of the fusion proteins under investigation. A possible explanation for the involvement of the membrane anchor in fusion that is in accordance with these results is as follows: The anchor is required to place mechanical strain on the donor membrane while it is tugged towards the opposing membrane. Being in contact with both membrane leaflets may be required to promote the breaking of the inner leaflet during pore formation and enlargement.

The transmembrane domains of some fusion proteins have a sequence-specific involvement in the membrane fusion process. The coronavirus, mouse hepatitis virus, fusion protein was unable to induce membrane fusion when its transmembrane domain was substituted (Chang et al., 2000). Cleverley et al. (1998) found that a glycine residue in the transmembrane domain of VSV-G is required for membrane fusion. It is possible that the specific requirements differ

for the various viral fusion proteins, as supported by the loss of fusion following substitution of the two adjacent glycines found within the transmembrane domain of HA of influenza subtype H2 despite a sequence-independent requirement for the transmembrane domain in the influenza subtype H3 HA protein (Melikyan et al., 1999; Armstrong et al., 2000).

The two reports demonstrating a change in fusogenic activity by a single amino acid substitution described in the previous paragraph both involve transmembrane glycine residues. Interestingly, both the avian reovirus (ARV) and Nelson Bay reovirus (NBV) p10 fusion proteins have a tri-glycine motif that is conserved despite an overall identity of only 33% between the two proteins (Figure 4.1b). Mutational analysis showed that this tri-glycine motif is important for membrane fusion induced by p10. P10 and all substituted p10 proteins were tagged with 2 HA epitopes at the N-terminus (2HAN). Mutation of glycines at positions 48 and 49 of p10 to alanines (G48A and G49A) resulted in a greatly reduced syncytium-inducing activity (Figure 4.2). Substitution of the glycine at position 50 to alanine (G50A) abrogated its fusogenic activity, as did the double mutation to glycines 49 and 50 (G49/50A). Interestingly, increasing the glycine stretch to four consecutive glycines (A47G) also abolished p10-induced fusion. For all mutants, protein expression was unaffected (Figure 4.2), and all mutants were shown to remain as integral membrane proteins (Figure 4.3). The mobility differences of p10 with various residue substitutions are presumed to reflect variable binding of amino acids to sodium dodecylsulfate (SDS). This

explanation is clearly demonstrated by the equal increase of mobility between 2HAN, G49A and G49/50A. The mutations made to the tri-glycine motif did not alter p10 protein stability, as the degradation of G49/50A was found to be slower than the HA-tagged p10 protein (2HAN) (Figure 4.3B). Finally, we found the loss of fusion activity due to the tri-glycine substitutions was not due to altered surface localization, since surface immunofluorescence was evident for G49A, G49/50A and A47G (Figure 4.4). These results strongly suggest that the tri-glycine motif is necessary for the fusogenic activity of p10 and does not influence membrane localization or surface expression.

Proposed functions of the p10 transmembrane domain in membrane fusion.

A possible role for transmembrane domains in the function of fusion proteins is an involvement in protein-protein interactions such as the involvement of the transmembrane domain of the Newcastle disease virus (NDV) hemagglutinin-neuraminidase fusion protein (HN) in tetrameric structure formation (McGinnes et al., 1993). Several strategies used to detect multimerization of transmembrane proteins failed to show that p10 was a multimer, suggesting that the p10 transmembrane domain is not essential for the formation of homo-oligomeric structures. Furthermore, transmembrane domains involved in protein-protein interactions share in common either a leucine heptad repeat or several glycine residues on the same face of the α -helix to permit intimate packing of residues at the interface of the helix dimer, and are sometimes further stabilized by charged

pairs (Cosson et al., 1992; Lemmon et al., 1992; Lemmon and Engelman, 1994; Cosson et al., 1991; McGinnes et al., 1993; Brosig et al., 1998; Gurezka et al., 1999). Such is not the case for p10 (Figure 4.5).

The role of the p10 transmembrane domain could be similar to that proposed for the transmembrane anchors of enveloped virus fusion proteins - to pull the donor membrane towards the target membrane with sufficient force to disrupt the lipid leaflets and create an enlarged fusion pore. The tri-glycine motif may function to extend the length of the p10 transmembrane domain. Glycine residues can adopt a much wider range of conformations than other residues and are therefore considered to be helix breakers. Although hydrogen bonds between main chain nitrogen and oxygen atoms stabilize the α -helix conformation in most transmembrane helices, the tri-glycine stretch could be forced to take non-helical conformations that would lengthen the transmembrane domain and permit optimal spanning of the membrane. To test this possibility, the transmembrane domain of p10 could be substituted with that of other viral or non-viral proteins with the assumption that if length was the main requirement, one of the p10 mutants should maintain fusogenic activity. Alternatively, p10 with the tri-glycine motif moved to other locations within the transmembrane or with amino acid additions that lengthen the anchor could be tested. The conservation of the tri-glycine motif between NBV and ARV, however, argues for a more specific function in the mechanism of p10-induced fusion.

An alternative function for the transmembrane domain of p10 and other fusion proteins is to directly contribute to the destabilization of the donor membrane, as suggested by studies on synthetic peptides modeled after the transmembrane domain of the VSV-G protein. Langosch et al. (2001) showed that these synthetic peptides were able to induce the fusion of liposomes. Mutations made to the synthetic peptides and to the VSV-G protein had similar effects on liposome-liposome and cell-cell fusion, respectively. The transmembrane of VSV-G is, therefore, proposed to have lipid destabilizing properties that favor formation of fusion intermediates similar to those described for the fusion peptides. Similar analysis should be performed with synthetic peptides resembling the transmembrane domain of p10. It seems reasonable to predict that interactions that decrease membrane stability would be favorable in both donor and target membrane. The conformational flexibility of the tri-glycine motif in p10 may translate into more flexibility of the lipid fatty acid chains. Movement of acyl- chains would increase the acyl-chain –to- polar head group ratio for lipids that surround p10, favoring negative curvature of the outer leaflet as depicted in figure 4.5. Studies on the transmembrane domain of p10 could be instrumental in understanding the contribution of changes to donor lipid bilayers involved in membrane fusion.

In addition to the tri-glycine motif, the alignment of all aromatic residues along one side of the transmembrane domain, with the other side consisting of aliphatic amino acids, may play an important role in p10-mediated fusion (Figure 4.5A).

Not only do the aromatic groups have the largest molecular mass and hydrophobicity, but also they are capable of stacking interactions such as hydrogen bond formation. Similar to the tri-glycine motif, the aromatic face may distort the shape of the transmembrane anchor and change the packing behaviors of nearby lipids. Studies on enveloped virus fusion proteins show that the oblique insertion of the fusion peptide into the lipid bilayers necessary for membrane perturbation and fusion is a consequence of a hydrophobicity gradient along the peptide (Epanand et al., 1995; Slepushkin et al., 1993). The activities of other proteins involved in membrane alterations such as ion channels, antimicrobial peptides, and lipoproteins require membrane-seeking peptides with variable distribution of hydrophobicity and volume that interact with lipids in a specific manner corresponding to their function (Brasseur, 1991; Brasseur et al., 1997). Although the transmembrane anchor cannot behave identically to these membrane-seeking peptides that do not span the membrane, unequal distribution of hydrophobicity, size, and hydrogen bonding potential could be responsible for unique interactions with the lipid bilayers that are involved in destabilization of the donor membrane. Studies should be undertaken to assess the importance of the unique arrangement of aromatic and aliphatic residues in the membrane anchor. Substitution of each aromatic group with the most conservative non-aromatic amino acid or additions that would alter the arrangement of each amino acid within the α -helix could be made and their effects on the fusogenic activity of p10 evaluated.

For enveloped viruses, the membrane destabilizing potential of the transmembrane domain would have to be minimized to ensure stability of the envelope during viral passage. Since NBV and ARV are non-enveloped viruses, there may be a reduced limitation to the destabilizing potential of the transmembrane domain. Interestingly, previous analysis of the p10 fusion peptide showed that it is less potent at inducing liposome fusion and is less hydrophobic when compared to the fusion peptides of enveloped virus fusion proteins (chapter 3). These studies suggested that because the p10 fusion peptide is exposed within the cell and attracts the degradation machinery, the hydrophobic potential may be restricted. A possibility is that the transmembrane domain of p10 compensates for the reduced fusion potential of the fusion peptide. If membrane fusion requires a minimum sum of forces that effect donor and target membranes, this could explain why various enveloped virus fusion proteins differ in their specific transmembrane and/or fusion peptide requirements.

4.C.2. Direct involvement of the transmembrane- adjacent intracellular cysteine residues in fusion

The transmembrane-adjacent cysteine residues of p10 are involved in fusion.

Both ARV and NBV contain two conserved cysteines adjacent to the transmembrane domain (Figure 4.1b). Mutational analysis showed that these

cysteines were essential for syncytium formation (Figure 4.6). The substitution of each cysteine alone to serine (C63S and C64S) or mutating them both to either alanines or serines (C63/64A and C63/64S) produced p10 proteins that were unable to induce cell-cell fusion when transfected into quail cells, despite continued expression (Figure 4.6), membrane integration (Figure 4.3A), and cell surface localization (Figure 4.4A and B). Transmembrane-proximal cysteines on the intracellular face of cellular membranes have the potential to be palmitoylated. We were interested to determine whether these conserved cysteines within p10 are palmitoylated, and whether syncytium formation required the cysteine residues or the palmitate additions.

The transmembrane-adjacent cysteine residues of p10 are palmitoylated.

The post-translational addition of palmitic acid through a thioester linkage is commonly associated with cysteines within or close to the transmembrane domain of integral proteins. Palmitoylation has been demonstrated for the fusion proteins of several enveloped viruses including the influenza HA fusion protein (Naeve et al., 1990; Naim et al., 1992) and the measles virus fusion protein (Caballero et al., 1998). In contrast, the small hydrophobic (SH) protein of human respiratory syncytial virus is not palmitoylated at the membrane-proximal cysteine residue (Collins et al., 1993). Furthermore, addition of cysteine residues to the nonacylated Sendai virus F protein at positions expected to undergo palmitoylation were not acylated (Ponimaskin et al., 1995). Interestingly, Ponimaskin and Schmidt (1998) found that sequences from the influenza HA

transmembrane and/or cytoplasmic domains could promote acylation of the cysteine-containing Sendai virus F protein suggesting that acylation depends on characteristics that surround the intracellular cysteine residues (Ponimaskin, E., and Schmidt, 1998). In view of the potential palmitoylation of membrane-proximal cysteine residues, we directly examined the acylation of p10.

[³H]-palmitic acid-labeling of cells transfected with the authentic p10 (p10auth) showed that p10 was palmitoylated (Figure 4.7A). The mutant p10 protein that contained a cysteine substitution to serine (C63S) showed reduced [³H]-palmitic acid incorporation, while both cysteine residues, when replaced with alanines (C63/64A), showed a complete loss of palmitoylation. The authentic p10 and cysteine substituted p10 proteins were still expressed in transfected cells, as demonstrated using [³H]-leucine labeling (Figure 4.7A). To confirm these results, palmitic acid labeling was repeated with the p10 constructs containing the remaining modifications to the transmembrane-proximal cysteines. Again, 2HAN was intensely labeled, both C63S and C64S showed reduced [³H]-palmitic acid incorporation, and C63/64A and C63/64S were not labeled with radioactive palmitic acid (Figure 4.7B). Future experiments should aim to confirm that labeling was not a consequence of incorporated [³H]-palmitic acid breakdown products. Soaking the SDS-PAGE gels in 1 M hydroxylamine, pH 7.5 for 14 hours before processing for fluorography to hydrolyze the thioester linkage of palmitate should remove [³H]-labeled-p10 if it is in fact labeled with [³H]-palmitic acid. These results demonstrated that the two conserved cysteines found

directly adjacent to the transmembrane domain of p10 are palmitoylated, and suggest that palmitoylation may be essential for the fusogenic function of p10.

The function of palmitoylation of p10.

To confirm the requirement of palmitoylation for p10-induced fusion, the effects of inhibitors of the palmitoylation reactions should be assessed. These experiments would ensure that the loss of palmitate is responsible for the abrogation of p10 fusogenic activity and eliminate the possibility that cysteines are essential residues independent of palmitoylation. Tunicamycin, a nucleoside antibiotic, inhibits protein glycosylation and palmitoylation (Patterson et al., 1994; Buckley and Whorton, 1997). Previous studies showed that tunicamycin did not inhibit syncytium formation when added to quail cells infected with avian reovirus (Duncan et al., 1996). These studies would suggest that palmitoylation is not essential for p10-induced fusion and that loss of syncytium formation with C63S, C64S, C63/64/A and C63/64S were a consequence of the amino acid substitution directly. When compared to other studies using tunicamycin to block palmitoylation, however, the studies with p10 used a lower concentration of inhibitor (2 μ m) and different solubilization techniques, in addition to not using fresh tunicamycin. For these reasons, and in light of the results showing a loss of fusion when cysteines at positions 63 and 64 of p10 were mutated, the tunicamycin experiments should be revisited. Additionally, cerulenin, another compound that blocks palmitoylation, should be assessed for its effect on fusion (Hurley et al., 2000). Palmitate analogues such as 2-bromopalmitate inhibit

palmitoylation while long chain polyunsaturated fatty acids such as arachidonic acid and eicosapentaenoic acid inhibit fatty acid acylation (Webb et al., 2000). Inhibitors such as these would be useful for further analysis of the involvement of palmitoylation in p10-induced fusion. Interestingly, in all studies on the fusion proteins of enveloped viruses described below, investigators assumed that when cysteines were mutated, it was the loss of palmitoylation and not the mutation itself that accounted for their results.

Palmitoylation has been shown to have different effects on the activity of fusion proteins from various sources. The fusion protein of Ebola virus containing a substitution of the palmitoylated cysteine to alanines was still capable of conferring viral infectivity through membrane fusion (Ito et al., 2001). In contrast, the syncytium-inducing activity of the measles virus (MV) fusion protein (F) was reduced as a consequence of elimination of the palmitoylated cysteine (Caballero et al., 1998). Studies on the influenza HA, however, have produced variable opinions as to the involvement of palmitoylation in membrane fusion. Substitutions and deletions that removed the three conserved cysteine residues in the HA fusion protein of influenza virus subtype H3 did not prevent lipid-mixing between virus and cell or content-mixing between infected cells (Jin et al., 1996). These results suggest that for influenza virus subtype H3, palmitoylation is not necessary for fusion. Studies completed by other investigators suggest that the dependence of palmitoylation on membrane fusion induced by influenza HA must depend on either the subtype of the virus, or the method of membrane fusion

assessment. When the HA of influenza subtype H7 was assessed for its fusogenic activity following loss of palmitoylated cysteines, it was found that palmitoylation was not required for lipid-mixing and pore formation (Schroth-Diez et al., 1998). These investigations used transfer of octadecyl rhodamine B chloride (R18) from the lipid bilayers of labeled red blood cell ghosts (RBC) as an indication of lipid-mixing, and the transfer of the small fluorophore calcein trapped within RBC to suggest pore formation. Other investigations demonstrated that the influenza HA (H7) devoid of palmitoylation was unable to induce the transfer of larger molecules or to bring about complete cell-cell fusion (syncytium) (Fischer et al., 1998; Kozerski et al., 2000). Studies on enveloped virus fusion proteins have not provided a unified hypothesis on the essentiality and role of palmitoylation for membrane fusion. It may be that palmitoylation plays a beneficial but accessory role in the activity of fusion proteins that is overlooked when optimal membrane fusion conditions are used in experimentation. The extent to which palmitoylation benefits the activity of fusion proteins may also depend on the efficiency of other domains involved in membrane fusion such as the fusion peptide.

Possible role for palmitoylation in p10-induced fusion.

The beneficial role of palmitoylation for the membrane fusion activity of p10 may be more apparent, as compared with fusion proteins of enveloped viruses, owing to the slow and inefficient activity of p10 and its relatively weak fusion peptide. Palmitoylation of p10 could serve several functions. Membrane fractionation

analysis of mutants deficient in one, or both, palmitoylation sites showed that palmitoylation was not necessary for p10's membrane association as an integral protein (Figure 4.8A). Similar to the authentic HA-tagged p10 (2HAN), C63S, C64S, C63/64A, and C63/64S were found to be absent within the soluble fraction of cells but present within the membrane fraction as integral proteins resistant to treatments that separate peripheral proteins. Furthermore, palmitoylation did not contribute to the stability of p10. Rapid degradation of 2HAN, C63S, C64S, C63/64A, and C63/64S was evident from pulse-chase experiments (Figure 4.8B). Palmitoylation, therefore, does not contribute to the turnover rate and membrane integration of p10.

An alternative role for palmitoylation could be an association with specific lipid components of membranes. Palmitoylation of caveolin-1, a protein involved in the transport of newly synthesized cholesterol from the endoplasmic reticulum directly to the surface, has been shown to be necessary for binding to cholesterol (Uittenbogaard and Smart, 2000). Sindbis virus (SIN) and Semliki Forest virus (SFV), both members of the alphaviruses, have cholesterol requirements for membrane fusion (Kielian and Helenius, 1984; Lu, Y et al., 1999; White and Helenius, 1980). Although recent investigations showed that cholesterol is required for conformational changes of the SFV fusion protein (Chatterjee et al., 2000), cholesterol may also participate in promoting changes to the membrane that are optimal for fusion. Razinkov and Cohen (2000) showed that in liposome fusion, cholesterol incorporation into lipid bilayers significantly promotes fusion by

stabilizing pore formation. Interactions between palmitates of p10 with cholesterol may, therefore, augment membrane fusion.

There are other proposed roles for palmitate attachment to fusion proteins in promoting membrane merger. Fischer et al. (1998) suggest that following the insertion of the influenza HA fusion peptide into target membranes and formation of a coiled-coil hairpin, a tilting of the transmembrane domain forces partial immersion of the cytoplasmic tail into the inner leaflet of donor membranes. In this scenario, increased hydrophobicity through palmitoylation would favor this immersion process. Alternatively, when fusion proteins force opposing membranes into close proximity, palmitate chains could create local strain on the inner leaflet. Yet another possibility is that palmitoylation of fusion proteins is involved in targeting the proteins to specific lipid domains within the cell membrane called rafts, as will be discussed next. Fusion assays that distinguish between the ability to induce outer leaflet merger, pore formation, pore enlargement, and inner leaflet fusion would help address the function of palmitoylation for the activity of p10.

4.C.3. Raft association of p10

p10 is associated with detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains.

The transmembrane and cytoplasmic domains and/or the palmitoylation of p10 may participate in determining its lipid environment. Lipids with long saturated

acyl chains including sphingolipids and glycosylphosphatidylinositol (GPI)-linked proteins pack tightly together and confer a high degree of order to membranes. Along with cholesterol, sphingolipids form moving platforms on the cell surface called rafts that can be isolated from cells as glycolipid-enriched membranes (GEMs) (or detergent insoluble glycolipid-rich membranes (DIGs)) (Brown and London, 1998; Simons and Ikonen, 1997; Brown and London, 2000; Kurzchalia and Parton, 1999; Harder and Simons, 1997; Hooper, 2000). The tight lipid packing imparts a more ordered state (liquid-ordered phase) to membrane rafts conferring resistance to Triton X-100 detergent treatment at 4°C (Schroeder et al., 1998). Equilibrium centrifugation on a discontinuous sucrose gradient allows separation of DIG-associated proteins from other detergent-insoluble materials including cytoskeletal elements (Brown and Rose, 1992; Fiedler et al., 1993). The highly buoyant DIGs float on sucrose gradients and are found in fractions above 40% sucrose where detergent soluble proteins and contaminants remain. The presence of several fusion proteins including the HIV gp41 and influenza HA in detergent-resistant rich fractions has confirmed their association with lipid rafts (Simons and Ikonen, 1997; Nguyen and Hildreth, 2000). We, therefore, sought to determine whether p10 was also present within DIGs.

Quail cells transfected with 2HAN and treated with Triton X-100 at 4°C were mixed in an equal volume of 80% sucrose, overlaid with 30% and 5% sucrose layers and subjected to sucrose gradient centrifugation. Raft associated proteins such as CD59 have been previously shown to localize entirely to the top fractions

of the 30% sucrose layer while proteins that are excluded from membrane rafts such as CD45 remained entirely within the 40% sucrose layer (Nguyen and Hildreth, 2000). With p10, although a large fraction remained within the 40% sucrose fractions where the bulk of the detergent soluble proteins reside, a significant portion of p10 migrated up the 30% sucrose fraction towards the interface between 30% and 5% sucrose (Figure 4.9). Interestingly, previous characterization of influenza HA and other integral membrane proteins such as major histocompatibility complex 1 and CD63 showed a similar distribution to p10, with protein populations within both detergent-soluble and insoluble fractions (Zhang et al., 2000; Nguyen and Hildreth, 2000). Similar to p10, the influenza HA fusion protein is more abundant in the soluble fractions than within DIG domains and the HA protein can be identified within every fraction up to the 5% sucrose layer. These preliminary studies, therefore, suggest that a subpopulation of p10 may in fact localize to cellular rafts.

Several further investigations are necessary to confirm the presence of p10 within lipid rafts. First, isolation of DIGs should be repeated with proper controls for both detergent-soluble and insoluble proteins. Although most publications omit these controls, they would ensure that the detergent lysis conditions and sucrose gradients are successful at separating DIGs from the remaining membrane components. Additionally, confirming the localization of p10 within rafts could be accomplished using confocal fluorescence microscopy which has successfully colocalized the transmembrane subunit of the HIV-1 fusion protein

(gp41) with Thy-1, CD59, GM1, and 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine [DiIC₁₆], molecules that favor raft lipid domains (Nguyen and Dzung, 2000). Conversely, colocalization with CD45 or DiIC₁₂ which partition to the fluid domains of membranes would suggest that p10 is not predominantly raft associated. Finally, experiments described above could be repeated in the presence of raft-dissociating reagents (described in 'the significance of rafts' section), which would provide a good negative control.

Palmitoylation of p10 is not essential for raft association.

In addition to GPI-anchors, other acyl chain additions to proteins such as palmitoylation and myristoylation are sufficiently long and saturated to confer association with rafts. Proteins such as endothelial nitric-oxide synthase and the Src-related kinases Fyn and Lck have been shown to associate with the inner membrane leaflet of rafts through palmitoylation despite the absence of transmembrane domains (Dietzen et al., 1995; Shenoy-Scaria et al., 1994; Shaul et al., 1996; Webb et al., 2000). Furthermore, the membrane-spanning linker molecule (LAT) requires palmitoylation for raft association (Zhang et al., 1998). Palmitoylation at cysteines has also been suggested as the mechanism for targeting some of the fusion proteins to lipid rafts. Covalent additions of palmitate residues to cysteines of the influenza HA transmembrane and cytoplasmic tail were shown to be required for DIG association of HA (Melkonian et al., 1999). The two palmitoylated cysteines in the cytoplasmic tail of HIV gp41 are postulated to participate in conferring raft preference (Yang et al., 1995). On

the other hand, palmitoylation is dispensable for the association of other integral membrane proteins such as caveolin with lipid rafts (Dietzen et al., 1995). Given the significant data correlating palmitoylation with raft association, we were interested in determining whether palmitoylation of p10 was required for localization with DIGs.

The DIGs from cells transfected with p10 constructs containing mutations to the intracellular cysteines were isolated to determine whether palmitoylation was essential for p10 localization to raft domains. Substitution of each intracellular cysteine to serine (C63S and C64S) did not prevent p10 from floating into the top detergent insoluble fractions of the sucrose gradient (Figure 4.9). Furthermore, when both cysteines were substituted with alanines (C63/64A), the mutated p10 behaved similarly to wild type. Mutational analysis of p10 suggests that palmitoylation is not an essential requirement for association with DIG domains. To confirm these results, the effects of palmitoylation inhibition with palmitate analogues such as 2-bromopalmitate or inhibition of fatty acid acylation with long chain polyunsaturated fatty acids such as arachidonic acid and eicosapentaenoic acid on raft association could be assessed (Webb et al., 2000). Furthermore, although raft association is possible in the absence of p10 palmitoylation, a more quantitative analysis would be required to determine whether palmitoylation of p10 *augments* the favorable interactions with lipid raft environments.

It would be interesting to identify the features of p10 that provide the capability of associating with DIG domains. With other fusion proteins, in addition to palmitoylated cysteines, the cytoplasmic tail and transmembrane domain have been implicated in raft localization. Influenza HA constructs that lack the cytoplasmic tail but retain palmitoylation were found to have altered DIG association when expressed by virus infection or transfection suggesting a role of the cytoplasmic tail in raft localization (Zhang et al., 2000). Furthermore, mutations made within the transmembrane domain of influenza HA resulted in a large reduction in HA association with DIGS (Scheiffele et al., 1997; Lin et al., 1998). P10 could provide an additional example to the growing repertoire of proteins used to assess the determinants involved in protein association with lipid rafts.

The significance of rafts in membrane fusion.

Aside from confirming that p10 is associated with rafts, the significance of raft association for fusion would be interesting to assess. One could speculate on many different ways in which DIGs may impact membrane fusion. In their review, Brown and London (2000) suggest that rafts may affect processes by altering the environment of lipids and thus promoting alternative conformations of lipids and proteins. Altered lipid and protein conformations could play a role in promoting membrane fusion intermediates. The highly ordered lipid packing within rafts may be more susceptible to the destabilizing actions of fusion peptides and other domains within the fusion peptide. Alternatively, in excluding

large molecules such as CD45, rafts may permit more intimate contact between two opposing membranes. Proteins within rafts could be involved in intimate adhesion between cells and thus bring opposing membranes into close apposition for fusion protein function. Greater membrane access and cell-cell contact are two possibilities that would be especially useful for p10 due to the small size of its fusion peptide-bearing ectodomain. As mentioned previously, rafts may also serve to concentrate fusion proteins within the membrane to sufficient densities for efficient membrane destabilization and fusion. Rafts have been demonstrated to be the sites of high signal transduction activity through their preferential inclusion of molecules that participate in the signaling pathways. A hypothetical proposition is that membrane fusion requires signal transduction. Clearly, rafts could impact protein induced membrane fusion either directly or indirectly.

Evidence is growing for the importance of rafts in the life cycle of enveloped viruses. The abundance of cholesterol and sphingomyelin, the presence of the lipid raft-specific ganglioside (GM1) and GPI-linked proteins Thy-1 and CD59 and the exclusion of CD45, a molecule that is excluded from rafts, in HIV envelopes confirmed that donor membranes involved in HIV induced membrane fusion are composed mostly of DIGs (Aloia et al., 1993; Nguyen and Hildreth, 2000). In another study, partial analysis of the lipid envelope composition of influenza HA and the association of HA with DIG fractions of infected cells suggested that influenza buds from cholesterol-sphingomyelin-rich raft domains (Scheiffele et

al., 1999). These studies suggest that rafts play a role in the life cycle of enveloped viruses, but do not distinguish between the participation of rafts in providing a platform for virus assembly or budding and membrane fusion.

The finding that many fusion proteins localize to raft domains and that the lipid membranes of some enveloped viruses are composed of raft components does not necessarily imply that fusion proteins require DIG membranes for function. Alternatively, rafts may provide an efficient method of concentrating viral glycoproteins as launching sites for virus budding. A recent report, however, has found a correlation between the presence of DIGs within cellular membranes and human T-cell leukemia virus type 1 (HTLV-1) induced syncyium formation (Niyogi K and Hildreth, 2001). Conversely, depletion of cholesterol, a process known to disrupt lipid rafts (Scheiffele et al., 1997) had no effect on fusion induced by influenza HA (serotype H3) (Armstrong et al., 2000). Overall, there does not appear to be a general consensus on whether lipid rafts are necessary for enveloped virus assembly, budding, or membrane fusion.

Because p10 is not involved in the assembly or exit of avian reovirus, the evolution of an association with lipid rafts may suggest that DIGs do have consequence on the fusogenic function. Studies should be undertaken to determine whether the formation of DIGs in cells expressing p10 is essential for syncyium formation. The effect of treatments known to disrupt lipid rafts such as cholesterol depletion with β -cyclodextrin, fillipin and nystatin, cholesterol

oxidation with cholesterol oxidase, inhibition of cellular cholesterol biosynthesis with squalestatin or lovastatin, or inhibitors of cellular sphingolipid biosynthesis such as D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol on fusion could be assessed (Ilangumaran and Hoessli, 1998; Chang et al., 1992; Rothberg et al., 1992; Sheets et al., 1997; Smart et al., 1994; Stulnig et al., 1997; Taraboulos et al., 1995). Alternatively, monoclonal antibodies to proteins that are concentrated within rafts may also prevent membrane fusion through steric hindrance if raft domains are essential for the fusion mechanism (Niyogi and Hildreth, 2001). Using these and other strategies, the significance of lipid raft association of p10 should be determined.

In summary, preliminary studies demonstrated that p10 can associate with detergent-insoluble lipid domains and that palmitoylation is not essential for this association. Additional strategies have been suggested that could confirm these assessments. Future studies could determine whether the transmembrane and/or cytoplasmic domains of p10 are involved in p10-DIG association and identify the specific determinants. Furthermore, it would be especially interesting to determine whether raft association is significant to the fusogenic function of p10, and if so, whether lipid rafts play a direct or indirect role.

4.C.4. The membrane-proximal domain rich in positively charged residues is involved in membrane fusion

Positively charged residues are essential for p10-induced membrane fusion

The intracellular C-terminus of p10 occupies a large portion of the protein and is presumably involved in either protein processing or function. Previous analysis demonstrated that the last 20 residues within the endodomain were necessary for protein insertion into the cellular membrane in a sequence-independent manner (Shmulevitz and Duncan, 2000). The hypothesis proposed was that, in the absence of an N-terminal signal peptide to direct p10 insertion into the endoplasmic reticulum membrane, p10 utilizes the transmembrane domain as an internal signal-anchor sequence. This requires that at least 40 residues follow the transmembrane anchor to permit co-translational recruitment of the signal recognition particle that directs membrane insertion.

Using mutagenesis analysis, we found that the basic region of p10 is necessary for the function of p10. ARV p10 has lysine residues at positions 65, 67, and 69 and arginine residues at positions 74 and 79. Mutations made to lysines that conserved the basic charge (K67R and K69R) had no effect on the fusogenic activity of p10 (Figure 4.10). When these lysine residues were substituted with methionines (K67M, K69M), however, fusion was abrogated. Conservation of the positive charge at position 79 was not essential, as non-conservative mutations made to the Arginine (R79A) had no effect on p10 function.

Expression of all mutants was confirmed (Figure 4.10). These results suggested that the positive charges in the membrane-proximal basic domain are necessary for the processing and/or fusogenic activity of p10.

Transmembrane anchor-proximal basic charged residues are known to contribute to the determination of protein orientation within the membrane (Whitley et al., 1993, Matlack et al., 1998). Although we believe that the lysines in p10 are in fact involved in conferring a type I orientation to p10, our analysis has shown an additional role for these basic residues. All the p10 proteins containing substitutions within the basic region remained integrally associated with the membrane (see figure 4.3A). Furthermore, the p10 mutant K67M showed a similar rapid degradation profile as 2HAN suggesting that the loss of basic amino acids did not influence fusion by reducing the stability of p10 (see figure 4.3B). Most important were the results demonstrating that the basic domain mutants were successfully expressed on the surface of cells in a type I orientation, evident in figure 4.4A for mutant K69M. Surface staining was completed on live cells to ensure that no antibody crossed the cell membrane. Also, antibodies that only recognized the N-terminal HA epitope tag were used to confirm protein orientation. These analyses suggest that although the p10 proteins containing mutations within the basic domain are successfully expressed, assume the correct topology in the membrane, are transported to the cell surface, and exhibit similar protein stability, they are unable to induce complete cell-cell fusion.

Role of cationic residue in membrane interactions, destabilization, and fusion

Cationic amphipathic helices are common amongst proteins that destabilize membranes (Epanand et al., 1995). A family of viral proteins called viroporins that are involved in increased membrane permeability and virion release share in common a domain rich in basic residues (Carrasco, 1995). This family of proteins includes the Vpu of human immunodeficiency virus (HIV), the 6K protein of Semliki Forest virus, the small hydrophobic (SH) protein of human respiratory syncytial virus, the 2A and 2B proteins of poliovirus and NSP4 protein of rotavirus (Gonzalez and Carrasco, 1998; Sanz et al., 1994; Perez et al., 1997). A cationic amphipathic α -helix found within the 2B proteins of polioviruses and Coxsackie viruses and the NSP4 protein of togaviruses was shown to be essential for membrane permeabilization properties (Kuppeveld et al., 1997; Barco and Carrasco, 1998; Newton et al., 1997; Browne et al., 2000).

Synthetic cationic peptides have provided insight into their mode of action. Partial penetration of an α -helical peptide consisting of a three-lysine face was demonstrated using model membranes and synthetic peptides (Pecher et al., 1998). Studies on synthetic peptides with lipid- and bio-membranes suggest two mechanisms for the permeabilizing effects of cationic peptides on membranes. One proposed method of membrane permeabilization by positively charged, amphipathic α -helices is that these helices span the membrane and form

aqueous pores. If the positively charged domain of p10 is modeled as an α -helix, it would not be sufficiently long or amphiphilic to span the membrane to form aqueous pores (Figure 4.11). Furthermore, cross-linking by membrane permeable and impermeable reagents as well as complementation and dominant negative mutation screening failed to show any oligomeric interactions between p10 monomers, again suggesting that the aggregation of p10 domains to form pores is unlikely (see chapter on multimerization of p10). An alternative model describes these helices as lying parallel with the membrane and penetrating through the lipid head groups (Shai, 1995; Epand et al., 1995; Brasseur, 1991b). Penetration could have multiple effects on membranes such as the induction of degradation by phospholipases. In the case of fusion, phospholipase activity on the inner membrane lipids would not be favorable because it would decrease the head group-acyl chain volume ratio, and most likely does not explain the potential function of the p10 positive domain. Alternatively, interactions between the cationic peptides and the anionic phospholipid head groups of the inner leaflet would serve to increase the head group-acyl chain volume ratio, alter the spontaneous monolayer curvature, and promote the correct curvature necessary for fusion (Polozov et al., 1997). Measurements of the bilayers to hexagonal phase transition temperature of liposomes in the presence of peptides synthesized according to the amino acid sequence of the p10 basic domain could be used to assess the effect of the basic domain on membrane bilayers. In addition, methods to distinguish between fusion of the outer versus inner leaflets during cell-cell fusion would be instrumental in understanding the affects of the

basic domain. As an example, these studies may show that although p10 bearing mutations within the basic domain can not induce complete fusion, the intracellular domain is only required during the later stages of membrane fusion that involve changes to the inner leaflet of the lipid bilayers.

Although all studies to date have used α -helices to depict the interactions between cationic peptides and lipids, one study demonstrated that cationic peptide side chains bind to the anionic phosphatidylglycerol independent of structural propensity, where they disturb lipid head group organization (Dathe et al., 1996). Circular dichroism studies of the p10 basic domain peptide would indicate the secondary structure of this region, and help suggest the means of lipid interactions. It is known that the positions of lysine residues, the angle subtended by the hydrophobic residues, and the position of bulky residues all influence the effects of cationic peptides on membranes (Erand et al., 1995). Depending on the size of the hydrophobic and cationic face, peptides could immerse their hydrophobic face into the lipid bilayers while others may interact only between the anionic lipid head groups (Kiyota et al., 1995). The secondary structure of the p10 basic domain would permit more accurate predictions on potential lipid-peptide interactions.

The interactions between the basic domain of ARV p10 and the phospholipid bilayers can be predicted based on the depicted α -helix or β -sheet structures (Figure 4.11A). If the p10 cationic region is an α -helix, than the non-polar

alanines flanked by basic residues could insert parallel to the inner leaflet (Figure 4.11B). The non-polar alanines could interact with the acyl chains while the basic residues interact with the anionic phospholipid head groups. Alternatively, a β -sheet structure for the basic domain would also favor interactions with the inner leaflet. As depicted in figure 4.11B, a β -sheet could penetrate the inner leaflet at an angle that would allow both non-polar residue burying and ionic interactions with the lysine residues. When attempting to envision these interactions, it is important to consider that lysine and arginine residues have long aliphatic side chains that terminate with a positive charge, allowing these residues to 'reach out' to their destination and also confer some hydrophobic characteristic close to the peptide backbone. Interestingly, the insertion of an additional alanine residue between lysines 65 and 67 (A66/67) did not affect the fusogenic activity of p10 (Figure 4.10). This modification to the basic domain would have changed the original position of the first lysine residue in the predicted α -helix or β -sheet structure (Figure 4.11). Future mutations should attempt to make larger alterations to the placement of polar and basic residues within predicted secondary structure. In determining the role of the basic domain, it would be of pivotal importance to determine whether the basic residues are sufficient for this role, or whether the conformation of the basic domain is of equal significance. The basic domain of NBV p10, when depicted as either a β -sheet or an α -helix, has less substantial polar and non-polar faces (Figure 4.11). It would be interesting to make changes to the basic domain of NBV p10 and investigate their effects on membrane fusion. The similarity

between the domain organization of p10 proteins encoded by ARV and NBV suggests that these proteins function similarly, although they may differ in the extent to which each domain contributes to fusion.

Lastly, it is important to note that studies on the fusion proteins of enveloped viruses have excluded discussions on the possible participation of basic residues in the fusion mechanism. A quick assessment of the cytoplasmic region of the influenza HA, vesicular stomatitis virus, Semliki forest virus, Newcastle disease virus, and murine leukemia virus fusion proteins showed a variable quantity of basic residues ranging from two to seven, with no similarity in their arrangement or spacing. It is therefore likely that the basic residues are not directly involved in the fusion process of all fusion-inducing proteins. P10 may represent an amalgamation between the fusion proteins of enveloped viruses and other membrane-perturbing proteins such as the viroporins. This suggestion is supported by previously noted differences between the role of p10 and enveloped virus fusion proteins in the viral life cycle. While enveloped viruses may focus on destabilization of the target membrane to ensure that the integrity of the envelope is not jeopardized prior to cell contact and fusion, ARV and NBV do not incorporate p10 into the virus and may favor the destabilization of donor membranes. Furthermore, the exposed fusion peptide of p10 attracts the degradation machinery and therefore may be restricted in its potential activity on target membranes. This hypothesis presumes that membrane fusion requires a minimum sum of donor and target membranes instability, an assumption that

could be tested using synthetic membranes and peptides or domain swaps between fusion proteins.

4.C.5. The cytoplasmic tail of p10 can withstand only a threshold of elongation without loss of fusion.

Studies on enveloped viruses have demonstrated that the cytoplasmic tail of fusion proteins encoded by paramyxoviruses are essential for fusogenic activity (see examples; Dutch and Lamb, 2001; Sergel and Morrison, 1995), while the intracellular domains of orthomyxoviruses and retroviruses are dispensable (see examples; Schroth-Diez et al., 1998; Melikyan et al., 2000). Elongation of the influenza HA cytoplasmic tail however resulted in a decreased syncytium formation activity directly correlating with the number of added C-terminal histidine residues (Ohuchi et al., 1998). Consecutive addition of histidine residues did not effect protein expression, reduced but did not eliminate lipid mixing, but completely eliminated content mixing. Residues other than histidine, when added to the cytoplasmic tail, showed a similar effect on the activity of HA. This study supported a role in the cytoplasmic tail in permitting the formation of large pores between donor and target membranes.

It was interesting to discover that additions of more than 10 residues to the cytoplasmic tail of p10 were also detrimental to the fusion activity. Several constructs were made with additional insertions of HA epitope at the C-terminus of p10. The first HA epitope added replaced the last 11 residue within p10, and

was called 1HAC (one HA epitope at the C-terminus). 2HAC, 3HAC and 4HAC were then constructed through the insertion of 1, 2, or 3 additional HA epitopes at the C-terminus of 1HAC. Expression of all four modified p10 proteins was demonstrated by immunoprecipitation with antibodies that recognize p10 or the HA epitope (Figure 4.12A). Quail fibroblast cells transfected with 1HAC, 2HAC, 3HAC or 4HAC were stopped at 24, 36, 48 or 62 hours and assessed for the presence of syncytium by microscopy visualization following immunohistochemical staining (Figure 4.12B). Both 1HAC and 2HAC were capable of inducing cell-cell fusion by 24 hours. At all time points, 2HAC fusion activity is slightly reduced as compared to 1HAC. Elongation of p10 by 11 residues therefore reduces, but does not abrogate fusion activity. Both 3HAC and 4HAC did not induce cell-cell fusion even 62 hours post-transfection as indicated by only single cells expressing these proteins. This analysis suggests that similar to analysis of influenza HA, the cytoplasmic tail of p10 can not withstand significant elongation without loss of activity.

Several experiments are necessary to determine the means by which C-terminal additions interfere with the fusogenic function of p10. First, confirmation that these constructs successfully locate within the membrane fraction and are transported to the surface of cells in the correct orientation is necessary to eliminate the possibility that the changes at the C-terminus are effecting membrane insertion or protein transport. These constructs cannot be easily assessed for surface localization, as we do not presently have antibodies that

recognize the N-terminus of p10. Insertion of an alternate epitope tag at the N-terminus would be necessary. Alternatively, additions of other residue sequences at the c-terminus of 2HAN (p10 with optimized translation and 2 N-terminal HA epitope tags) would permit surface localization studies and would ensure that elongation, and not the acidic residues abundant in the HA epitope, is responsible for the effects on fusion. One could envision that the acidic residues added to the C-terminus would bind and interfere with the function of the membrane-proximal basic residues previously demonstrated to participate in the membrane fusion mechanism of p10. Additions to the C-terminus could affect the free mobility of the transmembrane domain by disfavoring tilted conformations or perhaps they could interrupt the activity of the basic residues within p10. Alternatively, additions to the C-terminus may have an indirect affect on fusion by disallowing the correct processing and trafficking of p10. Understanding the means by which progressive HA epitope additions to the C-terminus of p10 inhibit fusogenic activity may provide additional proof for the involvement of the cytoplasmic tail in p10-mediated cell-cell fusion.

4.D. CLOSING DISCUSSION

Our results have demonstrated that the transmembrane tri-glycine motif, the palmitoylated cysteines, and the basic domain are directly involved in the fusion activity of p10. Mutations to these regions prevented p10 induced syncytium formation without changing protein expression, stability, membrane insertion, or surface localization. These studies support that in addition to the requirement of a fusion peptide to interact with target membranes, there is a role for sequence-dependent interactions between p10 and the donor membrane.

We predict two methods by which the interactions between residues in p10 and the donor membrane could be involved in the fusion process. If membrane fusion requires the close opposition of two lipid bilayers with substantial stress perpendicular to the membrane surface, then the requirement of these domains would be to anchor p10 firmly within the donor membrane. The involvement of palmitate groups and basic residues interacting with the membrane lipids could ensure that the force is exerted on both lipid leaflets, making pore formation especially favorable. The tri-glycine motif could provide the transmembrane anchor with sufficient length or elasticity.

Several lines of analysis have suggested that there is no sequence-dependent requirement for the transmembrane and cytoplasmic domains of some enveloped virus fusion proteins. The cytoplasmic domain of influenza HA is dispensable without a loss of membrane fusion activity, and is believed to be important only

for permitting free mobility of the transmembrane domain within the lipid bilayer (Ohuchi et al., 1998; Fisher et al., 1998). The involvement of the cytoplasmic domain has only been demonstrated with paramyxoviruses such as Sendai virus and simian parainfluenza virus 5 (Dutch and Lamb, 2001; Bagai and Lamb, 1996). Furthermore, truncations within and exchange between the transmembrane domain of influenza HA suggest a length but not sequence requirement for the membrane anchor (Armstrong et al., 2000; Kozerski et al., 2000; Melikyan et al., 1999), although exceptions mentioned previously have been found. These studies suggest that the role of the transmembrane domain in membrane fusion is to anchor the protein firmly within the two leaflets of the membrane. Forces exerted by the pulling of these proteins towards target membranes are proposed to confer the changes on the donor membranes.

This first model is compatible with the hypothesis that structural rearrangements of enveloped virus fusion proteins are coupled with fusion (Bollough et al., 1994; Bron et al., 1993; Carr et al., 1997; Carr and Kim, 1993; Weissenhorn et al., 1997; Kozlov et al., 1998; Bentz, 2000; Baker et al., 1999; Bentz and Mittal, 2000). The formation of thermodynamically optimal protein structures could provide the energy for pulling two opposing membranes into close apposition. If p10 originates in a fusion active conformation and does not undergo further conformational changes, which is suggested by the small size of the ectodomain and the lack of heptad repeats that would favor coiled-coil structures, it is difficult to justify that its fusion peptide could freely interact with target membranes yet

exert a significant pulling force. We would need to impose the following changes to the present paradigm of the role of fusion peptides in the fusion process; that the hydrophobicity of the fusion peptide makes significant changes to the hydration layers surrounding the cell surface and promotes close membrane proximity. The fusion peptide could then insert into the target membrane and impose a short, unfavorable distance between membranes that would support membrane fusion over prolonged membrane contact.

The second model proposes a more direct role of the transmembrane and cytoplasmic domains in membrane fusion. Instead of functioning simply to anchor the fusion protein within donor membranes, these domains may create local destabilization within the lipid bilayer. The tri-glycine motif in the p10 transmembrane anchor could impose a non-helical conformation in the hydrophobic core of the bilayer that would permit more fatty acyl-chain disorder and promote negative curvature favored by lipids whose fatty acid chains occupy more area than the polar head group. The induction of negative curvature would promote the mixing of outer leaflet lipids and favor the formation of the hemifusion intermediate. The palmitate groups and basic residues within the cytoplasmic tail of p10 could be involved in destabilization of the inner leaflet to promote pore formation and enlargement. The inner leaflet is composed of lipids whose fatty acid chains occupy more area than the polar head groups. Insertion of the cylindrical palmitate residues or increased polar head group area through ionic interactions with basic residues would alter the characteristics of the inner

leaflet lipids that surround p10. Future studies should aim to distinguish whether the interaction between p10 and donor membranes is disruptive through analysis of the effects of synthetic peptides on liposomes.

The second model is also supported by studies on the fusion proteins of enveloped viruses. Repeated analysis has demonstrated that fusion peptides destabilize target membranes (reviewed in Pecheur et al., 1999; Martine et al., 1999; Durrel et al., 1997). Only recently has similar analysis commenced to demonstrate that peptides modeled according to the fusion protein transmembrane domain have membrane destabilizing and fusion activity on liposomes (Langosch et al., 2001). If destabilization of a single lipid bilayer favors membrane merging, then similar changes to donor membranes should also have favorable effects on fusion. With p10, donor membrane destabilization may be even more favorable because p10 is not incorporated into released virus particles and because of the low activity of its fusion peptide.

Developments of methods that distinguish between the hemifusion intermediate and complete fusion would help to confirm the involvement of p10 domains in different steps of protein mediated membrane fusion. Furthermore, the small size and simplicity of p10 makes future mutational studies easy and insightful. With the development of more analytical methods, p10 would become an excellent candidate for understanding the minimal membrane fusion machinery and lipid bilayer modification requirements. The analysis of p10 might contribute

to an improved understanding of membrane fusion by demonstrating the minimal requirements for biological membrane fusion, but would not necessarily relate directly to the function of complementary domains in the fusion proteins of enveloped viruses because of different constraints imposed on these two different fusion proteins.

4.E. CHAPTER 4 FIGURES

Figure 4.1. Structural model of p10 and sequence conservation in the transmembrane and cytoplasmic domains. (A) Locations of conserved cysteine residues, the hydrophobic patch/ fusion peptide, a stretch of conserved ectodomain residues, the transmembrane anchor (TM), the domain rich in basic residues, and the highly variable (non-conserved) domain in the integral membrane type I (N-terminus out) p10 protein are depicted. (B) Top panel indicates location of conserved structural motifs and the first and last residues in the ARV p10 sequence. The center panel shows the sequences of p10 from ARV strains 176 and 138, and NBV. The location of similar or identical residues, basic residues, and potentially palmitoylated cysteines are indicated according to the legend.

Figure 4.2. Substitution analysis in the transmembrane anchor of p10 suggests a sequence-dependent requirement for fusion activity. The identities and exact locations of specific substitutions are indicated using the single letter amino acid code to indicate the identity of the authentic residue (s), its position, and the identity of the substituted amino acid. All constructs contained two N-terminal HA epitopes (red box) and their sequence was confirmed. The modified proteins were transfected into cells and their fusogenic activity assessed by visualization of syncytium formation at 24, 48, and 60 hours post-transfection. Expression of proteins was confirmed by immunostaining and immunoprecipitation.

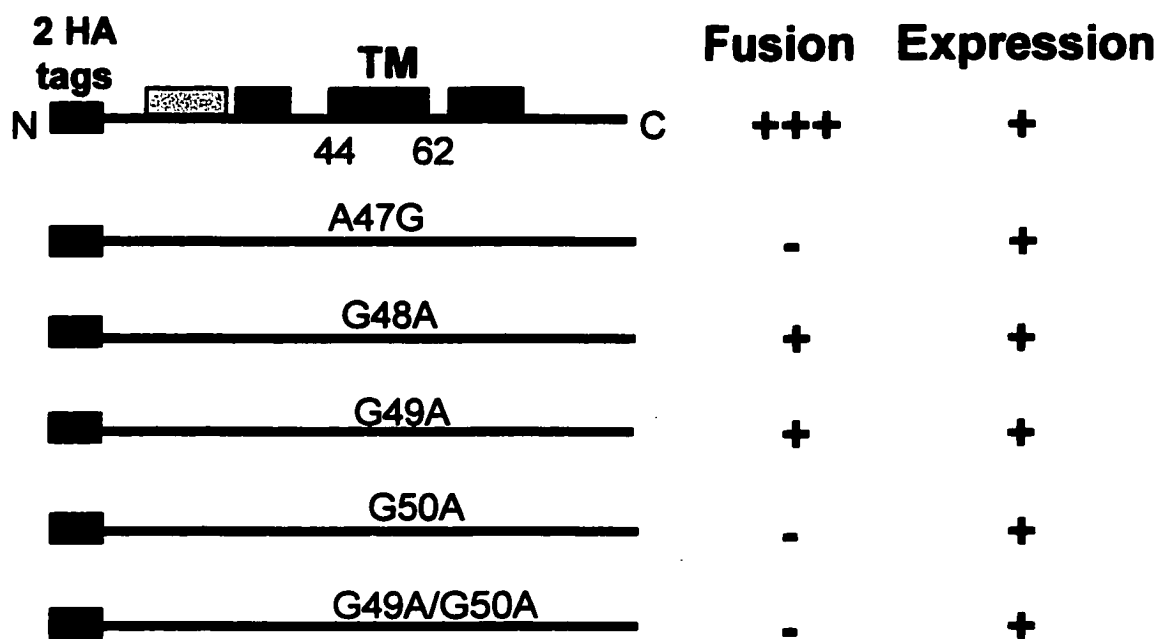
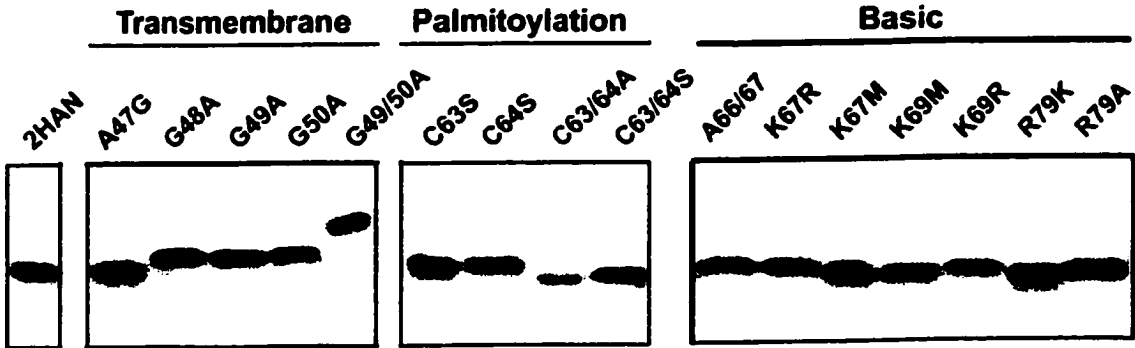


Figure 4.2

Figure 4.3. Membrane insertion and protein stability are not effected by mutations within the transmembrane and cytoplasmic domains of ARV p10.

(a) The ability of HA-tagged p10 (2HAN) or mutated p10 variants to localize to the membraneous fraction under conditions that remove peripheral proteins was assessed by cell fractionation. Changes in mobility are presumed to correspond to the substitutions, which alter SDS-binding potential of the proteins. (B) HA-tagged p10 containing substitutions within the transmembrane or cytoplasmic domains showed rapid degradation characteristic of 2HAN. Degradation of proteins was monitored in transfected cells by pulse-chase analysis. Following 10 minutes incubation with [³⁵S]-methionine, cells were chased for 0, 30, 60, or 120 minutes as indicated, followed by immunoprecipitation and gel electrophoresis.

A



B

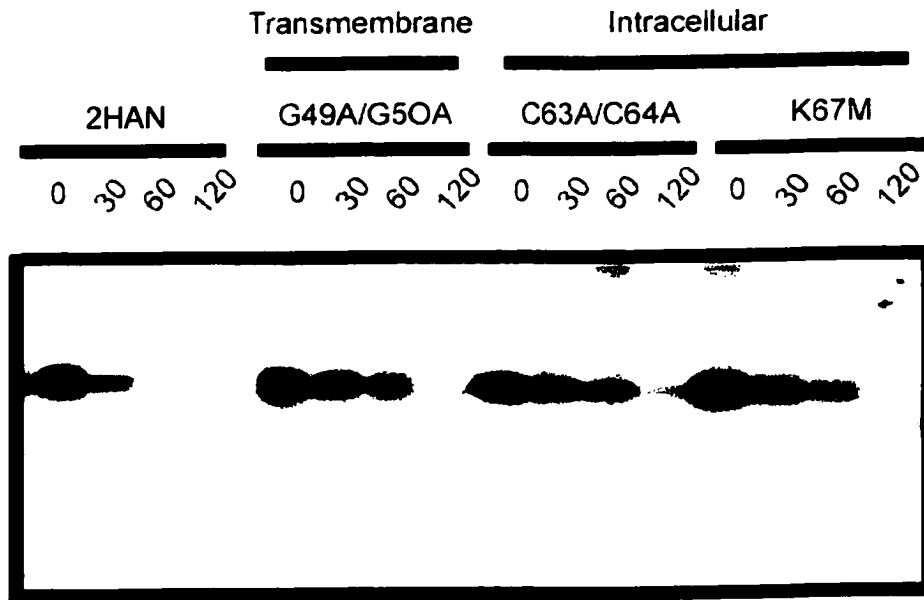


Figure 4.3

Figure 4.4. Substitutions made within the transmembrane and cytoplasmic domains of p10 did not prevent surface localization. Live transfected cells were bound with anti-HA monoclonal antibodies, fixed, and stained with FITC-conjugated secondary antibodies to reveal surface localized HA-tagged proteins. Because HA epitopes were added to the N-terminus of all p10 constructs, positive surface staining also indicated the correct type I orientation. Surface fluorescence of cells transfected with different p10 variants was captured at 20 X (A) or 40 X (B) magnification.

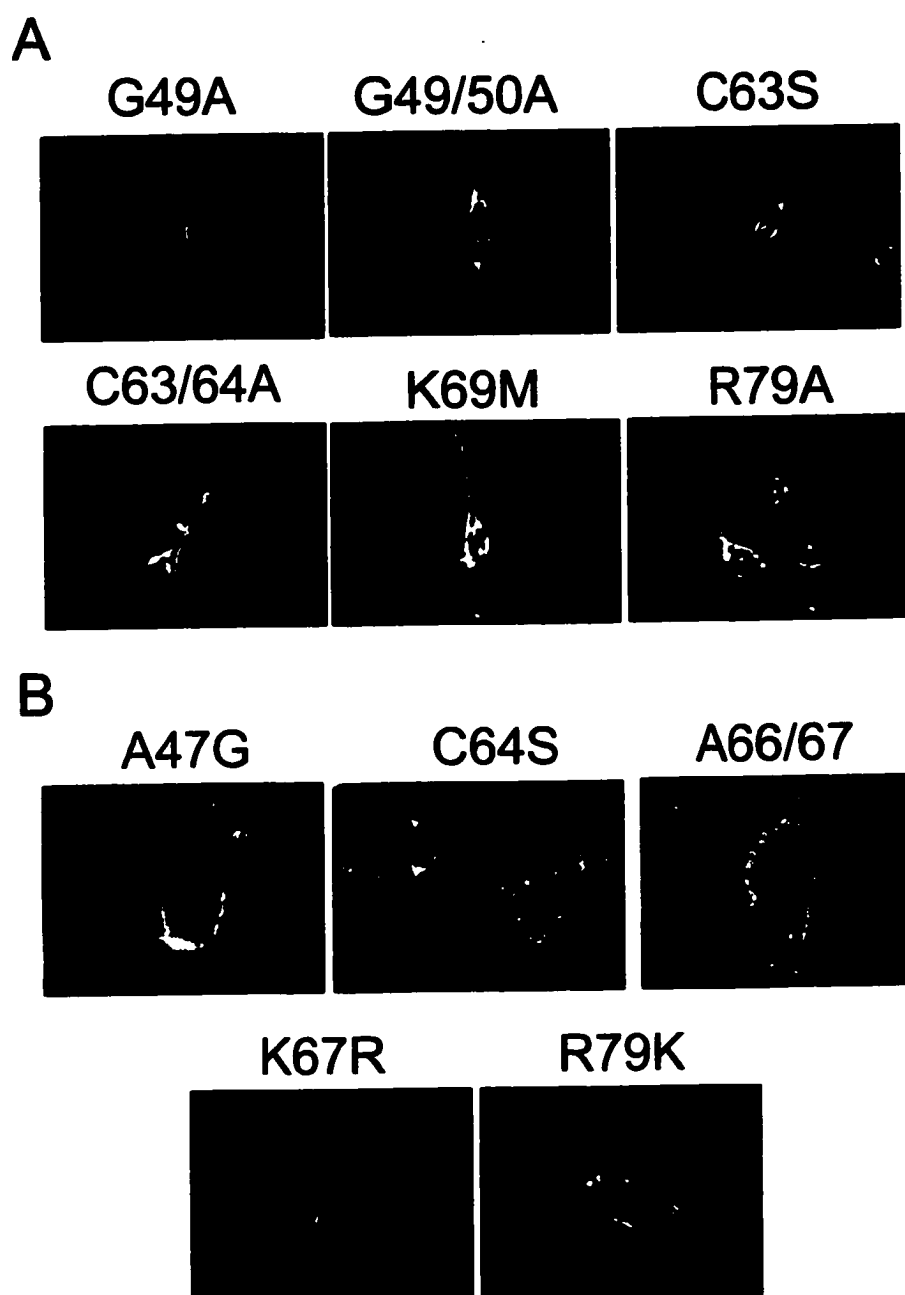
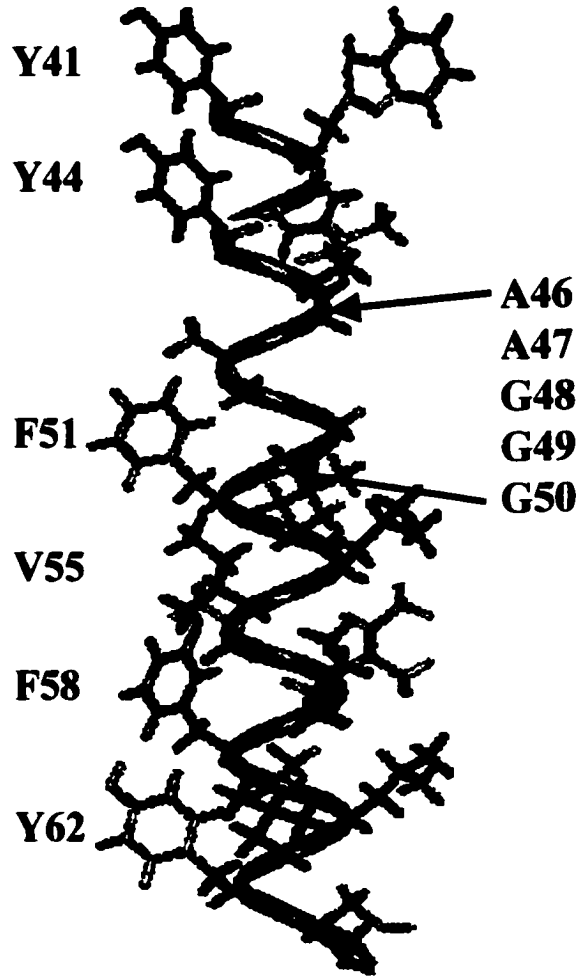


Figure 4.4

Figure 4.5. Model of the p10 transmembrane domain and the proposed mode of membrane perturbation. (a) An illustration depicting the α -helical transmembrane domain shows that aromatic groups (indicated at left of helix) line up on a single face. Furthermore, the gap created by the stretch of small, flexible glycine residues (indicated at right of helix) is evident. (B) Predicted local changes to the lipid organization by the triglycine motif is shown. Increased mobility of fatty acid chains is predicted to promote the negative curvature of the outer leaflet necessary for creation of the hemifusion intermediate.

A



B

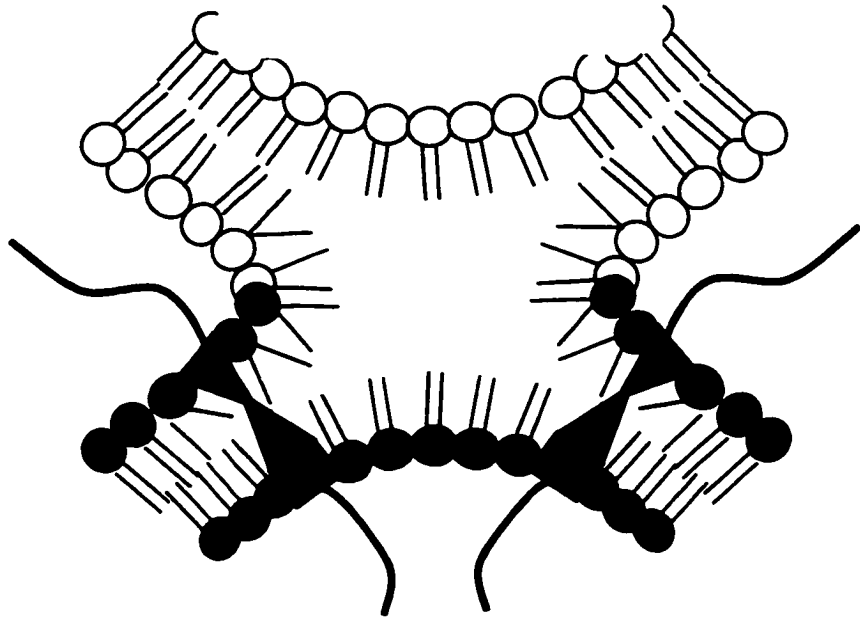


Figure 4.5

Figure 4.6. Substitution analysis of the conserved membrane-proximal cysteine residues suggests their involvement in fusion. The identities and exact locations of specific substitutions are indicated using the single letter amino acid code to indicate the identity of the authentic residue (s), its position, and the identity of substituted amino acid. All constructs contained two N-terminal HA epitopes (red box) and their sequence was confirmed. The modified proteins were transfected into cells and their fusogenic activity assessed by visualization of syncytium formation at 24, 48, and 60 hours post-transfection. Expression of proteins was confirmed by immunostaining and immunoprecipitation.

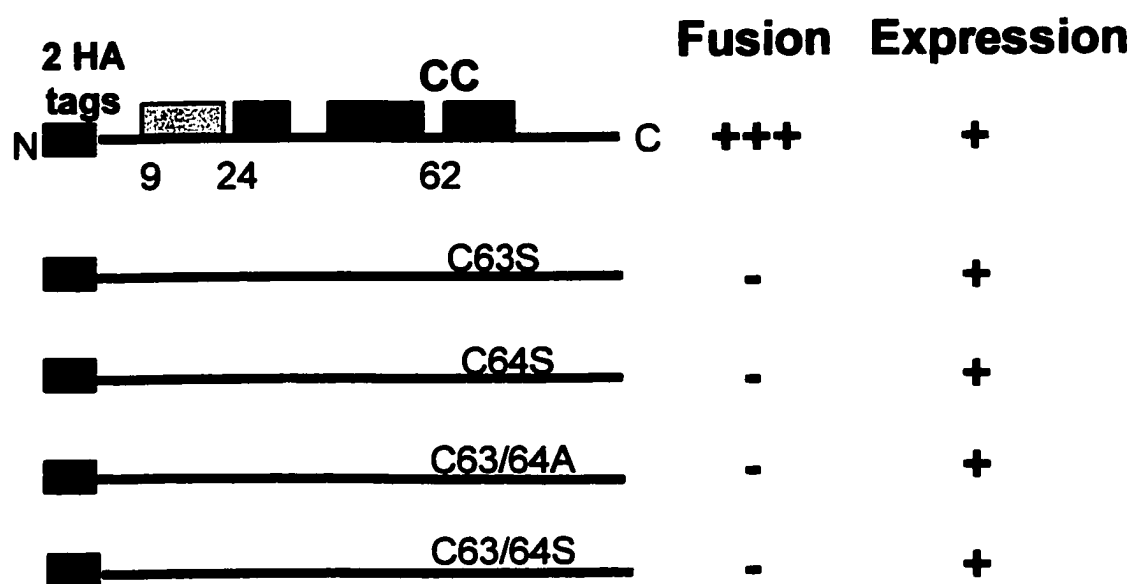


Figure 4.6

Figure 4.7. The membrane-proximal cysteines necessary for p10 induced fusion are palmitoylated. (A) Transfected cells were labeled with [³H]- palmitic acid or leucine as indicated in materials and methods. Immunoprecipitated proteins were analysed by SDS-PAGE (15%) and fluorography. (B) Palmitoylation of both cysteines was confirmed by assessing the palmitoylation of p10 proteins containing various cysteine mutations.

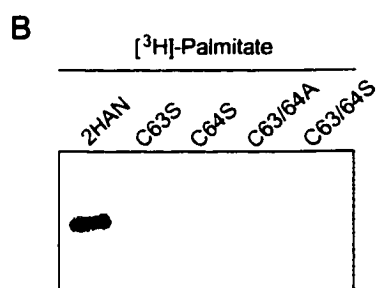
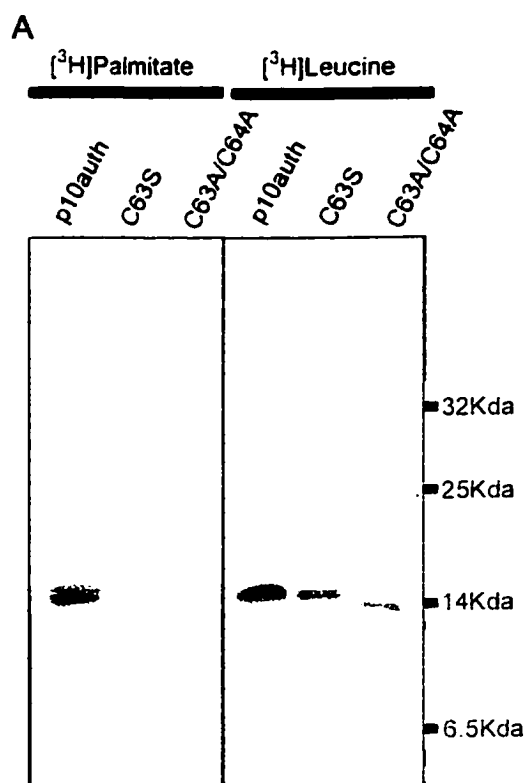


Figure 4.7

Figure 4.8. Loss of palmitoylated cysteines does not effect membrane insertion and protein stability of p10. (a). Soluble (sol.), peripheral (per.) and integral (int.) proteins were separated by cell fractionation under conditions that remove peripheral proteins. Each fraction was immunoprecipitated to determine the location of 2HAN or cysteine-substituted p10 following SDS-PAGE analysis. (B) Degradation of 2HAN or cysteine-substituted p10 proteins was monitored in transfected cells by pulse-chase analysis. Following 10 minutes incubation with [³⁵S]-methionine, cells were chased for 0, 30, 60, or 120 minutes as indicated, followed by immunoprecipitation and gel electrophoresis.

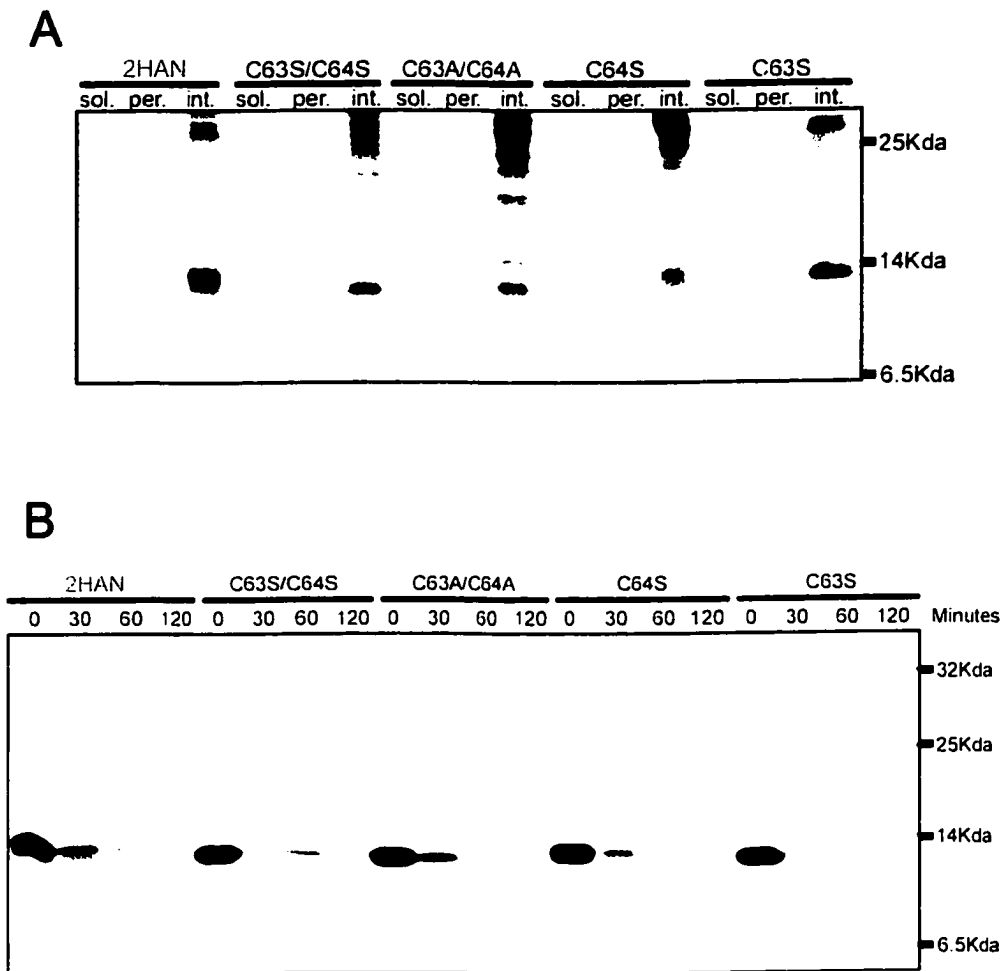


Figure 4.8

Figure 4.9. P10 association with detergent-insoluble glycolipid-rich membranes is independent of palmitoylation. Cells transfected with 2HAN with or without various cysteine substitutions were labeled with [³⁵S]-methionine and extracted with 1% Triton X-100 at 4°C. The lysates were subjected to equilibrium centrifugation through a discontinuous sucrose gradient and 10 fractions were collected from the top and labeled 1 to 10. Fraction 10 is the bottom fraction, while fraction 1 corresponds to the top of the gradient. Detergent soluble proteins remain within the 40% sucrose fractions (8-10) while DIG-associated proteins float through the 30% sucrose layer (4-7) towards the 5%-30% sucrose interface. Immunoprecipitation with anti-HA monoclonal antibodies and electrophoresis permitted detection of HA-tagged authentic and mutated p10 proteins.

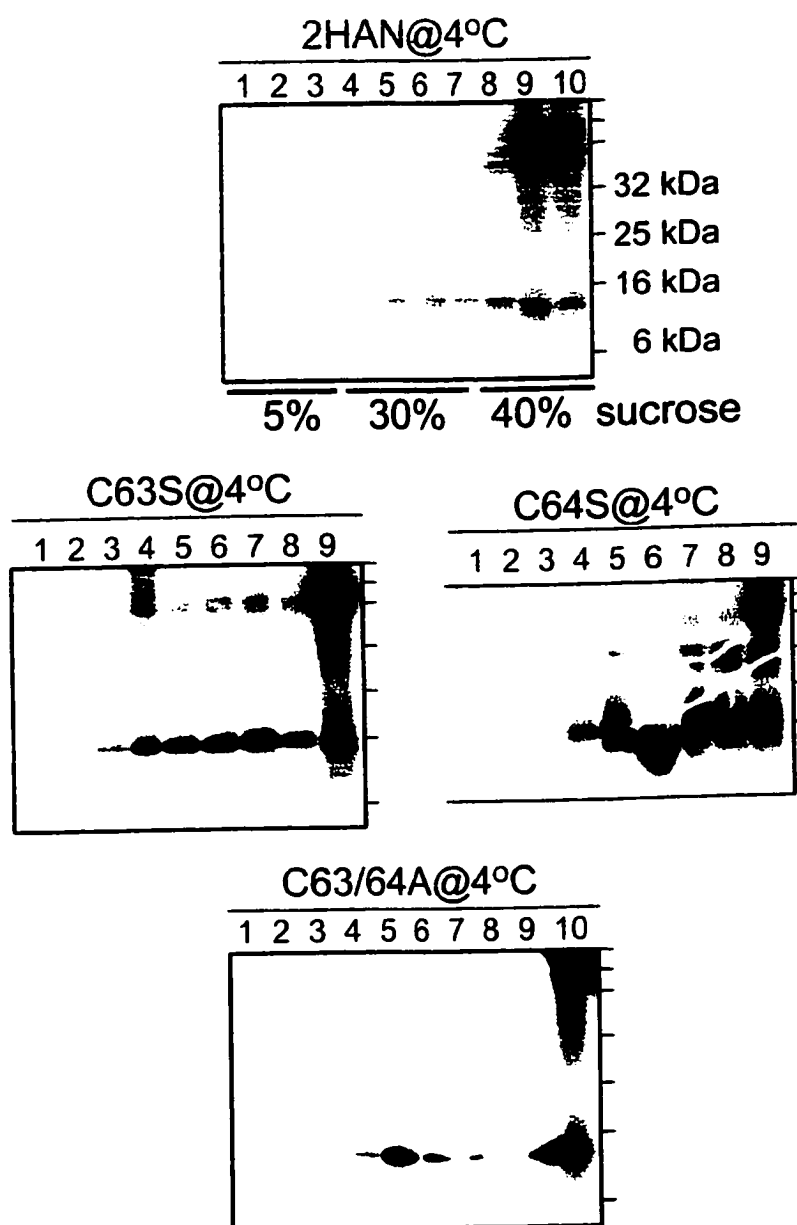


Figure 4.9

Figure 4.10. Substitution of basic residues in the cytoplasmic tail of ARV p10 suggests their involvement in fusion. The identities and exact locations of specific substitutions are indicated using the single letter amino acid code to indicate the identity of the authentic residue (s), its position, and the identity of substituted amino acid. All constructs contained two N-terminal HA epitopes (red box) and their sequence was confirmed. The modified proteins were transfected into cells and their fusogenic activity assessed by visualization of syncytium formation at 24, 48, and 60 hours post-transfection. Expression of proteins was confirmed by immunostaining and immunoprecipitation.

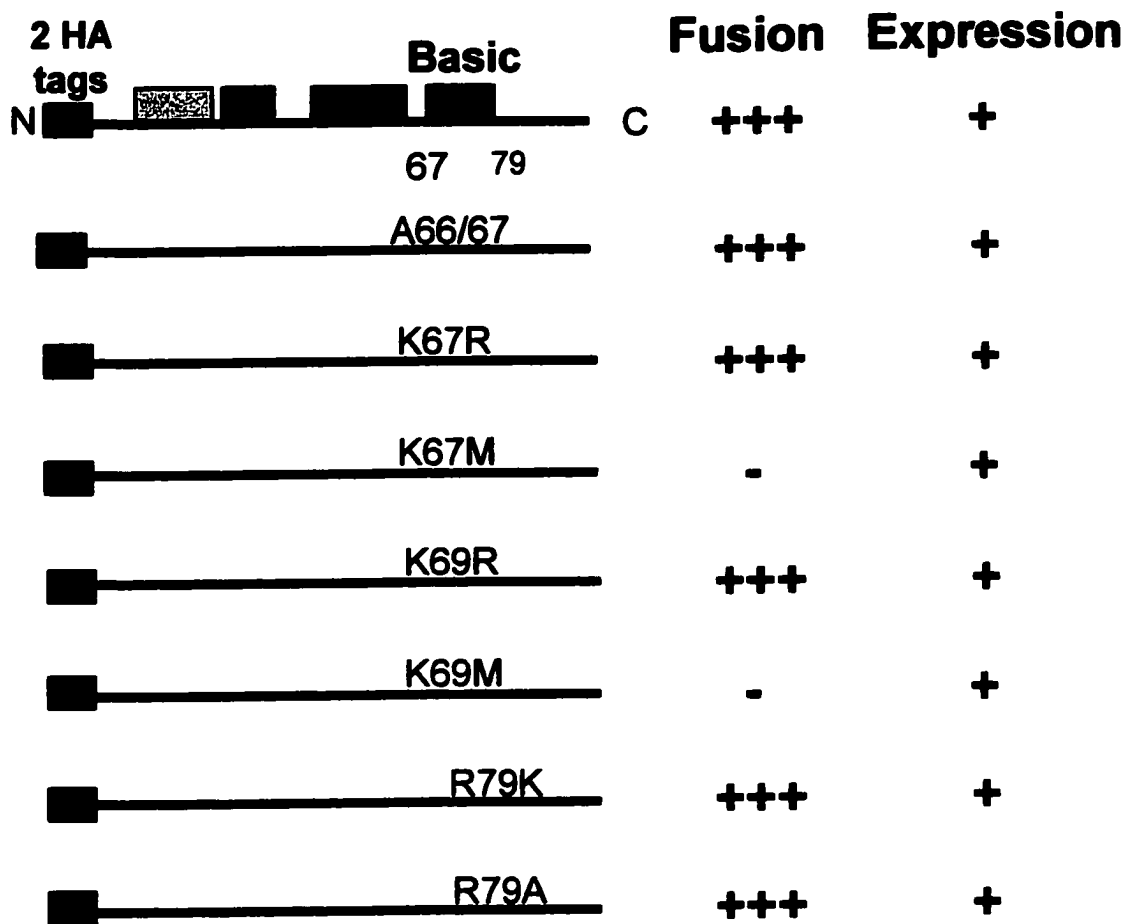


Figure 4.10

Figure 4.11. Models that depict how basic residues aligned in either an α -helix or β -strand interact with inner leaflet lipids to favor membrane fusion.

(A) Cartoon depictions of the basic domains of ARV p10, ARV p10 containing an alanine insertion at position 66 (A66/67), and NBV p10 modeled as an α -helix or β -strand. The locations of nonpolar, cationic and other polar residues is indicated by coloring according to the legend. (B) Cartoon demonstrating how interactions between basic residues of ARV p10 could impact the lipid bilayer. As either an α -helix or β -strand, interactions near the surface of the inner leaflet would induce fusion-promoting curvature by increasing the lipid head group volume.

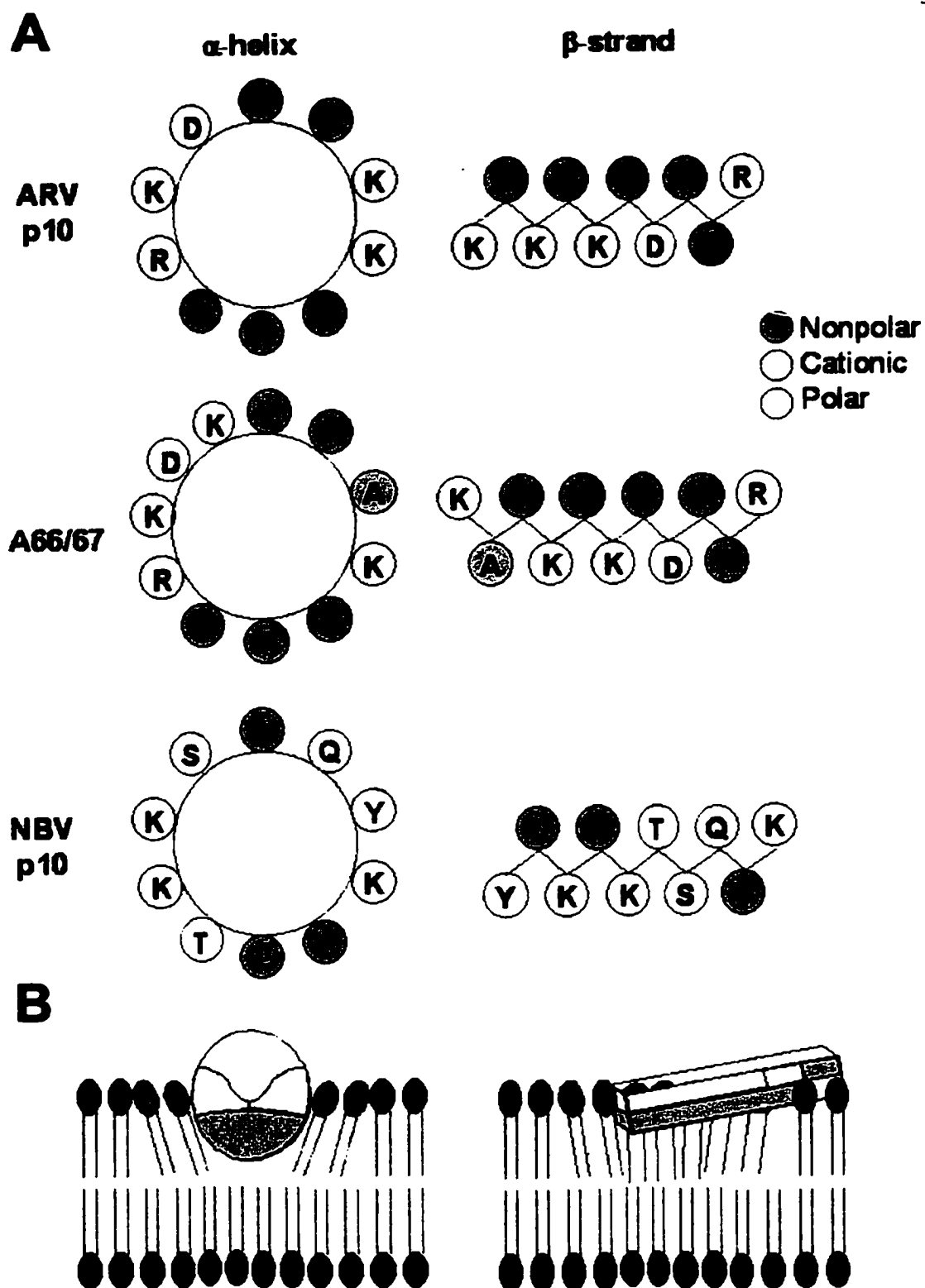


Figure 4.11

Figure 4.12. Progressive additions of HA epitopes to the C-terminus of p10 reduce fusogenic activity. Thirty-six hours following transfection with 1HAC, 2HAC, 3HAC, or 4HAC, cells were labeled with [³⁵S]-methionine and immunoprecipitated with p10- or HA-specific antibodies to ensure protein expression (A). Transfected cells were methanol fixed at 24, 36, 48, or 62 hours post-transfection and immunohistochemical staining with antibodies specific to the HA epitope was performed to visualize the presence, or absence of cell-cell fusion (B). Three arrows denote the perimeter of multinucleated syncytium, while 1 arrow indicates a single, positively stained cell.

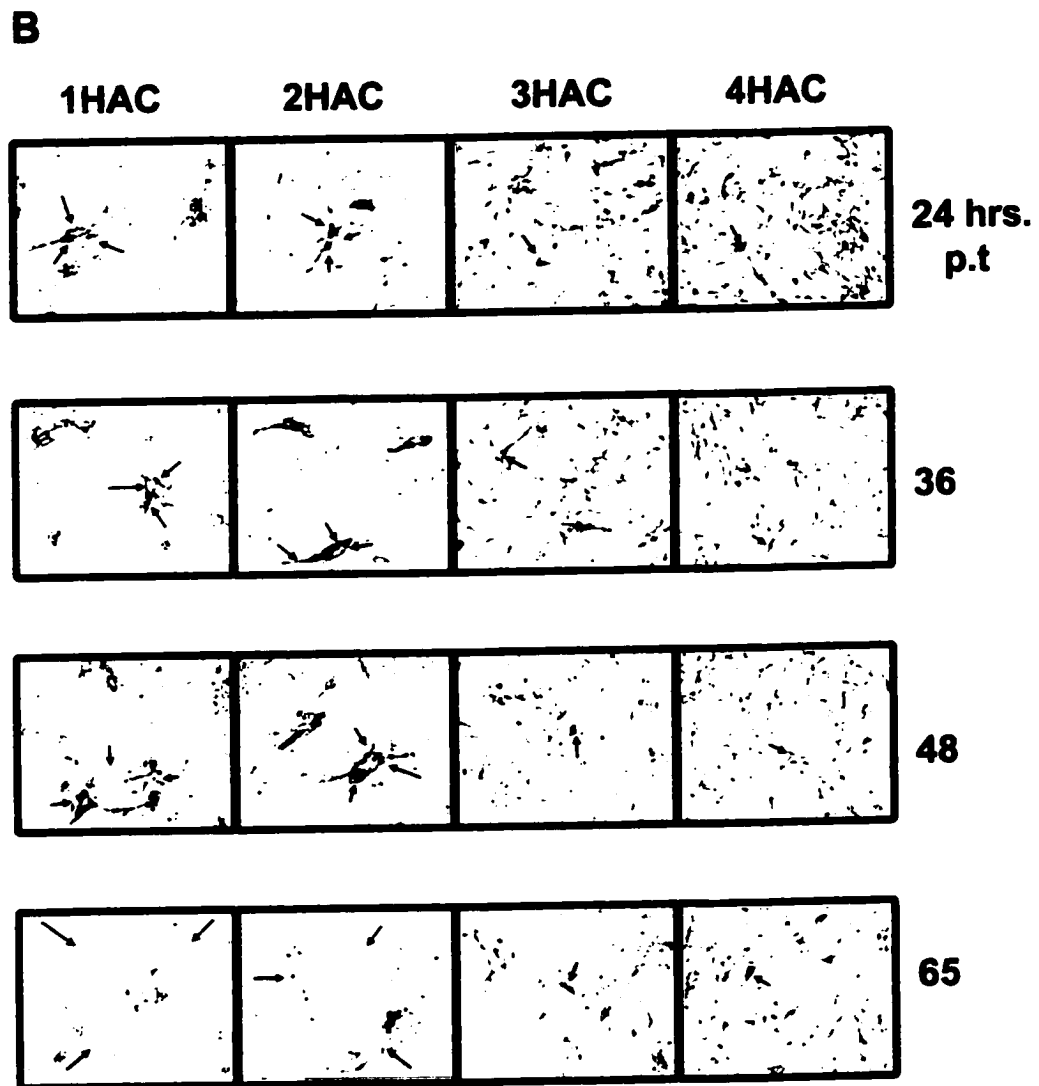
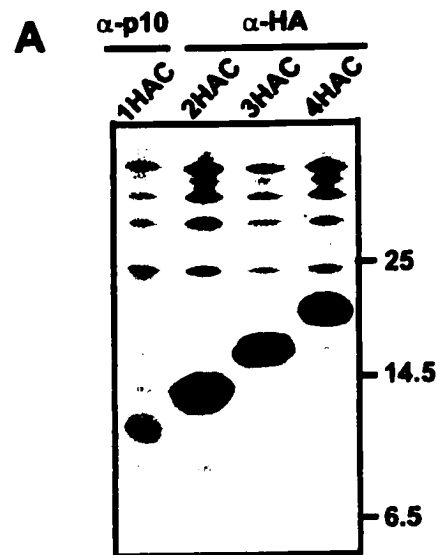


Figure 4.12

4.F. MATERIALS AND METHODS NOT PREVIOUSLY DESCRIBED

Palmitoylation assay

Thirty six hours post-transfection, six million QM5 cells transfected with HA-tagged p10 (2HAN) or cysteine mutants were prelabeled with MEM media in the absence of serum for 30 minutes. 500 μ Ci of [³H]-palmitic acid was dried under nitrogen, resuspended in 20 μ l of DMSO, diluted to 2 ml in MEM media devoid of serum, and added to cells for one hour. Note that recent evidence of rapid degradation of p10 suggests that future labeling experiments should either be done for only 30 minutes or in the presence of proteasome inhibitors to increase the yield of radioactive p10. Cells were lysed and immunoprecipitated with mouse monoclonal antibodies to the HA epitope as previously described. Proteins were diluted in protein sample buffer without reducing agents and were not heated prior to loading on 15% SDS-PAGE gels to avoid cleavage of the labile thioester bond. Fluorography with PPO-DMSO was used to enhance the signal of tritium. In parallel, a duplicate flask of transfected cells was pre-incubated with leucine free media, labelled with 100 μ Ci/ml [3H]-leucine diluted in leucine minus media, and processed similarly.

Isolation of detergent insoluble glycolipid-rich membranes (DIGs)

Six million QM5 cells transfected with HA-tagged p10 (2HAN) or cysteine mutants were labeled with [35S]-methionine (75 μ Ci/ml) in methionine free media for 1 hour at 32 hours post transfection. Cells were washed with PBS and placed on ice. Seven hundred and fifty microliters of cold 1% Triton X-100 in MNE

buffer (25 mM MES pH 6.5, 150 mM NaCl, 5 mM EDTA) and protease inhibitors (aprotinin, leupeptin and pepstatin) was added to cells on ice and incubated for 5 minutes. Cells were scraped, passed through a 30-gauge insulin syringe ten times on ice, and mixed with an equal volume of cold 80% sucrose/MNE. Solutions of 30% sucrose/MNE and 5% sucrose/MNE were layered on top of the sample (1.5 ml each) followed by high speed centrifugation (200 000 X g) for 18 hours. Ten consecutive fractions were carefully removed from the top, mixed with equal volume of 2 X lysis buffer and subjected to immunoprecipitation and gel electrophoresis as described previously.

CHAPTER 5. Multiple strategies substantiate that the avian reovirus encoded p10 is the first viral fusion protein to exist in non-oligomeric structures.

5.A. ABSTRACT

Multiple strategies suggest that unlike the membrane fusion inducing proteins of enveloped viruses, the p10 fusion protein encoded by the nonenveloped avian reovirus (ARV) does not form stable multimeric structures. Coexpression experiments demonstrated that an assortment of mutated, nonfunctional p10 proteins did not impose transdominant negative effects on the function of authentic p10. If p10 formed homo-multimers, transdominant negative phenotypes would be expected following nonfunctional heteromultimer formation between authentic and mutated p10. Furthermore, coprecipitation analysis failed to show association between p10 molecules using multiple nonionic detergents and long chase periods sufficient for the formation of slowly assembled oligomers. The possibility that p10 multimers were not stable in the presence of detergent was discounted using chemical cross-linking reagents, which also failed to reveal p10 oligomerization. Higher molecular weight species of p10 were absent from cells or membranes treated with several cross-linking reagents that varied in spacer arm, membrane permeability, and reactive group preference. P10 is the first viral fusion protein proposed to exist in a monomeric state.

5.B. INTRODUCTION

Oligomeric structures are common to the fusion proteins of enveloped viruses.

The formation of homo-trimers was first conclusively demonstrated for the most extensively studied fusion protein of influenza (HA) in the early 1980's using X-ray crystallography (Wilson et al., 1981). Fusion glycoprotein oligomeric structures for members of many other enveloped virus families have subsequently been decisively demonstrated. Fusion proteins of Semliki forest virus (togavirus), vesicular stomatitis virus (rhabdovirus), HIV (retrovirus) and simian virus 5 (paramyxovirus) all form similar core trimer structures (Baker et al., 1999; Chen et al., 1999; Chan et al., 1997; Fass et al., 1996; Weissenhorn et al., 1997; Caffrey et al., 1998; Russel et al., 1994; Gibbons et al., 2000). Multimers between fusion glycoproteins can be stabilized by covalent or non-covalent interactions. The human immunodeficiency virus (HIV) fusion glycoprotein (gp120), as an example, forms non-covalently linked oligomeric structures (Weiss et al., 1990; Earl et al., 1990), while the trimeric fusion protein of baculovirus (GP64) is stabilized by intermolecular disulphide bonds (Volkmand and Goldsmith, 1984; Monsma and Blissard, 1995).

The influenza HA fusion proteins form sodium dodecylsulfate (SDS)-resistant trimers in the inactivate state (Laver and Valentine, 1969), but also following conformational changes triggered by low pH (Doms and Helenius, 1980). The oligomeric structure is therefore required throughout the fusion reaction. Members of the *Flaviviridae* and *Alphaviridae* families such as Sindbis virus,

Semliki Forest virus, tick-borne encephalitis virus and Ross River virus have two or more different oligomeric structures depending on their state of activation. Sedimentation analysis, cross-linking studies, X-ray crystallography and antibody recognition analysis have been used to determine the shift from dimer to trimer for these viral glycoproteins. The inactive state consists of mature metastable dimers. Upon triggering by low pH or receptor binding, protein dimers undergo a quick transition to monomeric form followed by the formation of thermostable trimers (Rey, 2000; Heinz, 2000). Although the specifics of oligomer formation differs among the fusion proteins of enveloped proteins, the formation of higher-order oligomers is a feature common to them all.

The proper folding and formation of the correct oligomeric state is necessary for the transport of viral fusion proteins to the surface of cells (Doms et al., 1993). The fusion activity of enveloped virus fusion proteins, however, also depends on the correct formation of homo-oligomers (Russel et al., 1994; Fass et al., 1996; Chan et al., 1997). For human parainfluenza, the proper assembly of the envelope glycoproteins into trimers has been demonstrated to be essential for biological function (Doms et al., 1993; Takimoto et al., 1992). The oligomeric structures of these fusion proteins are closely linked to their function by permitting the correct structural changes required for activation. As an example, it is thought that the fusion peptides responsible for interacting with, and destabilizing, membranes are primarily buried for all enveloped virus fusion proteins. Only after the correct triggers are encountered that favor the fusion

between the virus and cell are the fusion peptides exposed and active in promoting fusion. The exposure of fusion peptides necessitates conformational change. For alphaviruses and flaviviruses, the fusion peptide initially buried at the interface of the dimer molecules is placed at the apex of the newly formed trimers. For fusion proteins that initiate in the trimeric conformation such as HA and gp41 of influenza and HIV, respectively, low pH or receptor binding result in a re-shuffling of coiled-coil interactions that move the fusion peptide from its initially buried location towards the opposing membrane, subsequent conformational changes pull the N-terminal fusion peptide and transmembrane domains into close proximity. In addition to imposing correct re-folding events, multimer formation of enveloped virus fusion proteins may be involved directly in the final stages of fusion by allowing cooperative activity between multimer subunits. Clearly, preventing the formation of oligomers for these fusion proteins would impose gross changes to both structure and function.

The fusion proteins of enveloped viruses have *evolved* to function as multimers. The tertiary structure of these proteins clearly restricts their functionality as monomers. Fusion proteins play a pivotal role in the life cycle of enveloped viruses, ensuring timely entry into specific host cells. Enveloped virus fusion proteins require the optimal structures to permit rapid and efficient membrane fusion but only upon encountering the correct conditions. Have these fusion proteins evolved oligomeric structures because they are *absolutely essential* for their lipid bilayer fusion activity, or because they provide an efficient method of

conferring multiple, stabilized conformations that permit strict regulation of activity? Dissection of the structural requirements for fusion, as opposed to regulation, is extremely difficult for these highly evolved fusion proteins.

The p10 fusion proteins encoded by the nonenveloped avian and Nelson Bay reoviruses share some common features with the fusion proteins of enveloped viruses. The p10 proteins are type I integral membrane proteins present on the surface of cells where they induce membrane fusion. Previous analysis demonstrated that like other viral fusion proteins, p10 contains a fusion peptide within the ectodomain. In contrast, however, the p10 proteins are non-structural accessory proteins that are not necessary for viral entry or exit from cells. Because the p10 proteins are only produced and functional in viral infected cells, infection-dependent expression could provide the means of regulation in the absence of additional triggers. The absence of need for strict regulation and rapid function by p10 fusion proteins may explain their small size. The p10 proteins are significantly smaller than other viral fusion proteins, containing only 40 amino acids within the ectodomain. The small molecular size of p10 argues against the presence of complex conformational changes similar to those found in enveloped virus fusion proteins. Also, rapid degradation of p10 was shown to be dependant on the hydrophobicity of the fusion peptide, suggesting that the fusion peptide may not be buried in the primary conformation. Unlike the fusion proteins of enveloped viruses whose primary function is to induce rapid and specific membrane fusion between viral envelope and cell membranes for entry,

the p10 proteins mediate the membrane merger of infected and neighboring cells over the course of infection. Although p10 and the fusion proteins of enveloped viruses have evolved to serve the same final function, membrane fusion, they clearly function under different limitations. We were therefore interested in determining whether the function of p10 also requires formation of higher-order oligomers.

5.C. RESULTS

5.C.1. Fusion-deficient p10 mutants do not have a transdominant effect over authentic p10, and cannot complement the defects of other coexpressed mutants.

Mixed oligomers consisting of both mutated and authentic proteins are often nonfunctional. The transdominant inhibitory activity of the mutated protein can be a result of structural changes to the multimer or the requirement for cooperation between functional subunits for activity. Coexpression experiments have been used to identify oligomeric structures for the fusion proteins of enveloped viruses. In these experiments, the mutated enveloped virus glycoproteins dominantly interfered with the function of the wild-type glycoprotein and indicated that formation of oligomeric structures is necessary for membrane fusion activity. Cells infected with HIV-1 containing mutations or deletions in the N-terminal fusion peptide sequence of the transmembrane glycoprotein (gp41) dominantly interfered with syncytium formation and infectivity of wild-type virus demonstrating that mixed oligomers of gp41 exist and are incapable of inducing membrane fusion (Freed et al., 1992; Schaal et al., 1995). Deletion of the CD4 binding sequence or mutations made to the leucine zipper-like heptad repeats of the HIV-2 glycoprotein also produced a transdominant nonfunctional phenotype (Stefy and Wong-Staal, 1993; Chen et al., 1998). Similarly, the Newcastle disease virus fusion glycoprotein containing mutations that prevented proper cleavage had dominant negative effects if co-expressed with the authentic

glycoprotein with respects to syncytium formation activity, as expected for oligomeric proteins (Li et al., 1998). The positive-dominant inhibition effect of coexpressed mutants confirmed the requirement of multimers for the function of these enveloped virus fusion glycoproteins.

There are several advantages of using the transdominant effect for the study of oligomeric structures. For membrane proteins that form noncovalently attached multimers, purification conditions can disturb oligomeric structures. Coexpression experiments provide a functional assay for assessing the interactions between multimer subunits under biological conditions where they are favored. In addition, using statistical analysis, the proportion of homo- and hetero-multimers can be estimated depending on the fraction of each molecule (mutated or authentic) that is transfected into the cells and the number of subunits within the multimer. By varying the ratio of mutant to authentic molecules transfected, and measuring the proportion of functional oligomers formed through a functional assay, it is possible to calculate the number of subunits within the oligomer. Using such statistical analysis, Li et al. (1998) showed that the Newcastle disease virus fusion glycoprotein formed a trimeric structure within cells.

In an attempt to determine whether the ARV p10 protein functions as a multimer, and to estimate the size of such multimers, we performed co-expression experiments. Previous studies demonstrated that p10 containing three additional

HA epitopes at the C-terminus was non-functional. We therefore transfected both p10opt (the authentic p10 with an optimized translation start site) and 3HAC (p10opt with three HA epitopes) at various ratios and assessed the effects of 3HAC on the activity of p10opt molecules. A third construct (called 'other' in figure 5.1) unrelated to p10 function was also transfected into cells to produce a constant quantity of DNA used for transfection and ensure that transfection conditions were identical. Figure 5.1A summarizes the quantity of p10opt, 3HAC, and 'other' DNA used for transfection. To ensure that changes in the quantity of DNA construct transfected correlated with protein expression levels, we monitored the expression of 3HAC, which is most easily detectable using HA-specific antibodies. When radiolabeled 3HAC from transfected cells was immunoprecipitated and analyzed by SDS-PAGE, the intensity of protein band (s) clearly corresponded with the quantity of 3HAC-encoding construct transfected (Figure 5.1B). Experimental wells transfected with the same proportion of 3HAC-encoding plasmids produced identical p10 expression levels (lanes 5-9) while increasing the proportion of 3HAC DNA resulted in increased protein expression (lanes 1-5).

When syncytium formation was assessed, the addition of increasing amounts of 3HAC relative to p10opt had no effect on the fusion-inducing ability of p10opt (Figure 5.1C, first row). Because transfected cells were stained with HA-specific antibodies, cells transfected with only p10opt did not show positive staining, although visualization of syncytium confirmed that they were the same size as

the remaining wells containing an equal amount of p10opt. The finding that the activity of p10opt was not changed in the presence of increasing amounts of non-functional 3HAC suggested that non-functional mixed oligomers did not form. When the amount of 3HAC was held constant, in the presence of increasing amounts of transfected p10opt, syncytium formation correlated with the quantity of p10opt, again suggesting that p10opt functioned independent of 3HAC. Overall, the expression of p10opt, but not 3HAC, correlated with the membrane fusion activity of p10 and suggested that 3HAC did not produce a dominant negative effect.

The inability of 3HAC to inhibit the activity of authentic p10 may have been unique. The gross addition to the C-terminus may have prevented the formation of mixed oligomers. We therefore performed analogous studies using other p10 constructs containing mutations throughout the protein. Mutations in the ectodomain (C9S, A14/15, V15M, C21S, G29A, L32A), the transmembrane domain (G49/50A), the palmitoylation site (C63S), and the intracellular basic domain (K67M) of p10 were all previously characterized to be deficient in membrane fusion activity. All of these mutants, however, do integrate into the cell membrane and would therefore be within the endoplasmic membrane where oligomerization occurs. Except for G29A and L32A, all mutants were also known to be cell surface localized in the correct type I (N-terminus out) orientation. The two HA epitopes present at the N-terminus of these mutants did not interfere with protein function when added to authentic p10 (2HAN) and therefore are not

expected to interfere with multimerization if it is essential for function. When only 2HAN was transfected into cells, large syncytium were visible at 36 hours post-transfection (Figure 5.2, shaded boxes). When mutated p10 proteins were co-transfected with 2HAN at a 1:1 ratio, the syncytium size resembled that of 2HAN cotransfected with mock DNA (Figure 5.2, shaded boxes). Again, these results demonstrated that p10 functioned independently of co-transfected nonfunctional p10 constructs. If p10 did require dimer formation for function, then only 25% of the dimers within a 1:1 cotransfection experiment would be homomultimers of p10opt and therefore only 25% of the original syncytium activity should be expected. If p10 requires trimer formation, only 12.5% of the p10 activity should have persisted in these cotransfection experiments. The inability to detect any changes to the activity of p10 in the presence of cotransfected altered p10 proteins clearly indicates an absence of dominant negative effects. Given the large panel of mutants used for the coexpression assay, the speculation that altered p10 proteins were unable to hetero-oligomerize and continued to form only nonfunctional homo-multimers is unlikely.

If p10 molecules do, in fact, form multimers but function independently within the oligomeric structure, then complementation between p10 molecules carrying substitutions in different domains may be seen. For example, if one molecule is deficient in destabilization of the donor membrane inner leaflet, while the other is unable to destabilize the target membrane, than a heterodimer composed of these two altered molecules may succeed in the fusion process. For p10,

destabilization of the donor membrane inner leaflet is likely attributed to the transmembrane and cytoplasmic domains, while destabilization of the target membrane is proposed to be a consequence of the fusion peptide present within the ectodomain of p10. A mixed oligomer composed of p10 bearing mutations within the intracellular or transmembrane domain and within the ectodomain may successfully destabilize the two membranes and therefore retain fusogenic function. When the panel of p10 mutants were cotransfected with each other in various combinations, transcomplementation between mutants was not seen (Figure 5.2, unshaded boxes). These coexpression experiments aimed at finding dominant negative or complementary mutations suggest that p10 molecules act independently for function. These experiments alone, however, cannot conclusively eliminate the possibility that independent p10 molecules do reside within a larger oligomeric structure.

5.C.2. Co-precipitation failed to show multimer formation between p10 molecules.

Another method to identify multimeric structures is to lyse cells under mild conditions and determine whether molecules within the complex coprecipitate with one another. Many fusion proteins of enveloped viruses are resistant to solubilization with nonionic detergents. The Hepatitis C virus glycoprotein (E1 and E2) and the human parainfluenza F protein multimers are stable in 0.5% NP40 (Dubuisson et al., 1994; Tong and Compans, 2000), while the Rous sarcoma virus envelope protein oligomers remain stable in octyl- β -D-glucoside

(Einfeld and Hunter, 1997) and the vesicular stomatitis virus envelope glycoprotein (G) multimers remain intact within solutions containing 0.5% TritonX-100 (Doms et al., 1987).

Coprecipitation analysis is only useful if molecules within the multimer can be resolved and distinguished from each other by SDS-PAGE analysis and if antibodies are specific to only one of the multimer subunits. Receptor-ligand interactions are, therefore, commonly demonstrated using coprecipitation experiments, such as the interactions between the measles virus hemagglutinin protein and the CD46 receptor (Nussbaum et al., 1995). Similarly, the dimeric glycoproteins of hepatitis C virus (E1 and E2), varicella-zoster virus (gpl and gpIV), and rubella virus (E1 and E2) consist of two different molecules and can therefore be analyzed by coprecipitation with antibodies to only one of the dimer subunits (Dubuisson et al., 1994; Hobman et al., 1993; Yao et al., 1993). In order to use coprecipitation for analysis of p10, we coexpressed authentic p10 and 2HAN. Both p10opt and 2HAN are fusion competent and, therefore, must be capable of forming multimeric structures if essential for p10 structure or function. 2HAN and p10opt could easily be distinguished by molecular weight following SDS-PAGE analysis. Furthermore, monoclonal antibodies to HA specifically bind 2HAN but not p10opt and can be used to identify p10opt-2HAN interactions by coprecipitation.

Cell lysis and immunoprecipitation was completed using three different detergent environments; 0.1% NP40, 0.1% Triton x-100, or RIPA (1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS). P10-specific antibodies that recognize both p10opt and 2HAN showed that transfected cells coexpressed both species of p10, as expected (Figure 5.3B). Although HA-specific antibodies clearly bound 2HAN in 0.1% NP40 or Triton x-100 as indicated by the intense bands corresponding to 2HAN under these conditions, p10opt did not coprecipitate with 2HAN (Figure 5.3B). Similar results were demonstrated in cells cotransfected with p10 containing two HA epitopes at the C-terminus (2HAC) and p10opt (Figure 5.3A). The inability of p10opt to coprecipitate with 2HAN and 2HAC suggests that multimers are not formed, or that they are unstable under the solubilization conditions used.

Several other solubilization buffers were assessed in order to address the possibility that p10 multimers were unstable when mild and nonionic detergents disrupt cells. P10opt did not coprecipitate with 2HAN when Tween 20 (0.1%), or octyl- β -D-glucoside (0.5%) was used to solubilize transfected cells, (Figure 5.4A). In addition, reduction of the final concentration of Triton X-100 to 0.02% did not stabilize potential p10 multimers (Figure 5.4B). To increase the likelihood of p10opt coprecipitation with 2HAN, the p10opt :2HAN transfection ratio was increased. Even at a three-fold excess of p10opt over 2HAN, p10opt did not coprecipitate with anti-HA antibodies (Figure 5.4B). Although the coprecipitation analysis could be tested using other mild detergents such as 1% Brij97 (Lapham

et al., 1996), the already exhaustive search for conditions that permit coprecipitation between p10 molecules supports that p10 may exist as monomers within cells.

The time required for oligomerization of proteins varies amongst different enveloped virus fusion proteins. The human parainfluenza and vesicular stomatitis viral fusion proteins (F and G, respectively) were shown to form trimers by 10 minutes (Tong and Compans, 2000; Doms et al., 1987). The $t_{1/2}$ for the assembly of HA into trimers was also found to be between 5 and 7 minutes (Copeland et al., 1986; Doms and Helenius, 1986; Gething et al., 1986). Other viral glycoproteins, however, require more time for correct folding and maturation that culminates with the formation of stable multimers. The envelope proteins of Rous sarcoma and hepatitis C viruses necessitate 1-2 hours for maximal complex formation (Einfeld and Hunter, 1997; Dubuisson et al., 1994). To ensure that the inability of p10opt to interact with 2HAN or 2HAC during coprecipitation experiments was not a consequence of insufficient time allowed for multimer formation, we reattempted coprecipitation of radiolabelled proteins following a 30, 60, or 120-minute chase (Figure 5). Rapid degradation of p10 due to the N-terminal hydrophobic fusion was previously demonstrated, and prevented detection of proteins following chase periods longer than 120 minutes. Two hours however, should be sufficient time for multimer formation. Coprecipitation was performed on cells solubilized with 0.1% NP40 (Figure 5.5A and B) or 0.1% saponin (Figure 5.5C). Cells transfected with only p10opt were

immunoprecipitated with HA-specific antibodies to ensure that no cross-reactivity occurred (Figure 5.5A, B, and C, lanes 1). Similar to previous coprecipitation results, although p10-specific antibodies recognized p10opt, 2HAN and 2HAC confirming their expression in cotransfected cells (lanes 2), p10opt was not coprecipitated with HA-specific antibodies (lanes 3) following the three chase periods. Even after very long exposures, no trace of p10opt was found associated with immunoprecipitated 2HAC or 2HAN (Figure 5.5, 30 and 60 minute chase periods).

5.C.3. Multimeric forms of p10 are not found following protein cross-linking reactions.

Addition of cross-linking reagents to stabilize protein-protein interactions through covalent bonds is a common technique used to identify oligomeric structures that may be unstable under cellular solubilization conditions. Identification of chemically cross-linked homomultimers is possible, as monomers, dimers, trimers, or tetramers can be easily distinguished by molecular weight through SDS-PAGE analysis. Cross-linking reagents have been used to detect and analyse oligomeric structures of many enveloped virus glycoproteins including those of HIV-1, human respiratory syncytial virus, and human parainfluenza virus (McInerney et al., 1998; Collins and Mottet, 1993; Tong and Compans, 1999; Russel et al., 1994; Anderson et al., 1992; Tong and Compans, 2000). Similarly, cross-linking reagents could stabilize the interactions between p10 molecules within homo-multimers and permit their detection if they exist.

Cross-linking analysis of p10 in transfected cells was attempted under several conditions. Multiple cross-linking reagents were used with variable reactive group preference and spacer arm distance to enhance the probability that p10 molecules within potential multimers could be cross-linked together (summarized in figure 5.6A). Furthermore, except for DSS, all reagents were membrane permeable and, therefore, could react towards residues in the ectoplasmic, transmembrane and cytoplasmic domains in p10. The membrane permeable reagents were used to increase the reactive group availability, but also to account for the possibility that the transmembrane or cytoplasmic domains of p10 are involved in protein-protein interactions. Most importantly, the rapid degradation of p10 results in a very low surface localized pool that cannot be detected by surface immunoprecipitation and would, therefore, not be sufficient for detection following cross-linking reactions. Membrane permeable reagents would access and modify p10 proteins within the cell prior to degradation, thereby increasing the protein yield. Except for DSS, all reagents were also cleavable by differential treatments that would allow dissociation of multimers into monomers. If any high molecular weight products were found after treatment with these cross-linking reagents, cleavage would restore the original monomeric molecular weight corresponding to the size of p10 and confirm that the new species are composed of homomultimers of p10. Lastly, cross-linking reagents were added to live cells while still adherent, to live cells in suspension to prevent cross-linking of cellular receptors with neighboring cells or coated plastic which

might produce aggregates that absorb p10, or to membranes containing p10 purified from cells in the absence of detergents (Figure 5.6B).

Cross-linking reactions failed to show that p10 forms dimer, trimer, or tetramer structures under all conditions tested (Figure 5.6B). DSP, EGS, DST, DSS and DTME, when added to suspended live cells, adherent live cells, or cellular membranes (lanes 2-6) did not produce any additional high molecular protein bands that were absent from the negative control devoid of cross-linking reagents (lane 8). Interestingly, bands that migrate similar to predicted molecular weights of multimeric p10 are present in all lanes. We assume that these are background bands and not highly stable p10 multimers because if the latter was correct, we would expect these bands to have higher intensities in the presence of multimer-stabilizing cross-linking reagents. Furthermore, the high molecular weight bands are of approximately equal intensity in almost all conditions despite the obvious differences in p10 immunoprecipitation following variable cross-linking conditions. The monomeric p10 was still present in most cases. The absence of monomeric p10 in some conditions (lanes 1-3 in membrane cross-linking) was a result of the formation of large aggregates that were removed prior to immunoprecipitation. The inability to demonstrate that p10 forms multimeric structures by cross-linking provides further evidence that p10 exists as monomers within cells.

5.D. DISCUSSION

Several independent lines of evidence implied that p10 does not function as a higher-order oligomer. Various nonfunctional p10 mutants did not exert a dominant negative effect on the activity of authentic p10 in cotransfection experiments. Furthermore, no complementation was found between p10 proteins deficient in function as a result of mutations in independent domains. Coprecipitation of two functional p10 proteins with distinguishable epitopes also did not reveal heteromultimer formation. Even when several detergents and prolonged chase periods were used to permit sufficient time for stable multimer formation, p10 multimerization was not evident. The use of several cross-linking reagents under different conditions also failed to reveal p10 multimers. These studies, together, support that p10 may function as a monomer to induce membrane fusion.

The inability of altered p10 constructs to exert a transdominant negative effect on coexpressed authentic p10 may suggest that p10 does not require multimer formation. Authentic p10 may function as an independent monomer and syncytium formation may correlate strictly with the abundance of functional p10 monomers within cells. Alternatively, if p10 is in fact a multimer, these results could be explained if the mixed oligomers of p10 remain functional. If multimer formation is essential for conferring the correct conformation to p10 but a single functional p10 molecule within the multimer is sufficient for fusion activity, then it would be expected that one of the many mutants would have sufficient alteration

to the structure of the multimer and therefore impose a dominant negative effect over the function of authentic p10.

The assay designed by Li et al (1996) could be attempted to ensure that heteromultimers between active and inactive molecules become inactive. Insertion of the lysosomal protease cathepsin B at the N- or C- termini of p10 would produce a non-functional monomer that would have dominant negative effects on the wild-type p10 if heteromultimers were formed. Proteolytic digestion of the heteromultimer and/ or lysosomal targeting and degradation would ensure that heteromultimers are nonfunctional.

We were unable to detect oligomerization between functional p10 constructs with variable size by coimmunoprecipitation analysis. We propose three possibilities for why p10_{opt} did not coprecipitated with 2HAN or 2HAC; (1) p10 functions as a monomer in cells and therefore oligomers will not be identified by any technique, (2) p10 cannot form heteromultimers and, therefore, only functional homomultimers form in cells coexpressing mutant and wild-type p10 proteins, or (3) p10 multimers are not stable under conditions used to solubilize cells. To address the second possibility, that p10 never forms heteromultimers, sucrose gradient centrifugation analysis of p10-expressing cells could determine whether different molecular weight species of p10 are formed. This technique is commonly used for enveloped viral fusion proteins that are composed of homomultimers and cannot be analyzed by coprecipitation unless new proteins

are created with unique epitopes. Rather than creating new protein subunits, high-speed centrifugation through sucrose gradients in the presence of mild detergents is often the preferred method of confirming the formation of oligomeric structures for these proteins. Analysis of p10 centrifuged through sucrose gradients could identify homomultimers. However, since sucrose gradient centrifugation experiments would require the use of detergents similar to those used in the coprecipitation analysis, it would also fail to detect p10 multimers if they are unstable under solubilization conditions.

Detergents could dissociate multimeric subunits if protein-protein interactions depend on the hydrophobic transmembrane domain. There is increasing evidence that the transmembrane domain of surface glycoproteins are involved in the formation and/or stabilization of oligomeric structures. Tetramer formation of the hemagglutinin-neuraminidase (HN) glycoproteins of Newcastle disease virus requires the transmembrane domain (McGinnes et al., 1993). The transmembrane domains of HIV and influenza virus fusion proteins also stabilize the oligomeric structures (Laver and Valentine, 1969; McInerney et al., 1998). Interactions between transmembrane helices are expected to be specific enough to ensure that proteins do not aggregate within membranes. For example, the transmembrane helices of human glycoporphin A or the Fc-gamma receptor of natural killer cells interact through a motif containing evenly spaced glycines that produce a glycine face within an α -helix (Bormann, 1989; Kurosaki et al., 1991). Since glycines are small residues, they permit more intimate contact between the

partnering helices (Lemmon and Engelman, 1994). Other motifs found within transmembrane domains involved in oligomeric interactions include a hepad-repeat of leucine residues or oppositely charged amino acids that form ionic bridges (Cosson et al., 1991). The transmembrane domain of p10 does not contain these motifs common to transmembrane domains that are involved in protein-protein interactions.

The presence of transmembrane involvement in oligomer formation does not necessarily make multimers susceptible to destabilization by detergent solubilization techniques. The Fc-gamma subunit of the T-cell and natural killer cell receptors were coprecipitated with remaining subunits in 0.12% Triton x-100/1% digitonin despite a dependence on transmembrane domain interactions (Kurosaki et al., 1991). Proper dimer assembly of MHC II molecule alpha and beta chains also requires transmembrane domain interactions that are stable during coprecipitation experiments using 0.5% Triton x-100 as the lysis buffer (Cosson and Bonifacino, 1992). We cannot exclude, however, that p10 multimers are dependent on transmembrane interactions, and that these interactions are highly susceptible to detergent disruption. For this reason, we used cross-linking reagents to stabilize protein-protein interactions.

A drawback to the cross-linking experiments was the absence of a true positive control for these reactions. The use of a nonrelated multimeric protein under the same assay conditions would confirm that the cross-linking conditions used were

effective. However, because proteins vary in size, structure, and availability of reactive groups, the ability to cross-link nonrelated proteins would not eliminate the possibility that p10 was not a target for the cross-linking reagents. Alternatively, reagents that modify the same reactive groups as the cross-linking reagents could be used to ensure that the reactive groups are available in p10 for modification. Biotin-conjugated chemicals that react to amino or silyfhydryl groups were also added to p10-transfected cells to ensure that these reactive groups were available for modification (Figure 5.6A and B, lanes 1 and 7). Unfortunately, the biotin-containing chemicals would only increase the size of p10 by 500daltons and therefore the presence of modified p10 was not visible by SDS-PAGE. In future experiments, the p10 conjugated with membrane permeable biotinylation reagents could be detected by western blot analysis or double immunoprecipitation with streptavidin-agarose and HA-specific antibodies to ensure that p10 is modified by these reagents. The use of water insoluble biotin-conjugated reagents capable of reacting with intracellular p10 will increase the yield of immunoprecipitated protein. Demonstrating that the cross-linking reagents function on other multimeric proteins and that p10 does contain reactive groups would substantiate the negative results.

Another consideration is that the dominant p10 species immunoprecipiated from transfected cells is located within the endoplasmic reticulum (ER) en route to the degradation pathway. A substantial proportion of the ER-localized p10 species may not be multimerized while the small fraction of p10 that exits the ER may in

fact have oligomeric structure. Previous analysis demonstrated that degradation of p10 is proteasome dependant (Shmulevitz et al., submitted) and can be prevented with specific proteasome inhibitors. The use of these inhibitors in future cross-linking experiments may favor the intracellular trafficking of p10 to the cell surface and the formation of p10 multimers.

Another method to analyze protein-protein interactions between p10 molecules would be the yeast two-hybrid system. Two constructs would be necessary for this analysis; the p10 protein fused to either the Gal 4 DNA-binding domain or the Gal 4 activation domain. Activation of Gal 4 responsive reporter genes such as HIS3 or lac2 would indicate protein-protein interactions between the two p10 constructs (See example of this method in Rende-Fourier et al., 1997). Unfortunately, the transmembrane domain of p10 would have to be deleted from the constructs to prevent false positive results as a consequence of non-specific hydrophobic interactions. Because the p10 fusion peptide is also relatively hydrophobic and may aggregate, results of the yeast two-hybrid experiments should be interpreted carefully. Another strategy to identify oligomeric structures is the disulphide cross-linking technique (See examples of this method in Pakula, 1992; Cao et al., 1992). Single amino acid mutations within the transmembrane or ectodomains of p10 to cysteine residues could be made and mutated proteins could be assessed for intermolecular disulphide bond formation under nonreducing conditions. The benefit of this technique is that it would allow close interactions between transmembrane domains to be identified. An assortment of

mutants would have to be used to ensure that they would line up along the face of protein-protein interactions. If multiple additional strategies fail to identify oligomeric structures within p10, the assumption that p10 functions as a monomer would be more compelling.

Several explanations for the involvement of p10 monomers are conceivable. One possibility is that p10 exists predominantly as a monomer and higher-order oligomers are formed prior to the membrane fusion event. Because we can not analyse p10 molecules during their fusogenic activity on lipid bilayers in isolation, we cannot exclude the possibility that multimers are required for p10 induced fusion. ARV p10 could function like alpha- and flaviviruses that form trimers immediately prior to membrane fusion, except that the first mature metastable state of p10 would be a monomer rather than a dimer. In order to assess this possibility, p10 proteins capable of remaining poised for fusion but awaiting exogenous signals would need to be constructed. As an example, cleavable extensions could be added to N-terminus of p10, which would prevent p10 fusion peptide insertion into target membranes and membrane fusion until specific proteases were added to p10-transfected cells. Regulatable p10 proteins could be allowed to build up on the cell surface and assessed for the formation of multimers upon stimulation.

The alternative interpretation of our results would be that monomers are sufficient for induction of protein-mediated membrane fusion. The tertiary structure of

enveloped virus fusion proteins could be involved in providing the correct conditions required for regulating the fusion event. Oligomeric structures may permit multiple fusion protein states where fusion peptides are buried or exposed, outstretched towards target membranes or in close proximity to the donor membrane. If p10 is expressed in the fusion active conformation (see chapter 3) then oligomeric structures involved in burying the fusion peptide would not be essential for p10-mediated membrane fusion. Alternatively, multimer formation may support efficient and rapid membrane fusion. The influenza HA fusion protein, for example, was shown to fuse membranes with a half-life of 15-30 seconds (White et al., 1982). These studies allow HA trimers to accumulate on the cell surface to large concentrations (similar to those found in viral envelopes) before assessing their fusogenic activity directly following activation with low pH. Once HA-expressing cells are exposed to low pH, syncytia formation continues rapidly until few, but very large multinucleated cells remain. In contrast, p10 proteins accumulate slowly on the cell surface during ARV replication and p10-mediated syncytia formation continues slowly so long as expression and cell viability persist (see chapter 2). A reduced rate of membrane fusion may, therefore, be favorable for ARV, and may have prevented the evolution of more optimally fusogenic oligomeric structures. Assuming that monomers are capable of inducing membrane fusion, though less efficiently, the above discussion provides a rationale for why p10 may have evolved to remain as a monomer unlike the fusion proteins of enveloped viruses.

It is important to note that the absence of p10 multimers does not exclude the possibility that aggregation of many p10 monomers may occur during the fusion process. In addition to the formation of trimers, larger oligomeric complexes have been predicted for influenza HA and HIV gp120 glycoproteins. Based on the effects of different concentrations of cell surface HA on fusion activity, Danieli et al. (1996) proposed that the initial stages of HA-mediated membrane fusion require a complex of at least three HA trimers. Other research proposed that even larger HA trimer complexes are required (Blumenthal et al., 1996). The baculovirus fusion protein gp64 forms transient large oligomers upon contact with target membranes (Markovic et al., 1998). Similarly, a requirement for a ten-fold excess of authentic vs. altered HIV gp120 molecules to overcome the dominant negative effects of mutated gp120 molecules suggested that several oligomers are required for fusion (Freed et al., 1992). These results have led to the hypothesis that fusion is a cooperative event that requires multiple oligomers to assemble into ring like structures, in which 2-3 multimers undergo conformational change while the remaining oligomers promote correct lipid bilayer conditions to promote fusion (Blumenthal et al., 1996; Danieli et al., 1996; Bentz et al., 1990; Blumenthal et al., 1995; Danieli et al., 1996; Zimmerberg et al., 1993; Bentz, 2000). For Newcastle disease virus however, only trimers are required according to the same protocol (Li et al., 1998) suggesting that cooperation between several fusion protein oligomers may not be a universal requirement for membrane fusion.

Apart from determining whether p10 functions as a multimer, it would be interesting to ascertain whether p10 aggregates on the cell surface. The previously demonstrated partitioning of p10 within detergent-insoluble glycolipid rich membranes may serve to concentrate p10 within localized environments. Since techniques are presently unavailable to look specifically at a population of p10 while it induces membrane fusion, the usual biochemical and biophysical approaches used to assess fusion protein aggregation prior to function are not feasible. With some innovative thought, however, alternative methods could be designed. As an example, two new species of p10 could be created, one having an enzymatic function such as transphosphorylation, and the other possessing the site for activity, such as phosphorylation site. Using these constructs in co-transfection assays, aggregation could be assessed by the presence of modified p10 species. Although these p10 constructs may be dysfunctional, their aggregation would likely reflect the potential of authentic p10 and suggest that cooperative activity of p10 should not be excluded despite the absence of multimerization.

P10 could function as a monomer and rely solely only on interactions with donor and/or target membranes to increase the favorability of membrane fusion. In this way, the mechanism of p10 would be similar to membrane fusion- or lytic peptides that induce significant destabilization of lipid bilayers. The fusogenic activity of p10 would resemble the late stages of membrane fusion induced by enveloped virus fusion proteins (i.e. where correct triggers and conformational

changes are complete). In the second mechanism, the fusion peptide of p10 would insert into donor and/or target membranes followed by aggregation of p10 monomers and their cooperation in creating dimples or hydrophobic voids within the lipid bilayers, as described by Bentz (2000). Conclusive determination of the oligomeric status and aggregation potential of p10 is instrumental in understanding the mechanism of p10-induced membrane fusion. Monomeric p10 expressed in an active conformation may reflect the minimal requirements for biological protein-mediated membrane fusion.

5.E. CHAPTER 5 FIGURES

Figure 5.1: The nonfunctional 3HAC protein does not inhibit the activity of coexpressed p10opt. (A) The quantity of constructs expressing p10opt (p10 with an optimized translation start site), or 3HAC (p10opt with three C-terminal HA epitopes) that were co-transfected into quail cells is indicated. The total amount of DNA for transfection was brought to 2ug with unrelated (other) DNA. The changes in 3HAC protein expression and p10opt-induced syncytia formation for each transfection condition are summarized on the right. (B) Expression levels of 3HAC following immunoprecipitation with HA-specific antibodies. Lanes labeled 1-9 correspond to the conditions described in (A). (C) Immunohistochemical staining of cotransfected cells with HA-specific antibodies. Blue arrows indicate the cell surface. A single arrow suggests the absence of syncytium and points to the surface of a single cell, while three arrows define the periphery of multinucleated syncytium. Ratios of 3HAC:p10opt (first column) or p10opt:3HAC (second column) are indicated for each of the five rows.

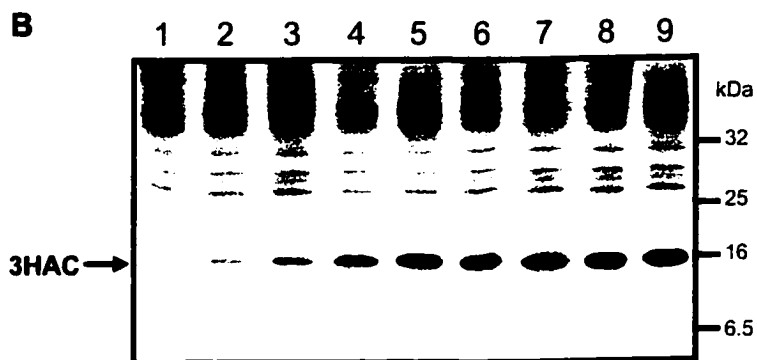
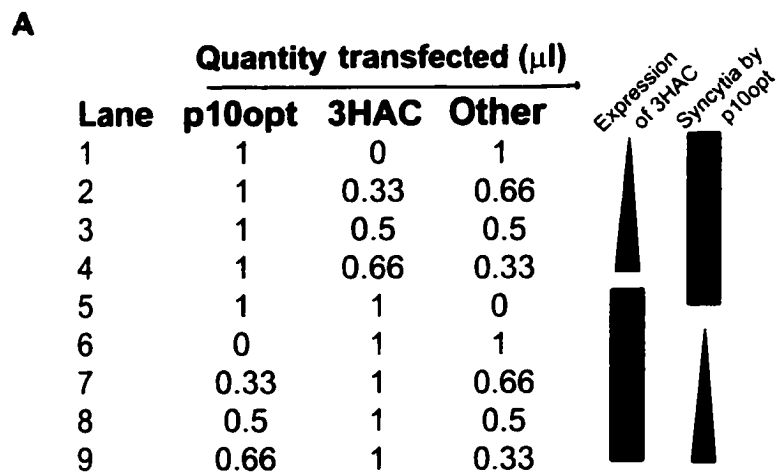


Figure 5.1

C

3HAC : p10opt

p10opt : 3HAC

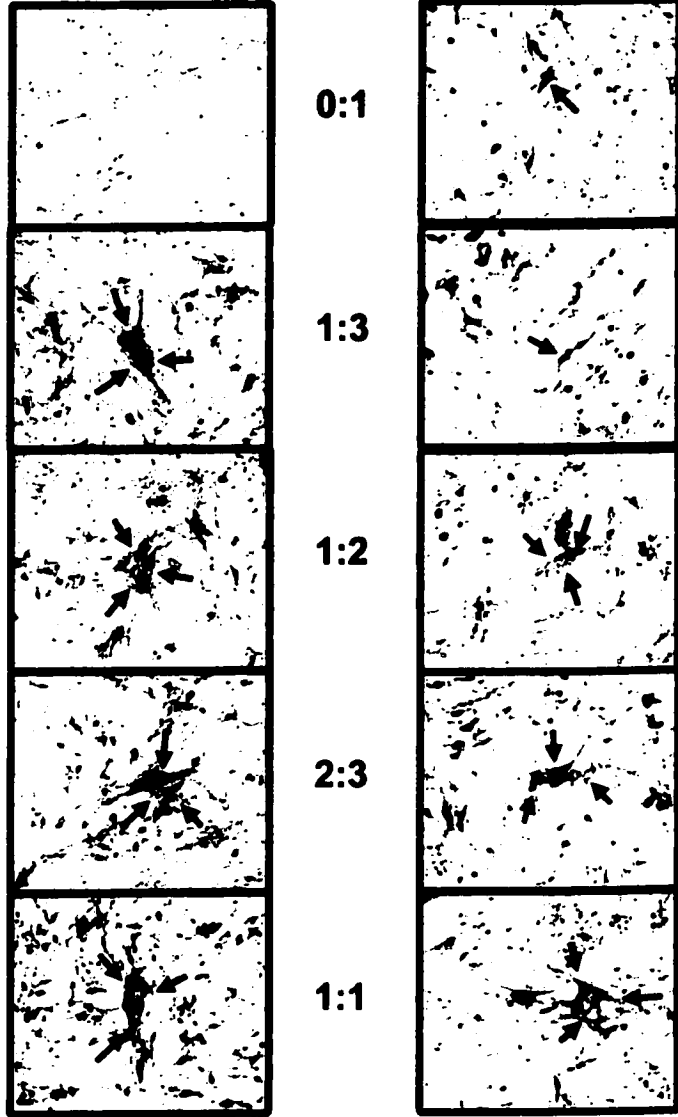


Figure 5.1

Figure 5.2. The absence of dominant negative or complementation effects between phenotypically different p10 mutants.

Quail fibroblast cells were cotransfected with equal proportions of two p10-containing constructs. Following immunohistochemical staining, monolayers were visualized for the presence of large (++++), medium (++) or no (-) multinucleated syncytium. P10 containing substitutions within the ectodomain hydrophobic patch (C9S, A14/15, V15M and C21S) or conserved region (G29A, L32A), the transmembrane domain (G49/50A), the palmytylation sites (C63S), or the basic intracellular domain (K67M) abolished the fusion activity of 2HAN (grey boxes) in cotransfection experiments. Note that when 2HAN was transfected alone, the two-fold increase in functional 2HAN protein accounted for the improved fusion (++++) relative to cotransfection conditions with additional nonfunctional p10 constructs (++) . There was no complementation of fusion activity between mutants (white boxes).

	2HAN	C9S	A14/15	V15M	C21S	G29A	L32A	G49/50A	C63S	K67M
2HAN	++++	++	++	++	++	++	++	++	++	++
mock	++	-	-	-	-	-	-	-	-	-
C9S	++	-	-	-	-	-	-	-	-	-
A14/15	++	-	-	-	-	-	-	-	-	-
V15M	++	-	-	-	-	-	-	-	-	-
C21S	++	-	-	-	-	-	-	-	-	-
G29A	++	-	-	-	-	-	-	-	-	-
L32A	++	-	-	-	-	-	-	-	-	-
G49/50A	++	-	-	-	-	-	-	-	-	-
C63S	++	-	-	-	-	-	-	-	-	-
K67M	++	-	-	-	-	-	-	-	-	-

Figure 5.2

Figure 5.3. Co-immunoprecipitation studies failed to show an association between p10 molecules.

Equal ratios of p10opt and 2HAC (A) or 2HAN (B) were cotransfected into quail cells. Thirty hours post-transfection, proteins were [³⁵S]-labeled, lysed with the specified detergents, precipitated with antibodies to HA or to p10 as indicated, and subjected to SDS-PAGE analysis and fluorography. Bands corresponding to 2HAC, 2HAN and p10opt are designated. Molecular weight markers are indicated on the right.

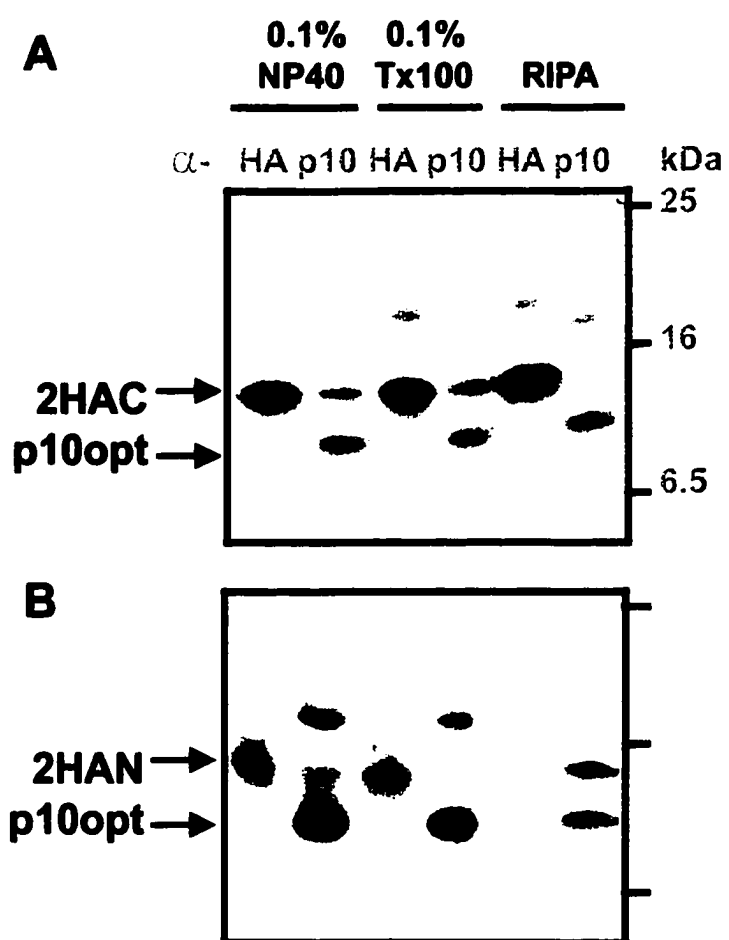


Figure 5.3

Figure 5.4. Different detergents and increased expression of p10opt did not enhance co-immunoprecipitation between p10opt and 2HAN.

In panel A, equal ratios of p10opt and 2HAN were cotransfected into quail cells, while the ratio was changed in panel B as indicated. Thirty hours post-transfection, proteins were [³⁵S]-labeled and lysed with the detergent specified. Cell lysates were precipitated with normal rabbit serum (NRS) or antibodies to HA or to p10 as indicated, and subjected to SDS-PAGE. Bands corresponding to 2HAN and p10opt are designated.

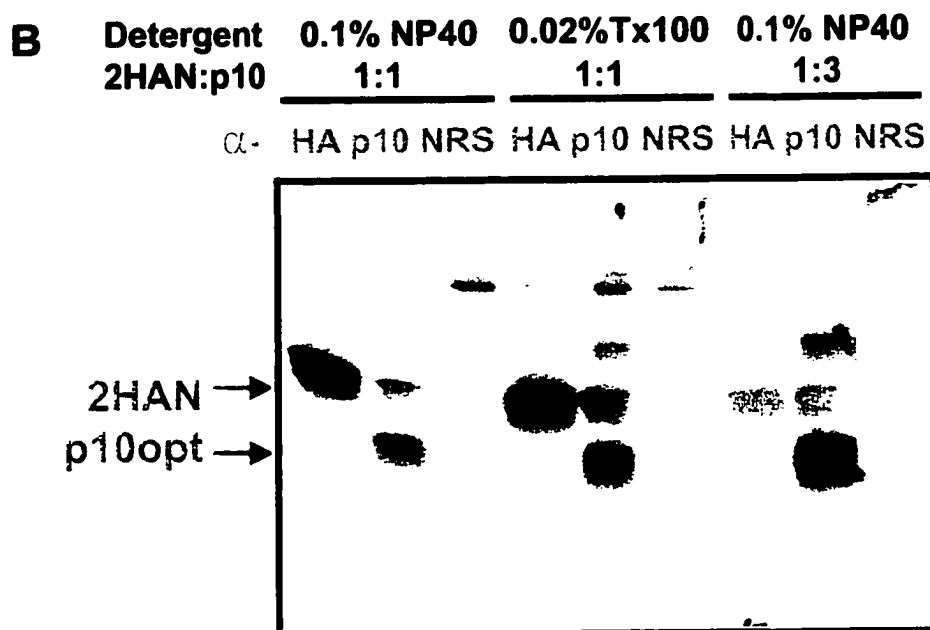
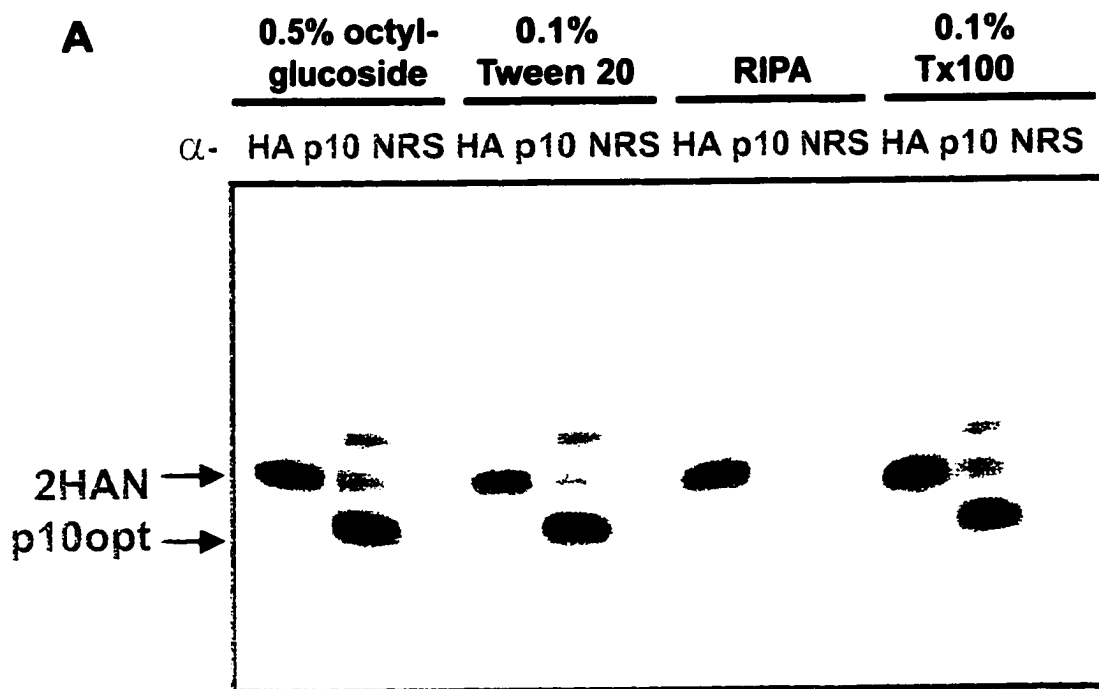


Figure 5.4

Figure 5.5. Coprecipitation between p10 molecules was not evident within the two hours permitted for multimer formation.

Quail fibroblasts were transfected with p10opt (lane 1), or co-transfected with p10opt and either 2HAN (A, lanes 2 and 3) or 2HAC (B, lanes 2 and 3). At 30 hours post transfection, cells were pulsed with [³⁵S]-Methionine and chased for 30, 60, 120, or 240 minutes (as indicated) to permit sufficient time for intracellular protein transport and assembly. Cells were lysed with 0.1% NP-40 (A and B) or 0.1% saponin (C) and immunoprecipitated with HA specific (lanes 1 and 3), or p10-specific (lane 2) antibodies. Proteins were analyzed by 15% SDS-PAGE. Prolonged exposure was necessary to compensate for the extensive degradation of p10 between 30 and 120 minutes.

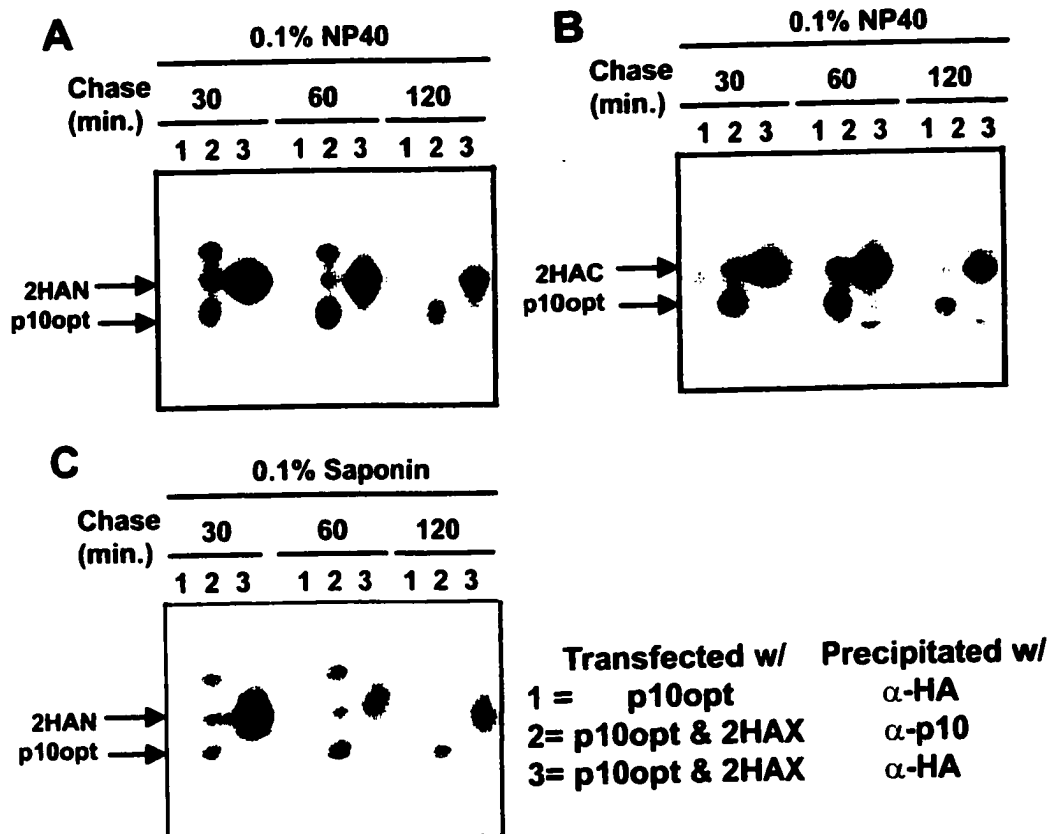


Figure 5.5

Figure 5.6. Cross-linking analysis did not reveal multimeric p10 species. (A) The characteristics of each cross-linking or biotinylation reagent used are indicated. The tube numbers correspond to the lanes numbered in panel B. **(B)** Quail fibroblasts were transfected with 2HAN and [³⁵S]-Methionine labeled at 36 hours post transfection. Cross-linking reagents were either added directly to cells (Live/adherent), after cell- suspension with PBS/EDTA (Live/Suspension), or to membrane fractions of quail fibroblasts (Membranes) at 2mM final concentration for 1 hour at room temperature. HA-specific antibodies were used to immunoprecipitate 2HAN and SDS-PAGE analysis was performed under non-reducing conditions to prevent cleavage of labile reagents. Arrows indicate the expected molecular weights of 2HAN as a monomer (17KDa), dimer (34KDa), trimer (51KDa) or tetramer (68KDa). Lighter molecular weight species are also present in control lane (8) and therefore did not represent p10-oligomers.

A

Tube	Reagent name	Reactive towards	Membrane permeable	Cleavable by	Spacer arm (Å)	MW (g/mol)	Conc. Final
1	Sulfo-NHS-SS-Biotin	amino	no	reduction	24.3	606.69	2mM
2	DSP [Dithiobis(succinimidylpropionate)]	amino	yes	reduction	12	404.42	2mM
3	EGS [Ethylene glycol bis(succinimidylsuccinate)]	amino	yes	Hydroxylamine	16.1	456.37	2mM
4	DST [Disuccinimidyl tartrate]	amino	yes	Oxidation	6.4	344.24	2mM
5	DSS [Disuccinimidyl suberate]	amino	no	no	11.4	368.35	2mM
6	DTME [Dithio-bis-maleimidoethane]	sulphydryl	yes	Reduction	13.26	312.37	2mM
7	PEO-Maleimide Activated Biotin	sulphydryl	no	no	29.1	525.62	2mM
8	Negative control: DMSO alone						

B

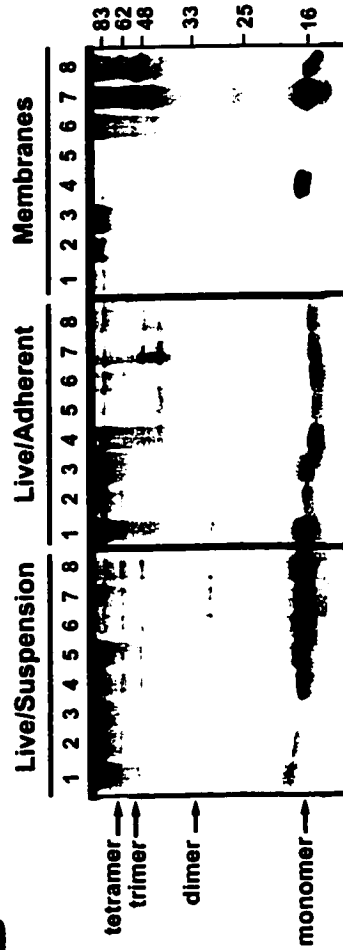


Figure 5.6

5.F. MATERIALS AND METHODS NOT PREVIOUSLY DESCRIBED

Cotransfection

Cotransfection with Lipofectamine (Life Technologies) was performed according to manufacturer's instructions. For every two million cells, the total amounts of DNA and lipofectamine were always 2ul and 6ul, respectively.

Coimmunoprecipitation

At 30 hours post-transfection, transfected cells were labeled with [³⁵S]-methionine (75μCi/ml) for 30 minutes. For pulse-chase experiments, cells were pulsed for 15 minutes with [³⁵S]-methionine (75μCi/ml), washed extensively with PBS, and chased for 30, 60, 120, or 240 minutes with Earles 199 media. Cells were lysed with 0.1% NP40, 0.1% or 0.02% Triton X-100, 0.5% octyl-β-D-glucoside, 0.1% saponin or 0.1% Tween 20 dissolved in 50mM tris-HCl pH 8.0, 300mM NaCl, containing the protease inhibitors aprotinin, leupeptin, and pepstatin. Alternatively, cells were lysed in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 50mM tris-HCl pH 7.5, 150mM NaCl, 0.1% SDS, and protease inhibitors). The same detergent mixtures were used in all steps of the immunoprecipitation with p10- or HA- specific antibodies. Proteins were analyzed by 15% SDS.

Cross-linking reactions

Two million cells for each condition were transfected and labeled with [³⁵S]-methionine (75 μ Ci/ml) for 30 minutes at 30 hours post-transfection. Incubation for 20 minutes at room temperature with PBS was used to suspend cells in solution, and cell pellets were obtained following centrifugation at 700 g for 7 minutes. Membranes were purified from suspended cells by high-speed centrifugation as described previously. All cross-linking reagents, purchased from Pierce biochemical, were resuspended in water (membrane impermeable reagents) or DMSO (membrane permeable reagents) at a concentration of 200mM, and diluted to 2mM in cross-linking buffer (Hanks balanced salt solution with 10mM HEPES pH 7.0), and added to live adherent cells, to membrane fractions, or to suspended cell pellets. The cross-linking reaction was incubated at room temperature for 60 minutes. Suspended cells and membranes were kept shaking for the incubation to prevent them from collecting at the bottom of the tubes. Reactions were stopped with 40mM glycine/PBS. Live adherent cells were washed with PBS and lysed with RIPA buffer. An equal volume of 2 X RIPA solution was added to suspended cells and membranes to produce a 1 X RIPA solution.

CONCLUSION: Mechanism of p10-induced membrane fusion

C. CONCLUSION

We have found that the nonenveloped viruses ARV and NBV encode homologous membrane fusion-inducing protein that we have named p10 based on their molecular weight. P10 is surface localized in a type I orientation similar to the fusion proteins of enveloped viruses (see chapter 1). Direct involvement of p10 in the fusion process is suggested by the ability of p10-bound antibodies to inhibit p10-mediated cell-to-cell fusion (syncytium) (chapter 1). The aim of my research has been to characterize p10 proteins and develop a hypothesis of the mechanism by which p10 mediates membrane fusion.

Chapter one describes the identification and sequence based characterization of p10. Chapter two discusses the unique limitations imposed on p10 activity as compared to the fusion proteins of enveloped viruses and suggests that p10-mediated fusion is controlled at the expression level rather than by structural changes triggered by specific conditions. Chapter two supports that p10 may represent the minimal fusion machinery devoid of complexities involved in specificity, regulation, rapidity and efficiency of the fusion reaction. Chapter three demonstrates that similar to the fusion proteins of enveloped viruses, p10 contains a fusion peptide capable of associating with membranes and inducing liposome fusion. Evidence suggests that the fusion peptide of p10 is internal, hydrophobic, and exists as a disulfide stabilized loop structure composed of antiparallel beta-strands. The fusion peptide is responsible for rapid intracellular degradation of p10, which suggests that it is exposed in the initial state of p10. If

p10 does not necessitate regulation of fusion activity, then burying of the fusion peptide would not be essential. Chapter four demonstrates that the transmembrane domain, two palmitoylated membrane-proximal cysteines and the intracellular basic region are important in p10-mediated fusion. That fusion proteins impact the stability of donor membranes is a newly developing concept. P10 may provide the best candidate for studies on donor membrane destabilization during membrane fusion because instability of donor membranes would not be deleterious to ARV and may actually favor ARV release from cells through lysis. In contrast, donor membrane instability would have deleterious consequences on released extracellular enveloped viruses because it would reduce their stability/longevity. The last chapter discusses evidence for the monomeric structure of p10. Since enveloped virus fusion proteins are all multimeric, the monomeric structure of p10 is a distinguishing feature. Chapters one through five provide detailed conclusions that will not be restated in this section to prevent further redundancy. Instead, I will provide a hypothesis for the mechanism of p10-mediated fusion and a comparison between p10 and the fusion proteins of enveloped viruses.

We hypothesize that p10 contains the minimal requirements for membrane fusion. The domain organization of p10 is portrayed in figure D.1. P10 is expressed from the first of three sequential open reading frames of the S1 genome segment. While the host translation machinery is still bound to the non-conserved region, p10 is co-translationally translocated across the endoplasmic reticulum membrane using the transmembrane domain as the signal anchor

sequence. The hydrophobic patch/ fusion peptide folds into a disulphide-bonded loop within the reducing environment of the endoplasmic reticulum. The hydrophobicity of the fusion peptide results in rapid intracellular, proteasome-dependent degradation of a large proportion of p10. Only a small population of p10 with the fusion peptide exposed is successfully transported to the cell surface where it locates both within and outside of lipid raft domains. The raft domains may help concentrate functional p10 proteins at a focal point to augment the destabilizing effect on the lipid bilayers. Cell-to-cell interactions bring opposing membranes into sufficiently close proximity to permit the merger of donor and target membrane following p10-induced bilayer destabilization. P10 is predicted to destabilize both donor and target membranes. The fusion peptide most likely inserts into the target membrane and produces local changes in lipid packing that disfavor the bilayer structure. The role of the transmembrane domain of p10 may include the destabilization of donor membrane through changes of outer leaflet lipid packing. The two palmitates and the basic domain are predicted to alter the local interactions between inner leaflet lipids. All together, the changes to the lipid bilayers induced by p10 reduce the activation energy necessary for spontaneous membrane merger. With accumulated p10 on the cell surface, the likelihood of membrane fusion is increased and cell-to-cell fusion ensues. This model for p10-induced membrane fusion suggests that bilayer destabilization is sufficient for membrane merger so long as membranes are close.

A comparison of the general features of p10 and the fusion proteins of enveloped viruses provides the rationale for why the hypothesis for p10-induced membrane fusion is a simplified version of the present paradigm of biological protein-induced membrane fusion (see introduction) based on studies of enveloped virus fusion proteins (Figure D.2). Both p10 and enveloped viruses share an N-terminal out orientation, an integral association within the membrane, and localization to the surface of cells. These characteristics are inherent requirements for proteins that induce membrane merger. The abilities to undergo structural changes, to have regulated, specific, rapid and efficient fusion, and therefore to initiate in- and culminate with- inactive states with intermediate activated structure are, however, not necessary for p10 function. The reduced requirements imposed on the function of p10 may explain why p10 is relatively small, lacks multimerization and glycosylation. The domains within fusion proteins would reflect the requirements imposed on their function (Figure D.3). That is to say, since enveloped viruses necessitate regulation, specificity, rapidity and efficiency, they would require domains that facilitate these general features. Heptad repeats and other domains that permit conformational changes, binding domains, and protein cleavage would be necessary for enveloped virus fusion proteins but not p10. What p10 shares in common with the fusion proteins of enveloped viruses are presumed to represent the minimal requirements of biological membrane merger. These minimal domains are believed to contribute to membrane fusion through their direct effects on lipid bilayers.

We hypothesize that the fusion active conformation and the minimal requirements for membrane fusion are shared between p10 and the fusion proteins of both enveloped viruses and intracellular SNARE protein-mediated fusion processes (Figure D.4). The active conformation is predicted to consist of domains capable of interacting with and destabilizing both donor and target membranes. The distance between donor and target membranes, if small, would favor the merger of destabilized bilayers. The means of attaining the fusion competent conformation differs between p10 and the fusion proteins of enveloped virus and intracellular fusion. The enveloped viruses encode fusion proteins that remain in an inactive conformation until correctly embedded in the viral envelope, the correct target cell is bound, and the specific environmental triggers are encountered. The enveloped viruses must undergo changes in structure that permit fusion peptide exposure as well as shortening the distance between donor and target membranes. Following such structural changes, the enveloped virus fusion proteins may resemble p10. The intracellular fusion proteins are held within inactive conformations which prevents their pairing until correct target and vesicle membranes are matched. The interactions between vesicle and target membrane SNARE proteins results in a shortened distance between membranes while the transmembrane domains presumably contribute to the changes in bilayer structure. P10 initiates with the fusion peptide exposed and is sufficiently small to maintain membranes in close apposition following interactions with the target membrane. Overall, p10 initiates in the fusion

competent conformation which reflects the minimal requirements for membrane merger.

The in-depth studies on p10-induced membrane fusion have used the ARV-encoded p10 as the model protein. Future studies on NBV-encoded p10 would help develop our hypothesis on the mechanism of p10-mediated membrane fusion. Domain swaps between p10 and the fusion proteins of enveloped viruses may also be useful. As an example, the transmembrane domain of p10 may enhance the fusion activity of enveloped virus fusion proteins by increasing the donor membrane destabilization activity. The latter experiments would help introduce p10 into the thoughts of investigators that study enveloped virus fusion proteins and membrane fusion. Because the characteristics of p10 and the constraints imposed on p10 during ARV evolution differ markedly from other membrane fusion proteins/processes, p10 will surely provide lucrative insights into the mechanism(s) of biological membrane merger.

CONCLUSION FIGURES

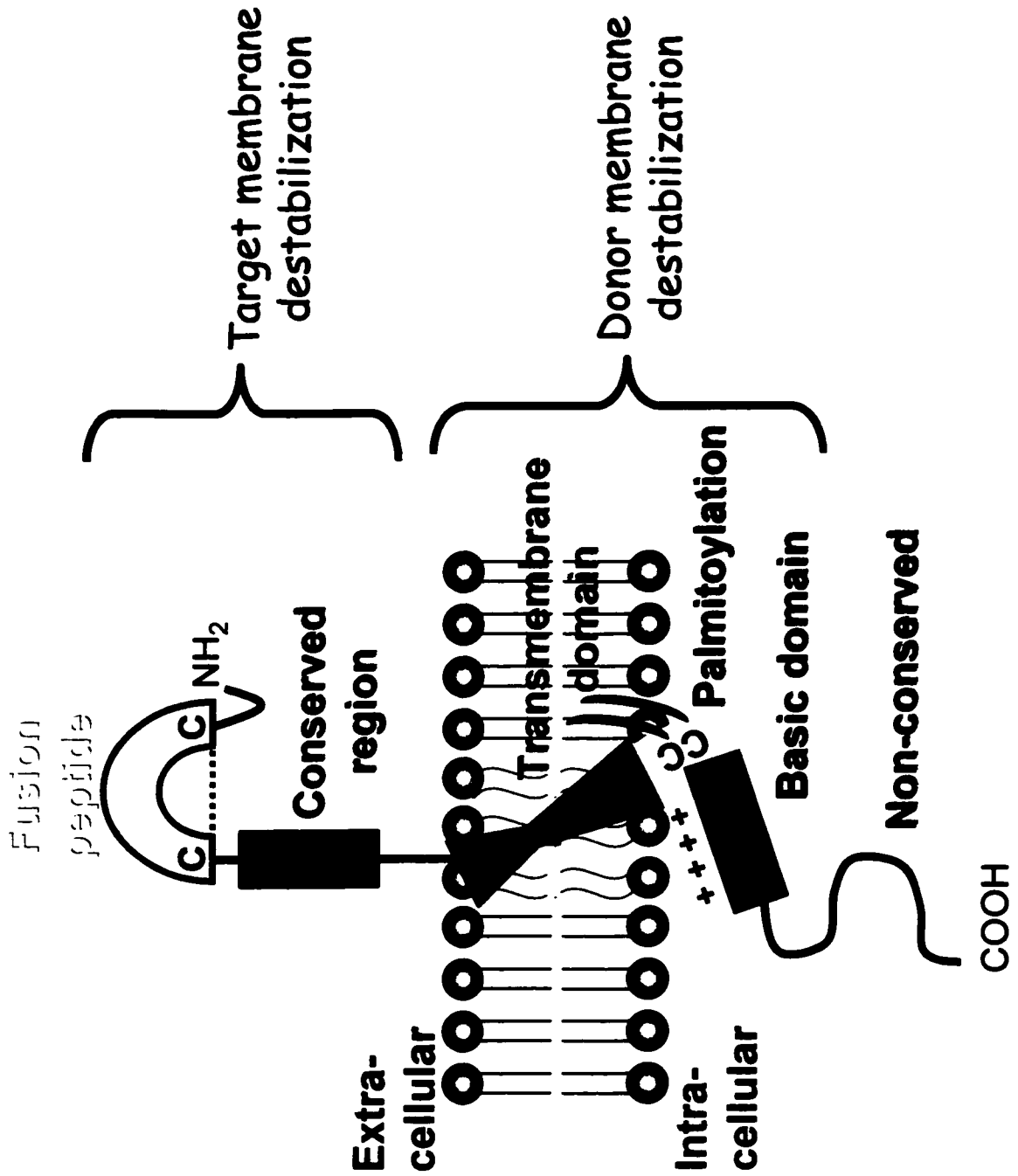


Figure D.1

GENERAL FEATURES OF ENVELOPED VIRUS FUSION PROTEINS

Feature	Proposed purpose for enveloped viruses	Shared by p10?	Rationale for presence in p10
Large	*consequence of complexity	no	* consequence of simplicity
Multimeric	*permits multiple states *permits fusion peptide burying *ensures rapidity/ efficiency	no	*exists in one state only *exposed fusion peptide *rapidity/ efficiency not needed
N-out orientation	*extracellular fusion peptide	yes	*extracellular fusion peptide
Glycosylated	*permits complex folding	no	*simple folding
Surface localized	*incorporation into envelopes	yes	*location of membrane fusion
Raft associated	*concentrates fusion protein *correct lipid environment	yes	*concentrates fusion protein *correct lipid environment
Structural changes	*permits regulation	no	*regulation not needed
Regulation	*ensures fusion with correct membranes (envelope membrane with correct target cell membrane)	no	*correct membranes are evident (infected cell membrane with neighbouring cell membrane)
Specificity	*ensures correct target cell binding and entry	no	*not involved in entry - target membrane is evident
Rapid & efficient	*timely entry	no	*not involved in entry - slow fusion preferable
Creation & preservation of primary inactive state	*prevents premature activation in infected cell *energetically favorable structural changes	no	*functions in infected cell *expressed as active
Fusion culminates with inactive state	*inactivation of incorrectly triggered proteins *permits close approach of membranes	no	*always correct activity *small size provides close membrane apposition

Figure D.2

DETERMINANTS OF ENVELOPED VIRUS FUSION PROTEINS

Feature	Enveloped virus fusion proteins		ARV and NBV encoded p10	
	Essential for fusion?	Proposed purpose	Common to p10?	Rationale
Fusion peptide	yes	*target membrane association *target and/or donor membrane destabilization	yes	*target membrane association *target and/or donor membrane destabilization
Cleaved	variable	*fusion peptide positioning *generates metastable state	no	*fusion peptide initially exposed
Binding domain	yes	*confers specificity *provides trigger / regulation	no	*specificity and regulation not needed
Heptad repeats	variable	*permits conformational change	no	*conformational change not needed
Transmembrane domain	yes	*membrane anchor *donor membrane destabilization	yes	*signal anchor *donor membrane destabilization
Acylation	variable	*anchoring *donor membrane instability	yes	*anchoring *donor membrane instability
Intracellular basic residues	no		yes	*membrane orientation *donor membrane instability

Figure D.3

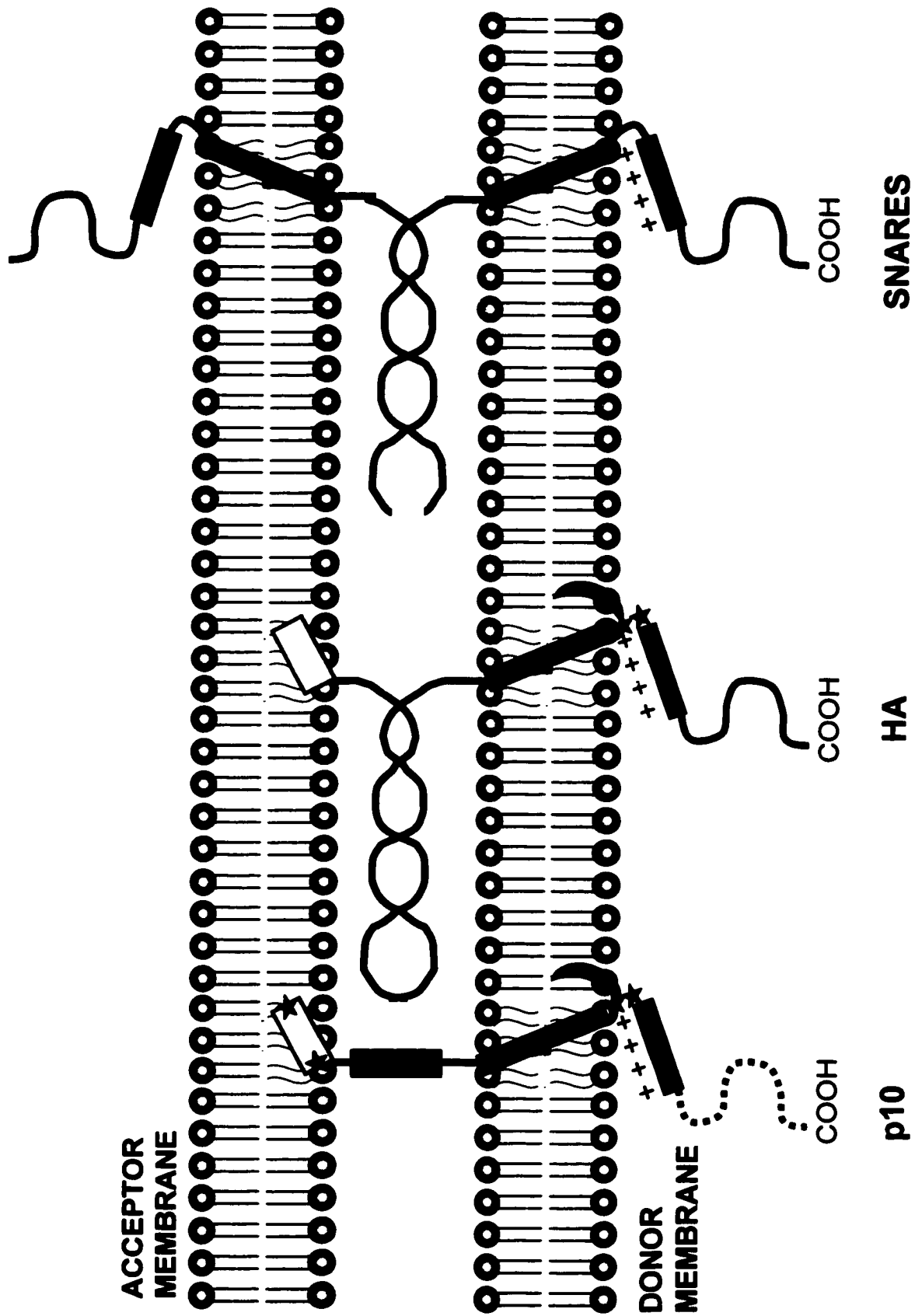


Figure D.4

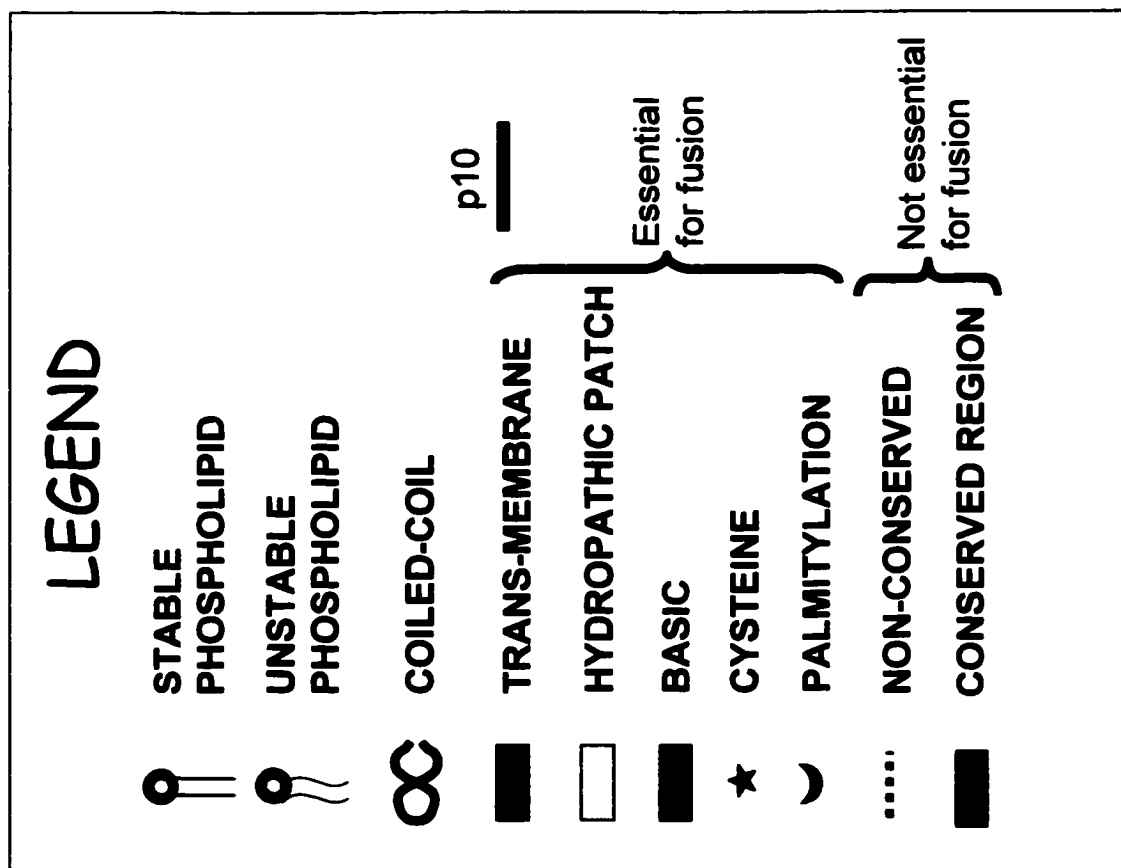


Figure D.4

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R.6. CHAPTER 5

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