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# Characterization of the Relationship Between Measles Virus Fusion, Receptor Binding, and the Virus-Specific Interaction Between the Hemagglutinin and Fusion Glycoproteins: a Dissertation

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# CHARACTERIZATION OF THE RELATIONSHIP BETWEEN MEASLES VIRUS FUSION, RECEPTOR BINDING, AND THE VIRUS-SPECIFIC INTERACTION BETWEEN THE HEMAGGLUTININ AND FUSION GLYCOPROTEINS

A Dissertation Presented
By

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Department of Molecular Genetics and Microbiology

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## ABSTRACT

Measles (MV) virions, like those of other enveloped viruses, enter cells by fusing their lipid membranes with those of the target host cells. Additionally, infected tissues often possess giant multinucleate cells, known as syncytia, which are formed by fusion of infected cells with uninfected neighbors. Expression of both the MV attachment (H) and fusion (F) proteins is required for membrane fusion. MV H mediates receptor binding in order to bring the two membranes into close proximity prior to F activation and is thought to trigger F activation through a specific interaction between the two proteins.

Although measles H and F are efficiently transported to the cell surface when expressed independently, evidence has been reported in support of an intracellular interaction between the two proteins that can be detected using an ER co-retention approach. However, it was not determined if the putative co-retention was specific to the two measles glycoproteins, as is their ability to complement each other for efficient fusion promotion. Thus, in this thesis, the formation of an intracellular complex between MV H and F was re-examined. Consistent with the formation of an intracellular complex, cell surface expression and receptor binding of untagged wt MV H is slightly reduced by co-expression of an excess of ER-tagged MV F compared to co-expression with wt F. However, the reduction in surface expression is non-specific in that it can also be induced with heterologous proteins of NDV, which lack significant homology with those of MV. Although this approach did not detect a specific intracellular interaction

between MV H and F, it cannot be ruled out that there is a weak association of the proteins that is undetectable by this method. This led to the use of an alternative approach to investigate the cellular site(s) of interaction between the measles H and F proteins.

Consistent with a cell surface interaction between MV H and F, the combination of surface biotinylation and co-immunoprecipitation detects formation of a virus-specific H-F complex. Approximately, 21% of the total amount of MV H at the cell surface can be captured with MV F using an antibody against the latter protein. Two complementary approaches were used to address the relationship between this cell surface interaction and receptor recognition by MV H. First, the proteins were co-immunoprecipitated from the surface of Chinese hamster ovary (CHO) cells, which do not express either MV receptor, CD46 or CD150. Similar levels of MV H can be co-immunoprecipitated with F from the surfaces of parental CHO cells and stably transfected cells that express human CD46 (CHO-CD46), indicating that binding to CD46 is not the trigger for the H-F interaction. Second, MV H proteins, carrying mutations that dramatically reduce CD46 binding, were shown to co-immunoprecipitate efficiently with F from the surface of HeLa cells. Significantly, these results indicate that MV H and F interact in the absence of, and thus prior to, receptor binding. This is in direct contrast to the NDV HN-F cell surface interaction, which is thought to be triggered by receptor binding.

Identification of the domains of the paramyxovirus attachment and fusion proteins that mediate membrane fusion activities is an essential part of understanding the mechanism of fusion. As a result of the H-F interaction prior to receptor binding, MV H attachment to its cellular receptor must result in conformational changes that trigger activation of the F protein. Site-directed mutagenesis analyses of two regions of MV H indicate that a HR domain in the stalk of the attachment protein is essential to the ability of H to activate F. However, either it is not the only region of H that interacts with F or it is indirectly involved in F activation because mutations in the HR do not disrupt MV H-F complex formation at the cell surface. Additionally, the functional interaction between MV H and F may be mediated, at least in part, by Loop 1 of the amino terminus of the C-rich region of the fusion protein. However, the exact role of this region of the F protein in fusion promotion remains to be determined. Importantly, the cell surface interaction between MV H and F proteins appears to be mediated by more that one region of each protein. In contrast to NDV, in no case has a definitive link between any single amino acid difference in MV H or F and an inability to form the cell surface H-F complex been established.

In conclusion, the data presented in this dissertation support a model of measles membrane fusion in which the H and F proteins form a complex prior to receptor recognition. This complex may hold F in its meta-stable pre-fusion state until binding of H to receptors at the cell surface triggers dissociation of the complex, releasing F to assume its fusogenic form. Importantly, these data also

indicate that, although paramyxoviruses may all use the same general process for promotion of membrane fusion, the mechanism may vary in multiple aspects. A more complete understanding of the means by which measles promotes membrane fusion may direct the development of specific strategies aimed at interfering with the early stages of infection.

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# TABLE OF CONTENTS

**ABSTRACT** ..... **iii**
**ACKNOWLEDGEMENTS** ..... **vii**
**TABLE OF CONTENTS** ..... **viii**
**LIST OF TABLES** ..... **x**
**LIST OF FIGURES** ..... **xi**
**LIST OF ABBREVIATIONS** ..... **xvi**
**CHAPTER I** ..... **1**
Introduction ..... 1
*1.1 Measles virus* ..... *1*
*1.2 Cellular receptors* ..... *11*
*1.3 Measles virus glycoproteins* ..... *16*
*1.4 Mechanism of membrane fusion promotion by MV H and F* ..... *29*
*1.5 Objectives of dissertation* ..... *43*
**CHAPTER II** ..... **45**
Materials and methods ..... 45
*2.1 Cell culture and transient transfection systems* ..... *45*
*2.2 Mutagenesis and chimera construction* ..... *48*
*2.3 Antibody production* ..... *49*
*2.4 Assays for measles virus glycoprotein expression and function* ..... *50*
**Chapter III** ..... **58**
Examination of the formation of an intracellular complex between MV H and F ..... 58
*Introduction* ..... *58*
*Results* ..... *60*
*3.1 MV glycoproteins are efficiently expressed and functional using the HeLa/ vTF7-3 system.* ..... *60*
*3.2 Characterization of the effects of ER retention of MV H and F expressed using the HeLa/ vTF7-3 system.* ..... *65*
*Summary* ..... *81*
**CHAPTER IV** ..... **82**
A MV H-F complex can be detected at the cell surface, independent of receptor binding ..... 82
*Introduction* ..... *82*
*Results* ..... *83*

*4.1 Interaction of MV H and F can be detected at the cell surface. ..... 83*
*4.2 MV H and F interact at the cell surface in the absence of receptors. 86*
*4.3 MV H lacking receptor binding interacts with MV F. ..... 94*
*Summary ..... 108*

**CHAPTER V ..... 109**

Heptad repeat in stalk of MV H is critical to its fusion helper function ..... 109
*Introduction ..... 109*
*Results ..... 110*
*5.1 Residues in the region between 244 and 250 in the globular head domain of MV H are not involved in mediating the interaction with F that is necessary for fusion promotion. ..... 110*
*5.2 Mutations in a heptad repeat (HR) in the stalk of MV H modulate the fusion helper function. ..... 114*
*Summary ..... 142*

**CHAPTER VI ..... 143**

Two regions of measles F involved in fusion protein cleavage and promotion of membrane fusion ..... 143
*Introduction ..... 143*
*Results ..... 144*
*6.1 Amino acid substitutions in HR-D of MV F cause decreased fusion promotion by altering stability of the trimer. ..... 144*
*6.2 Amino acid substitutions in the C-rich region of MV F cause decreased fusion promotion. ..... 151*
*Summary ..... 170*

**CHAPTER VII ..... 171**

*Discussion ..... 171*
*7.1 Re-examination of the detection of an intracellular MV H-F complex. 174*
*7.2 Detection of a MV H-F complex at the cell surface. ..... 177*
*7.3 The specific MV H-F interaction may be mediated by multiple regions of the glycoproteins. ..... 186*
*7.4 Mechanism of membrane fusion promotion by measles glycoproteins. 195*

**APPENDIX ..... 199**

Primers used for site-directed mutagenesis ..... 199

**REFERENCES ..... 205**

## LIST OF TABLES

Table 1. Examples of members of the Paramyxoviridae family. ..... 5

Table 2. Site-directed mutagenesis primers for generation of ER-tagged MV H and F. ..... 199

Table 3. Site-directed mutagenesis primers for generation of MV and CDV Fcsm proteins ..... 199

Table 4. Primers used for site-directed mutagenesis of putative MV H receptor recognition regions. ..... 200

Table 5. Primers used for site-directed mutagenesis of MV H region 244-250. ..... 201

Table 6. Primers used for site-directed mutagenesis of MV H heptad repeat.. 202

Table 7. Primers used for site-directed mutagenesis of MV F heptad repeat.. 203

Table 8. Primers used for site-directed mutagenesis of F protein C-rich region. ..... 204

## LIST OF FIGURES

Figure 1. Pathogenesis of measles infection. ..... 2

Figure 2. Diagram of linear arrangement of genes in the measles virus genome (adapted from Griffin, 2001). ..... 7

Figure 3. Measles virion structure..... 8

Figure 4. Diagram of measles replication cycle from attachment to the cellular receptor through assembly and budding (from Medical Microbiology, 5th edition)..... 10

Figure 5. Diagram of the structure of CD46, also know as membrane cofactor protein (MCP). ..... 13

Figure 6. Diagram of structure of CD150, also known as signaling lymphocyte activation molecule (SLAM) (adapted from Yanagi et al., 2002)..... 15

Figure 7. Diagram of the measles hemagglutinin (H) protein. ..... 17

Figure 8. Predicted structures of a measles hemagglutinin (H) protein monomer. ..... 19

Figure 9. Diagram of measles fusion (F) protein structure..... ....25

Figure 10. Predicted structure of measles fusion (F) protein trimer based on the crystal structure of NDV F (adapted from Plemper et al., 2003). ..... 28

Figure 11. Model for attachment protein (HN) conformation changes after receptor recognition (from Lamb et al., 2006)..... 32

Figure 12. Comparison of pre-fusion and post-fusion F structures (adapted from Yin et al., 2006). ..... 35

Figure 13. CSE of measles receptors and H by HeLa cells..... 61

Figure 14. Detection of expression of MV H and F in transfected HeLa cells.... 63

Figure 15. Membrane fusion promotion by virus glycoproteins in transiently transfected HeLa cells using the vTF7-3 expression system..... 64

Figure 16. ER retention of MV F. ..... 66

Figure 17. Syncytium formation in monolayers expressing ER-retention tagged MV F. ..... 67

Figure 18. CSE of MV H/ NDV HN co-expressed with MV Fcsm or F-ER. ..... 69

Figure 19. Hemadsorption (HAd) by MV H co-expressed with MV/ NDV Fcsm or F-ER. ..... 72

Figure 20. Intracellular processing of MV H is not altered by co-expression with MV Fcsm or F-ER. ..... 74

Figure 21. ER retention of MV H. ..... 76

Figure 22. Hemadsorption (HAd) by MV H co-expressed with H-ER. ..... 77

Figure 23. Syncytium formation in monolayers expressing ER-retention tagged MV H. ..... 79

Figure 24. Intracellular processing of MV F is not altered by co-expression with MV H or H-ER. ..... 80

Figure 25. Detection of an interaction between MV H and F at the cell surface of HeLa cells using co-immunoprecipitation assays. ..... 85

Figure 26. MV H does not co-immunoprecipitate with MV Fcsm when the proteins are expressed in separate monolayers, and it does not co-immunoprecipitate with CDV Fcsm. ..... 87

Figure 27. CSE of receptors and MV H by CHO and CHO-CD46 cells. ..... 89

Figure 28. Measles protein expression in CHO and CHO-CD46 cells. ..... 90

Figure 29. Detection of a MV H and F complex at the cell surface of CHO cell lines using co-immunoprecipitation assays. ..... 92

Figure 30. Diagram showing the locations of mutations created in regions of MV H thought to be involved in recognition of CD46. ..... 95

Figure 31. CSE and functional characteristics of MV H proteins carrying mutations at F431, V451, and Y481. ..... 97

Figure 32. IP of MV H proteins carrying mutations at F431, V451, and Y481. .. 98

Figure 33. CSE and functional characteristics of MV H proteins carrying the mutations S544G, S546G, and S548L/F549S. ..... 100

Figure 34. IP of MV H proteins with the mutations S544G, S546G, and S548L/F549S. ..... 101

Figure 35. CSE and functional characteristics of MV H proteins with mutations in the region 473-477. ..... 102

Figure 36. IP of MV H proteins with mutations in the region 473-477. ..... 103

Figure 37. Graph of hemadsorption versus fusion for MV H proteins with mutations in regions thought to be involved in CD46 binding. ..... 106

Figure 38. Co-immunoprecipitation of MV H proteins with defects in receptor recognition. ..... 107

Figure 39. Location of region 244-250 in the MV H ectodomain. ..... 111

Figure 40. CSE and functional characteristics of MV H proteins carrying mutations in residues 244-250. ..... 113

Figure 41. Plot of hemadsorption versus fusion activity of MV H proteins carrying mutations in residues 244-250. ..... 115

Figure 42. Amino acid sequence of heptad repeat (HR) in the stalk of MV H.. 116

Figure 43. Diagram of MV H showing the location and sequence of the heptad repeat domain in the putative stalk. ..... 118

Figure 44. CSE and functional characteristics of MV H proteins carrying mutations in heptadic residues of the HR domain. ..... 119

Figure 45. Comparison of the amino acid sequences in the HR domains in the stalks of paramyxovirus attachment glycoproteins. ..... 121

Figure 46. CSE and functional characteristics of MV H proteins carrying mutations in P94 and L95 in the HR domain. ..... 123

Figure 47. CSE and functional characteristics of MV H proteins carrying mutations in the intervening regions of the HR domain. ..... 125

Figure 48. Plot of hemadsorption versus fusion activity of MV H proteins carrying mutations in the HR region of the stalk. ..... 127

Figure 49. Sucrose gradient sedimentation profiles of MV H and MV H treated with 0.5% SDS. ..... 129

Figure 50. Sucrose gradient sedimentation profiles of MV H proteins carrying mutations in the HR domain. ..... 130

Figure 51. Promotion of hemifusion by MV H proteins carrying mutations in the HR domain. ..... 132

Figure 52. Co-immunoprecipitation of MV H proteins with mutations in the heptadic residues in the HR region of the stalk. ..... 134

Figure 53. Co-immunoprecipitation of MV H proteins with mutations P94A and L95A. ..... 136

Figure 54. Co-immunoprecipitation of MV H proteins with mutations at residues in the intervening region of the HR in the stalk including L92A, T93A, F96A, F96L, K97A, and I99A. ..... 138

Figure 55. Co-immunoprecipitation of MV H with MV Fcsm. ..... 139

Figure 56. Co-immunoprecipitation of MV H mutants with MV Fcsm. ..... 140

Figure 57. Location of HR-D in the MV F. ..... 145

Figure 58. Fusion promotion activities of MV F proteins carrying mutations in HR-D. ..... 147

Figure 59. IP of MV F proteins carrying mutations in HR-D. ..... 149

Figure 60. Comparison of the fusion promotion activities of MV F proteins carrying mutations in HR-D at 30°C and 37°C. ..... 150

Figure 61. Comparison of the IP of MV F proteins carrying mutations in the HR-D at 30°C and 37°C. ..... 152

Figure 62. Comparison of the fusion promotion activities of homologous and heterologous pairs of the MV and CDV glycoproteins. ..... 153

Figure 63. Location of the C-rich region in the MV F. ..... 155

Figure 64. Amino terminus of the C-rich region of MV F. ..... 156

Figure 65. Fusion promotion activities of MV F proteins carrying mutations in the amino terminus of the C-rich region. ..... 158

Figure 66. IP of MV F proteins carrying mutations in the amino terminus of the C-rich region. ..... 159

Figure 67. Comparison of the fusion promotion activities of MV F proteins carrying mutations in the amino terminus of the C-rich region at 30°C and 37°C. ..... 161

Figure 68. Comparison of the IP of MV F proteins carrying mutations in the amino terminus of the C-rich region at 30°C and 37°C. ..... 162

Figure 69. Expression and fusion promotion by MV/ CDV F protein chimeras carrying the C-rich domain of the heterologous virus. ..... 165

Figure 70. Fusion promotion by MV H and MV/ CDV F protein chimeras carrying the C-rich domain of the heterologous virus at 37°C versus 30°C. ..... 168

Figure 71. Two models of mechanism of paramyxovirus membrane fusion promotion. ..... 172

Figure 72. Models of mechanism of measles virus membrane fusion promotion. ..... 196

## LIST OF ABBREVIATIONS

| | |
|---|---|
| African green monkey | AGM |
| Canine distemper virus | CDV |
| Cell surface expression | CSE |
| Chicken embryo fibroblast | CEF |
| Chinese hamster ovary cells | CHOs |
| Constant region 2 | C2 |
| Cysteine-rich | C-rich |
| Cytopathic effects | CPE |
| Dimethyldioctadecylammonium bromide | DDAB |
| Dulbecco's Modified Eagle medium | DMEM |
| Endoplasmic reticulum | ER |
| Fetal calf serum | FCS |
| Fluorescein isothiocyanate | FITC |
| Fusion peptide | FP |
| Fusion protein | F |
| Hemagglutinin | H |
| Hemagglutinin-neuraminidase | HN |
| Heptad repeat | HR |
| Human parainfluenza virus | hPIV |
| Immunoglobulin | Ig |
| Internal fusion peptide | IFP |

| | |
|---|---|
| Intervening region | IR |
| Intervening sequence | IS |
| Immunoprecipitation | IP |
| Kilodalton | kDa |
| Large protein | L |
| Matrix protein | M |
| Measles virus | MV |
| Measles virus fusion protein | MV F |
| Measles virus fusion protein cleavage site mutant | MV Fcsm |
| Measles virus hemagglutinin protein | MV H |
| Membrane cofactor protein | MCP |
| Monoclonal antibody | MAb |
| Multiplicity of infection | MOI |
| Neuraminidase | NA |
| Newcastle disease virus | NDV |
| Nucleocapsid protein | N |
| Octadecyl rhodamine B chloride | R18 |
| Parainfluenza virus-5 | SV5 |
| pBluescript SK(+) | pBSK |
| Phosphate buffered saline | PBS |
| Phosphoprotein | P |
| Polyacrylamide gel electrophoresis | PAGE |

| | |
|---|---|
| Respiratory syncytial virus | RSV |
| Ribonucleoprotein particle | RNP |
| Rinderpest virus | RPV |
| Sendai virus | SV |
| Serine, threonine, and proline region | STP |
| Short consensus repeat | SCR |
| Signaling lymphocyte activation molecule | SLAM |
| Simian virus 5 | SV5 |
| Single-stranded DNA | ssDNA |
| Six-helix bundle | 6 HB |
| SLAM-associated protein | SAP |
| Sodium dodecyl sulfate-polyacrylamide gel electrophoresis | SDS-PAGE |
| Src homology 2 | SH2 |
| Subacute sclerosing panencephalitis | SSPE |
| Transmembrane | TM |
| Trichloroacetic acid | TCA |
| Untranslated region | UTR |
| Vaccine virus carrying T7 RNA polymerase gene | vTF7-3 |
| Variable region | V |
| Viral RNA-dependent RNA polymerase | vRNAp |
| Wild-type | wt |

# CHAPTER I

# Introduction

## 1.1 Measles virus

Measles virus (MV) is one of the most contagious human pathogens and was once a common childhood illness. While it has been eliminated from many areas of the world through extensive vaccination campaigns, outbreaks continue to occur. Measles remains one of the leading causes of death by a vaccine-preventable disease and is the leading cause of blindness in children in developing countries (Rima & Duprex, 2006, Semba & Bloem, 2004). It is estimated that, as recently as 2001, there were at least 40 million cases of measles per year resulting in approximately one million deaths (Rall, 2003).

Measles begins as a respiratory infection and is spread in aerosol droplets produced by coughing. The virus initially replicates in the upper respiratory tract, but it is not known if it infects the epithelial cells lining the lungs and trachea or the immune system cells residing within the tissues. The virus spreads from the respiratory tract into other tissues through infection of macrophages, lymphocytes, and dendritic cells. MV infection manifests as a fever and flu-like symptoms within 10-12 days after exposure (Fig. 1). During the asymptomatic phase, the primary sites of virus replication are lymphoid organs, including the lymph nodes, spleen, appendix, and tonsils. Infiltration of infected lymphocytes and macrophages into uninfected tissue distributes the virus throughout the host.

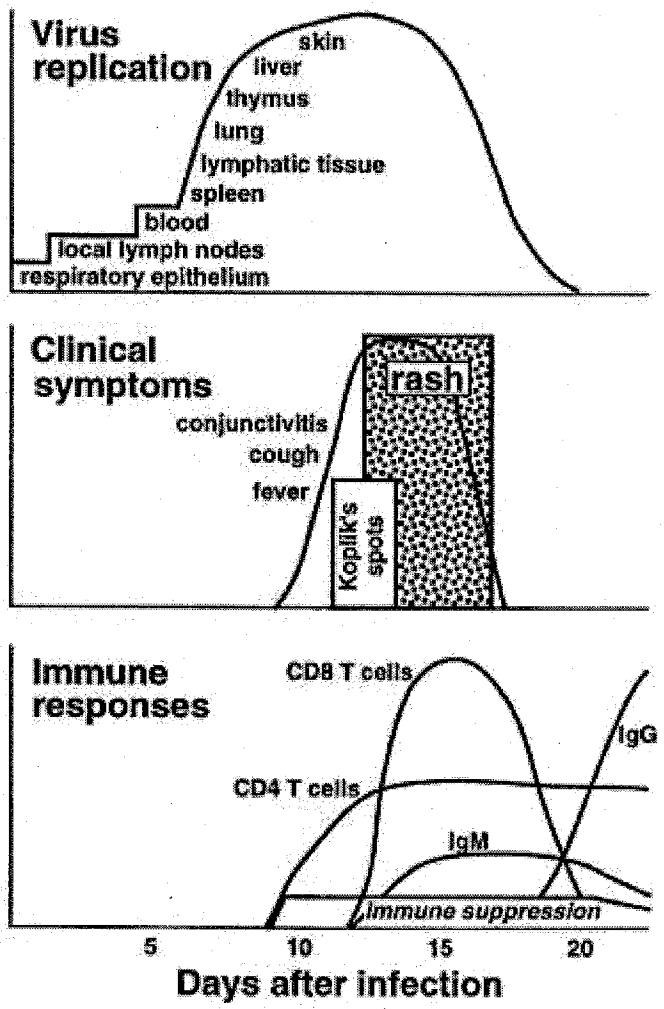



**Figure 1. Pathogenesis of measles infection.**

Time line comparison of virus replication, clinical symptoms, and immune response (Griffin, 2001).

At approximately 10 days post-infection, patients develop the characteristic symptoms of measles, including fever, cough, and conjunctivitis followed by the appearance of Koplik's spot on the oral mucosa and a maculopapular rash. Pathological examination of infected tissues often shows multinucleate giant cells known as syncytia that are formed by fusion of infected cells with uninfected neighbors. Although development of physical symptoms coincides with a MV-specific immune response that results in viral clearance, there is also an onset of a generalized immune suppression that leads to vulnerability to infection by other pathogens (Moss et al., 2004). Persistent infection can lead to infection of the central nervous system and result in an additional complication known as subacute sclerosing panencephalitis (SSPE) that manifests 7-10 years following resolution of the acute measles infection.

The dramatic reduction in the annual number of reported measles cases since the 1980s is the result of routine childhood immunization with live attenuated measles vaccines (Meissner et al., 2004). The Edmonston vaccine strain was first isolated in 1954 through serial passage in human cells and adaptation to chicken embryo fibroblasts (CEFs). It was licensed as the first measles vaccine in 1963, but was further passaged in CEFs to produce the Moraten vaccine strain currently used in the US, as well as the Schwarz and Edmonston-Zagreb strains used in other countries. Despite the effectiveness of the vaccines, MV has not yet been globally eradicated. Live attenuated vaccines require constant refrigeration, and thus, degradation of the vaccine occurs when

electricity is unavailable, resulting in sub-optimal potency. Additionally, in children less than nine months old, maternal antibodies are able to clear the vaccine resulting in an unprotected population of young children. These issues with the current vaccines have led to a continued search for alternative measles vaccination strategies and anti-viral treatments.

### 1.1.1 Classification and overview

Measles is the only human pathogen of the Morbillivirus genus within the subfamily *Paramyxovirinae* in the family *Paramyxoviridae*. Paramyxoviruses have envelopes derived from host membranes and negative sense non-segmented RNA genomes. Other viruses in the genus include animal pathogens, such as canine distemper virus (CDV), rinderpest virus (RPV), and peste-des-petits-ruminants virus (Table 1). There are several other important human pathogens within the paramyxovirus family, including mumps virus, respiratory syncytial virus (RSV), and the various parainfluenza viruses. There are also several well-studied animal pathogens in the family such as Newcastle disease virus (NDV), Sendai virus (SV) and parainfluenza virus 5 (SV5). The newly emerged Hendra and Nipah viruses have also been classified as paramyxoviruses. Morbilliviruses differ from most of the other paramyxoviruses in that they use specific protein receptors to enter cells, lack detectable neuraminidase activity, and form nuclear inclusion bodies.

The MV genome is 15,894 nucleotides in length and encodes 6 tandem genes, positioned 3′-N-P-M-F-H-L-5′, separated by short untranslated regions

**Table 1. Examples of members of the *Paramyxoviridae* family.**

- Family Paramyxoviridae
  - Subfamily Paramyxovirinae
    - Genus Avulavirus
      - Newcastle disease virus
    - Genus Henipavirus
      - Hendra virus
      - Nipah virus
    - Genus Morbillivirus
      - Measles virus
      - Canine distemper virus
      - Peste-des-petits-ruminants virus
      - Rinderpest virus
    - Genus Paramyxovirus
      - Sendai virus
      - Human parainfluenza virus type 1 and type 3
      - Bovine parainfluenza virus type 3
    - Genus Rubulavirus
      - Parainfluenza virus-5
      - Mumps virus
      - Human parainfluenza virus type 2, type 4a and 4b
  - Subfamily Pneumovirinae
    - Genus Pneumovirus
      - Human respiratory syncytial virus
      - Bovine respiratory syncytial virus

(UTRs) (Fig. 2). The six genes encode the nucleocapsid (N), phospho- (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins. The P gene also encodes three accessory proteins, V, C, and R. Unlike the other paramyxoviruses, measles has unusually long 3' and 5' UTRs after the M gene and before the F gene, respectively, that are thought to be involved in control of virus replication and cytopathogenicity (Takeda et al., 2005).

Measles virions are pleomorphic and vary in size from 100 to 300 nm (Fig. 3A). The host cell derived envelope consists of a lipid bilayer containing the two virus-encoded transmembrane glycoproteins H and F. The envelope encloses four additional viral structural proteins, including N, P, M, and L, along with the viral genome (Fig. 3B). The L and P proteins form a viral polymerase complex that comes together with the N protein-bound RNA genome to form a ribonucleoprotein particle (RNP). The hydrophobic M protein lines the inner surface of the membrane and is thought to mediate contact between the RNP complex and the lipid bilayer, as well as to interact with the cytoplasmic tail of the F protein (Cathomen et al., 1998).

Three non-structural proteins V, C, and R are expressed from the P gene after measles infection of a target cell. The V protein is produced from the P gene through RNA editing. It is an intracellular protein that inhibits interferon-α/β and – γ signaling, as well as cytokine-induced signal transduction (Ohno et al., 2004, Palosaari et al., 2003, Takeuchi et al., 2003). The C protein is produced by alternative translational initiation in a different reading frame. It co-localizes with

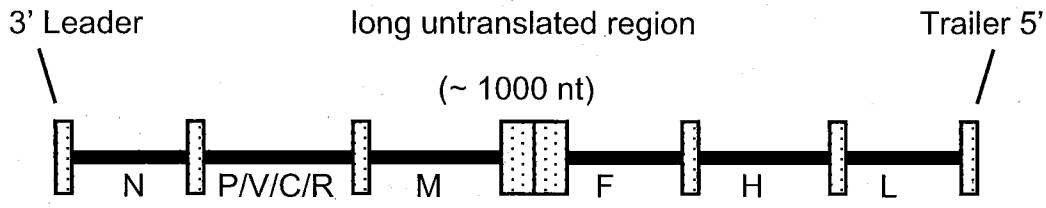
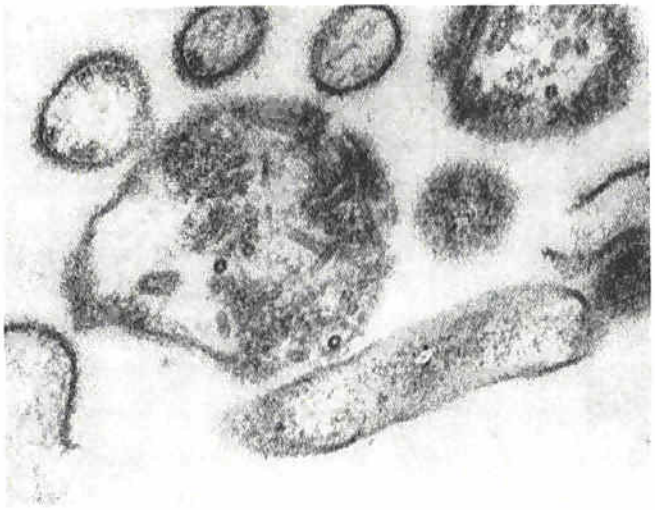



**Figure 2. Diagram of linear arrangement of genes in the measles virus genome (adapted from Griffin, 2001).**

A.



B.

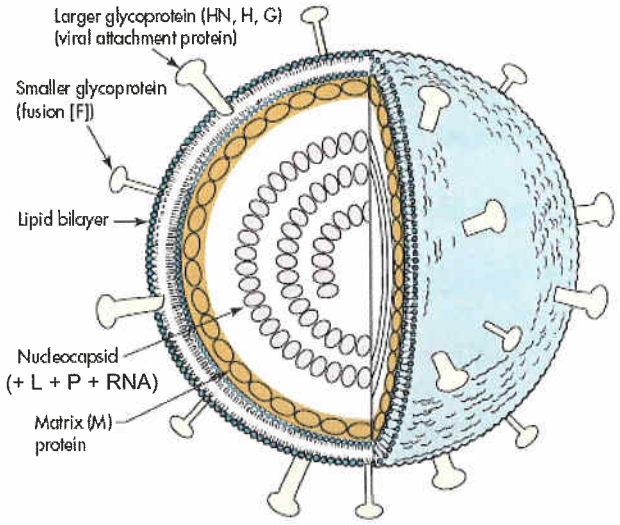



**Figure 3. Measles virion structure.**

(A) Electron micrograph of spherical and filamentous measles particles (from Nakai & Imagawa, 1969). (B) Diagram of paramyxovirus virion structure (from Medical Microbiology, 5th edition).

nucleocapsids and has been shown to interact with the L protein to regulate polymerase activity (Bankamp et al., 2005, Reutter et al., 2001). Additionally, the C protein has also been shown to inhibit interferon-α/β production and signaling (Shaffer et al., 2003). Although the V and C proteins are not necessary for replication in tissue culture, deletion of either protein results in reduced pathogenicity and virulence (Patterson et al., 2000). The R protein is a shortened form of the P protein that can be produced through ribosomal frame-shifting. It is almost identical to the V protein and may have a similar function (Liston & Briedis, 1995).

MV replication is initiated by virion attachment to the surface of a target cell, which is mediated by binding of MV H to one of its specific receptors, CD46 or CD150 (Fig. 4). Upon receptor binding, conformational changes of the H and F proteins are believed to mediate fusion between the viral and cellular membranes. It is thought that the M-nucleocapsid interactions are disrupted, and then, the nucleocapsid is released into the cytoplasm. The cytoplasm appears to be the primary site of replication for paramyxoviruses. However, morbilliviruses are unique within the family in that intranuclear inclusion bodies can also be detected (Llanes-Rodas & Liu, 1965, Oglesbee & Krakowka, 1993). The released nucleocapsid is competent for transcription and primary viral transcription begins almost immediately upon cell entry.

Following accumulation of the viral proteins in the cytoplasm, genomic replication is initiated. The antigenomes are encapsidated by N protein and

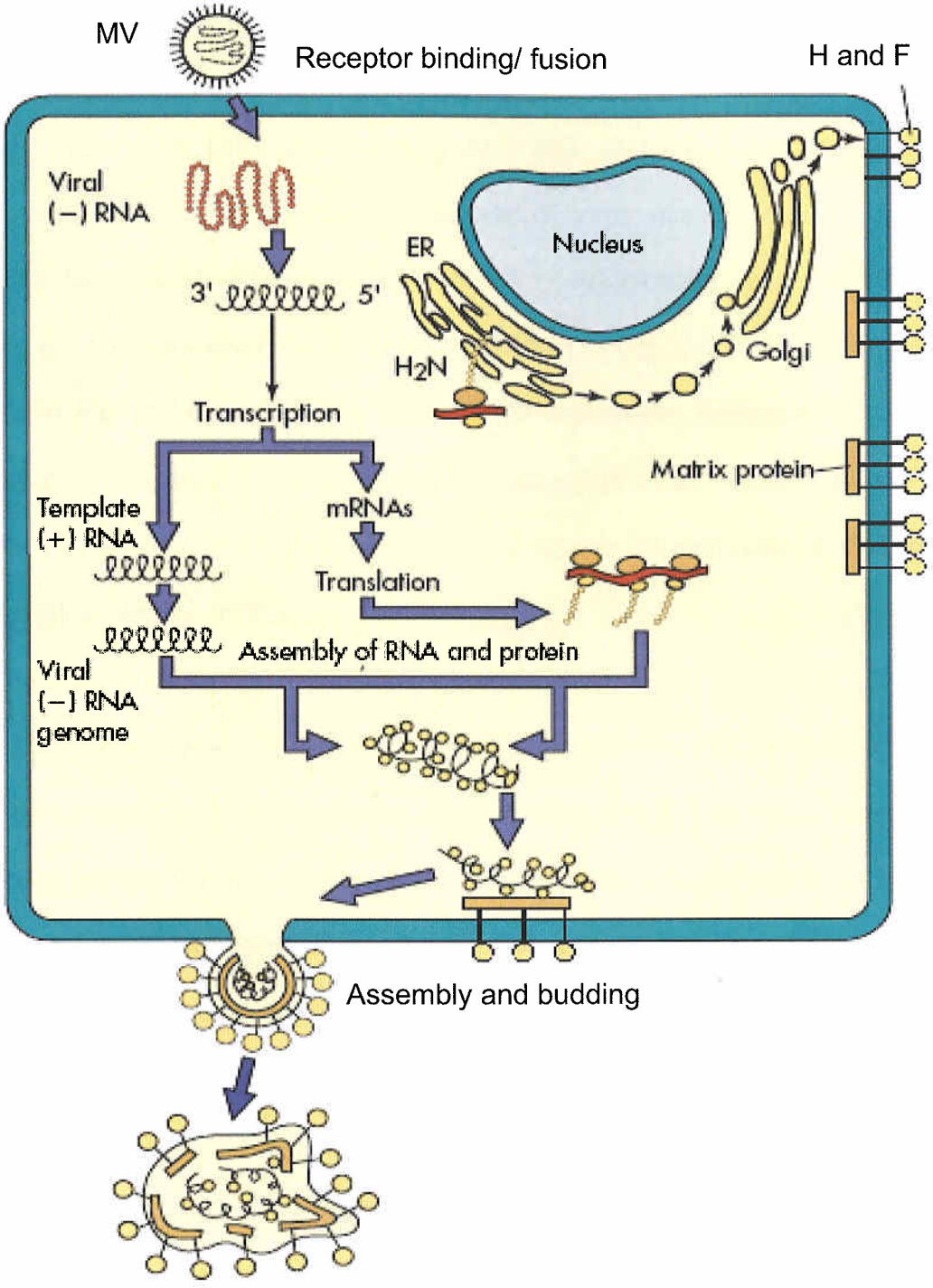



**Figure 4. Diagram of measles replication cycle from attachment to the cellular receptor through assembly and budding (from Murray et al., 2005).**

serve as the replication template to produce the full-length negative sense genomes that will be packaged into virions. Measles virion components are enriched in lipid rafts within the plasma membrane, and it has been suggested that these specific domains may be the site of virus assembly and budding (Manie et al., 2000, Vincent et al., 2000). Additionally, other host cell components may be involved in viral replication. Disruption of either actin filaments or microtubules prevents efficient MV replication, indicating a role for the cellular cytoskeleton in virion production (Berghall et al., 2004). Actin has also been found as a component of measles virions (Mountcastle & Choppin, 1977, Tyrrell & Norrby, 1978).

## 1.2 Cellular receptors

Despite its virulence, MV has a very limited tropism with humans serving as the only known natural reservoir. As receptor distribution is an important determinant of viral tropism, identification of the cellular receptors utilized by MV for infection has been important in understanding its pathogenesis. Unlike the majority of the other paramyxoviruses that bind to sialic acid on proteins, the morbilliviruses directly interact with specific cellular proteins. At this time, two receptors, CD46 and CD150, have been identified for MV. The complement regulatory protein CD46, also known as membrane cofactor protein (MCP), is a receptor for the Edmonston vaccine strain and its derivatives, as well as some clinical isolates (Dorig et al., 1993, Manchester et al., 2000a). The H-CD46 interaction is responsible for the ability of MV to promote aggregation of

erythrocytes isolated from some Old World primate species (Hsu et al., 1997). More recently, CD150, also known as signaling lymphocyte activation molecule (SLAM), was identified as a receptor for both wild type and vaccine strains of MV (Erlenhoefer et al., 2001, Ono et al., 2001a, Santiago et al., 2002, Tatsuo et al., 2000, Tatsuo et al., 2001, Yanagi et al., 2006).

### 1.2.1 CD46 (MCP)

CD46 belongs to the regulators of complement activation protein family, whose members control amplification of complement at the C3 step (Liszewski et al., 1991). Human CD46 is expressed on all cells with the exception of erythrocytes and it protects the cells from complement-mediated damage by restricting spontaneous complement activation (Barilla-LaBarca et al., 2002). Specifically, CD46 acts as a cofactor for the Factor I-mediated inactivation of C3b and C4b that leads to a block in the complement cascade at the C3 activation stage.

CD46 is a type I transmembrane protein that has eight known isoforms with tissue-specific expression patterns. All CD46 isoforms have four amino terminal short consensus repeats (SCRs) of approximately 60 aa each (Fig. 5). Each SCR has four invariant cysteines that form two intradomain disulfide bonds with a linker region of three to eight residues joining the adjacent domains. The SCRs are followed by a region rich in serine, threonine, and proline (STP), a region of unknown function, a transmembrane region, and a cytoplasmic tail. The H protein from the Edmonston vaccine strain is able to bind to all four of the

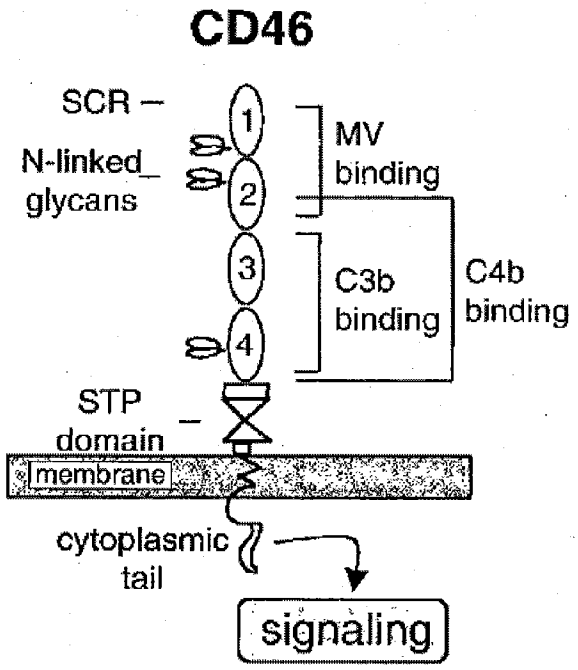



**Figure 5. Diagram of the structure of CD46, also know as membrane cofactor protein (MCP).**

CD46 (MCP) has four short consensus regions (SCRs) followed by a domain rich in serine, threonine, and proline (STP), as well as a region of unknown function, a transmembrane region, and a cytoplasmic tail (adapted from Manchester et al., 2000b).

primary isoforms to mediate infection (Maisner et al., 1994, Manchester et al., 1994). MV H binding to CD46 has been mapped to specific residues within SCRs 1 and 2 (Christiansen et al., 2000a, Hsu et al., 1999, Iwata et al., 1995, Manchester et al., 1995). Additionally, SCR 3 and 4 may influence the MV H – CD46 interaction by affecting the avidity of binding (Christiansen et al., 2000b, Devaux et al., 2004).

### 1.2.2 CD150 (SLAM)

Primary activated B and T cells, memory cells, activated monocytes and monocyte-derived dendritic cells express CD150. CD150 belongs to the CD2 family within the immunoglobulin (Ig) protein superfamily and functions as a co-receptor to modulate lymphocyte activation. Signaling through CD150 can promote T cell receptor-mediated cytotoxicity. Crosslinking of CD150 can enhance B and T cell proliferation and cytokine production.

CD150 is a type I integral membrane glycoprotein, and like other members of the Ig superfamily, has two extracellular domains, a variable (V) Ig-like domain and a membrane-proximal constant 2 (C2) domain (Fig. 6). A transmembrane region and a cytoplasmic tail follow the C2 domain. The region of CD150 required for measles binding and entry has been mapped to residues 60-63 within the N-terminal V domain (Christiansen et al., 2000b, Devaux et al., 2004, Ohno et al., 2003, Ono et al., 2001b). CD150 is also a receptor for other morbilliviruses, including CDV and RPV, which bind to canine and bovine CD150 respectively (Baron, 2005, Seki et al., 2003, Tatsuo & Yanagi, 2002).

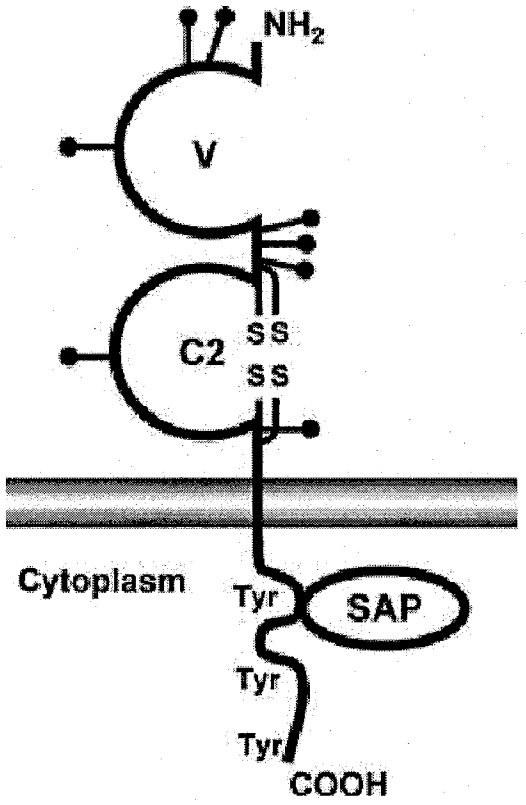



**Figure 6. Diagram of structure of CD150, also known as signaling lymphocyte activation molecule (SLAM) (adapted from Yanagi et al., 2002).**

CD150 has two extracellular domains including a variable (V) Ig-like domain and a membrane proximal constant domain (C2). It also has a transmembrane domain and a cytoplasmic tail, which binds to SLAM-associated proteins (SAPs).

## 1.3 Measles virus glycoproteins

The surfaces of measles virions and infected cells contain two types of viral transmembrane glycoproteins. The MV H glycoprotein binds to cellular protein receptors and is an important determinant of tropism. Unlike the attachment glycoproteins of most other paramyxoviruses, almost all of the morbillivirus receptor-binding proteins lack detectable neuraminidase (NA) activity, which mediates enzymatic cleavage of sialic acid from proteins. Thus, they are called hemagglutinin (H) rather than HN (hemagglutinin-neuraminidase) proteins. The MV F protein, with the aid of H, mediates virion-to-cell and cell-to-cell membrane fusion (Wild et al., 1991).

### 1.3.1 MV H

The MV H protein is a type II glycoprotein believed to form tetramers composed of pairs of disulfide-bound dimers. MV H has a short N-terminal cytoplasmic tail followed by a transmembrane domain and a large C-terminal ectodomain (Fig. 7). It is believed that the ectodomain consists of a stalk region that supports a globular domain containing the receptor recognition and antigenic regions of the protein. The measles H protein is multifunctional. It directly mediates receptor binding and the process of receptor recognition is thought to induce conformational changes in H that, in turn, activate fusion promotion by the F protein. Additionally, it is involved in post-infection down-regulation of surface expression of cellular receptors in order to prevent super infection of host cells, as well as progeny virion re-adsorption during assembly and budding.

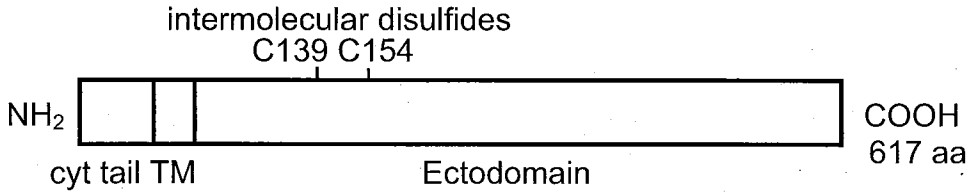


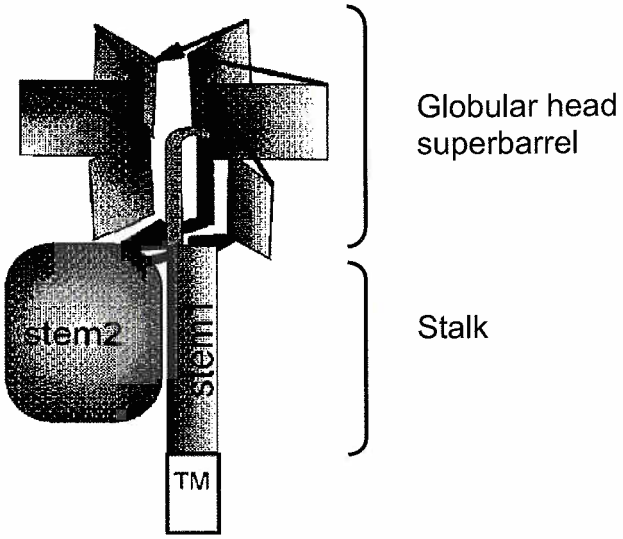

**Figure 7. Diagram of the measles hemagglutinin (H) protein.**

MV H has a short cytoplasmic tail followed by a transmembrane (TM) domain and a large ectodomain. It is believed that the ectodomain includes a stalk region that supports a globular domain containing the receptor recognition and antigenic regions. H dimers are held together by intermolecular disulfide bonds linking the cysteines at 139 and 154.

The morbillivirus H protein is thought to form a ß-propeller with six antiparallel ß-sheets arranged in a superbarrel cyclically around a center axis, analogous to the blades of a propeller (Langedijk et al., 1997). It is predicted that the ectodomain is initiated by a short helical stem that extends up from the transmembrane region to form the first ß-sheet and then a second helical stem that folds down to form part of the stalk before extending back up to form the remainder of the superbarrel (Fig. 8A). This unusual putative stem structure has not been confirmed experimentally. Although homology modeling predicts a NA active site for the H protein and slight enzymatic activity could be detected for RPV and peste-des-petits-ruminants virus, no activity was associated with MV (Langedijk et al., 1997).

Currently, no MV H protein crystal structures are available, but two different three-dimensional models of the globular domain of a monomer have been generated using sequence alignments along with the crystal structure of the NDV HN protein as a template (Fig. 8B) (Masse et al., 2004, Vongpunsawad et al., 2004). As a result of the crystallization method, the stalk region of the NDV HN protein is missing from the crystals, and thus, is also absent from the computer-generated MV H models. Although MV H has no detectable glycosylase activity, in the predicted structures, there is a conserved cluster of basic residues at the top center of the ß-propeller where the NA activity of other paramyxovirus receptor-binding proteins resides, suggesting that a species-

A.




B.

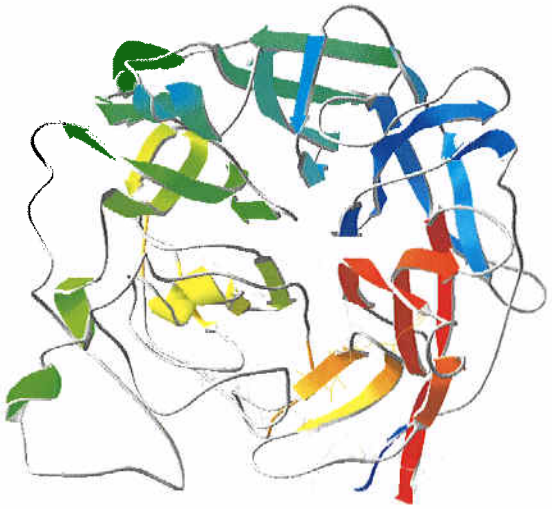


**Figure 8. Predicted structures of a measles hemagglutinin (H) protein monomer.**

(A) The morbillivirus H protein globular domain is thought to form a β-propeller with six antiparallel β-sheets arranged in a superbarrel cyclically around a center axis (adapted from Langedijk et al., 1997). The stalk is composed of two stem regions: stem 1 feeds into the first β-sheet from which stem 2 folds down and then extends back up to form the remainder of the superbarrel. (B) Three dimensional model of the globular region based on the crystal structure of NDV HN (from Vongpunsawad et al., 2004).

specific substrate may exist. However, although the two computer generated models differ from one another in other regions, the predicted NA catalytic site is absent from both.

The process of maturation of the H glycoprotein requires approximately five hours from translation to surface expression (Kohama et al., 1985). The first 154 residues are essential for MV H dimerization with cysteine residues at 139 and 154 forming intermolecular disulfide bonds (Plemper et al., 2000). MV H folds and oligomerizes in the endoplasmic reticulum (ER). It has five potential glycosylation sites at positions 168, 187, 200, 215, and 238. The first four sites are used, but glycosylation at 215 is heterogeneous. Glycosylation is essential for proper folding and dimerization of MV H, as well as its export from the Golgi (Hu et al., 1994a, Hu et al., 1994b).

Two types of the H protein can be detected on the surface of MV infected cells including one immature form with an apparent molecular mass of 74 kDa and another more complex 78 kDa form (Ogura et al., 2000). The mobility shift of the H protein results from addition of sialic acid to the oligosaccharide chains of the 74 kDa form in the trans-Golgi. The 74 kDa isomer can be isolated and is unable to agglutinate erythrocytes, suggesting that the addition of sialic acid is necessary for the interaction between MV H and CD46 (Ogura et al., 1991). However, other biological functions of the 74 kDa form of H, such as binding to CD150, receptor down-regulation and fusion promotion, have not yet been investigated.

Following the identification of CD46 as a cellular receptor for MV, several groups have attempted to map the regions of MV H involved in receptor recognition. Initially, deletions of MV H revealed that CD46 binding was dependent on the carboxyl terminal 18 amino acids (Blain et al., 1995). Subsequent studies demonstrated that mutations at MV H residues 451, 481, and 473-477 reduced CD46 recognition. Comparison of vaccine and clinical MV H protein sequences, in combination with site-directed mutagenesis, identified E451V and N481Y substitutions that transferred CD46 binding activities, including hemadsorption, CD46 down regulation, and fusion promotion, to the clinical strain (Lecouturier et al., 1996). The reciprocal V451E and Y481N mutations reduced these functions in the vaccine strain. It has also been demonstrated that mutation of residues 431 and 527, along with 451 and 481, results in a CD46-blind MV H protein, suggesting a role for these residues in CD46 recognition (Vongpunsawad et al., 2004). Additionally, chimeric proteins composed of segments from a MV H that binds to CD46 and the closely related RPV H, which does not bind CD46, revealed that the first 155 residues of MV H are not required for engagement of CD46 (Patterson et al., 1999). Further, peptide inhibition of binding, followed by alanine-scanning mutagenesis, was used to more finely map essential residues to 473-477.

More recently, through the use of a computer-generated model of MV H, three additional residues, 546-548-549, have been shown to form a potential CD46 binding site (Masse et al., 2004). Comparison of MV H sequences from

recently Vero-adapted strains and long-term passaged strains revealed a mutation at amino acid 546, suggesting a role for this residue in receptor binding activity (Shibahara et al., 1994). The involvement of this region in the CD46 binding activity of MV H was confirmed by comparison of MV H sequences from clinical isolates with those of their Vero-adapted progeny followed by site-directed mutagenesis of residue 546 (Li & Qi, 2002). Drastically reduced fusion promotion and CD46 down regulation was reported to result from mutation of the adjacent residues, S548L/F549S (Masse et al., 2002).

Two approaches have been used to identify MV H residues involved in CD150 binding. Both were based on the hypothesis that if all morbilliviruses use CD150 as a receptor, then the residues that interact with CD150 should be conserved. The first study also took into consideration that only vaccine strains of MV H are able to use CD46 as a receptor (Vongpunsawad et al., 2004). Three morbillivirus H sequences, including that of MV, CDV and RPV, were aligned and, initially, blocks of conserved and divergent residues were mutated. Site-directed mutagenesis of individual amino acids in the blocks that demonstrated the strongest decreases in promotion of fusion was used to identify the specific residues involved in binding to CD150. This approach led to identification of four residues, including 529, 530, 533 and 553, whose mutation decreased CD150-dependent fusion. In the second study, five morbillivirus H sequences were aligned and conserved residues were identified (Masse et al., 2004). Of the twelve residues characterized by mutagenesis, five, including 505, 507, 530, 533

and 536, were found to be involved in CD150-dependent fusion and down-regulation.

There is evidence of overlap between the MV H CD46 and CD150 binding sites. Several anti-MV H antibodies, as well as a CD46/CD55 chimeric receptor, can block binding of MV H to both CD46 and CD150 (Christiansen et al., 2002, Santiago et al., 2002). While it is possible that the inhibition results from a global steric hindrance of receptor binding by MV H, an overlap of CD46 and CD150 sites is consistent with more recent computer-generated models and mutagenesis studies (Masse et al., 2004, Vongpunsawad et al., 2004). In both models, the binding sites form a contiguous region along the rim of the top of the MV H monomer. In the models, amino acid 451 is not solvent-exposed and 481 is located on the side of the molecule, which suggests that these residues may not be directly involved in an interaction with CD46 or CD150. It is also possible that SCR1 interacts with 481 along the side of MV H, while SCR2 interacts with the top residues (Santiago et al., 2002). However, it has been proposed that mutation of residues 451, 481, or 473-477 disrupts receptor recognition by inducing conformational changes in the actual binding sites (Masse et al., 2004).

### 1.3.2 MV F

The MV F protein is a type I transmembrane glycoprotein believed to exist as trimers on the surfaces of virions and infected cells. The F protein is synthesized as an inactive precursor that is proteolytically cleaved into two subunits, $F_1$ and $F_2$, which are disulfide linked to form a single F monomer (Bolt &

Pedersen, 1998, Sato et al., 1988). The newly created amino terminus of $F_1$ is a hydrophobic domain known as a fusion peptide. It is thought that, prior to membrane fusion, receptor binding by MV H triggers conformational changes in the F protein that expose the fusion peptide for insertion into the target membrane to initiate the fusion process.

The MV F protein is translated as a 60 kDa precursor protein known as $F_0$ (Fig. 9). Processing of the precursor into the active form requires oligomerization, glycosylation, and proteolytic cleavage. Activation of $F_0$ occurs through proteolytic digestion by furin in the trans-Golgi network to form two subunits, $F_1$ and $F_2$. The cleavage site consists of a sequence of five basic amino acids, RRFKR (Bolt & Pedersen, 1998). Cleavage results in a new amino terminus for the $F_1$ subunit known as the fusion peptide. The fusion peptide is a highly hydrophobic stretch of 32 residues and is thought to penetrate target membranes. The MV F glycosylation sites are located in the $F_2$ subunit at residues 29, 61, and 67. Glycosylation of MVF is important for proper folding and surface expression of the protein and has been shown to influence fusion promotion (Alkhatib et al., 1994, Hu et al., 1995, von Messling & Cattaneo, 2003).

The MV F protein can be divided into multiple domains with putative roles in the membrane fusion process (Fig. 9). Three heptad repeats (HR), which consist of hydrophobic residues, such as leucine and isoleucine, separated from one another by six amino acids, have been identified in the MV F protein sequence, including HR-A, -B, and -C. HR-C is located in the $F_2$ subunit and has

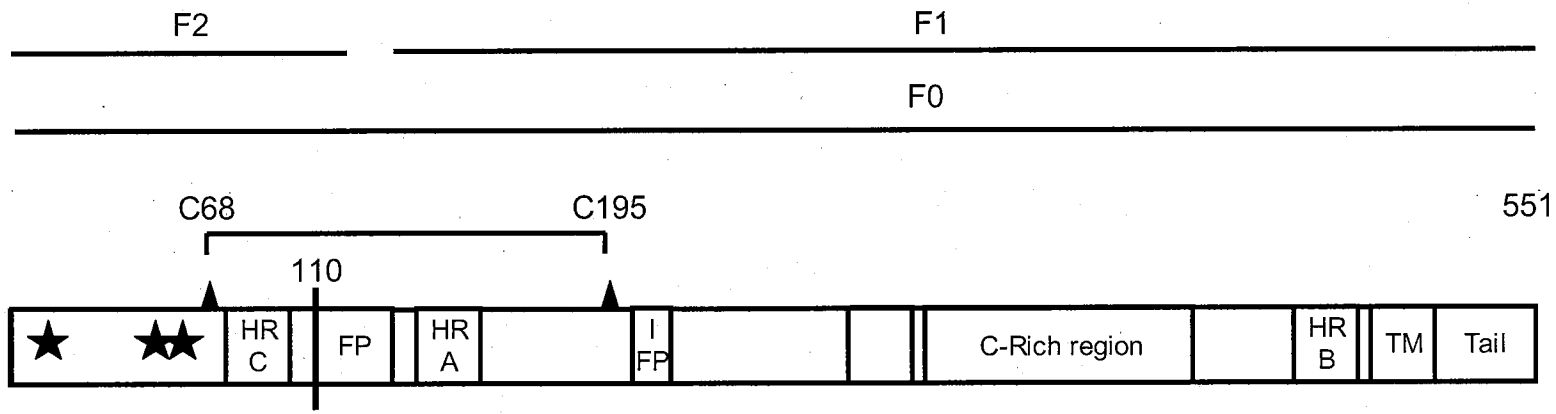



**Figure 9. Diagram of measles fusion (F) protein structure.** F is translated as a precursor $F_0$, which is cleaved into disulfide linked subunits $F_1$ and $F_2$. $F_2$ has a heptad repeat (HR-C) and three glycosylation sites (denoted by stars). Cleavage results in an amino terminus of $F_1$ known as the fusion peptide (FP). $F_1$ also has two heptad repeats (HR-A and HR-B), a potential internal fusion peptide (IFP), and a cysteine-rich (C-rich) region. $F_1$ and $F_2$ are held together by a disulfide bond between cysteine 68 and 195.

been found to play a role in fusion promotion (Plemper et al., 2003). While HR-A is located at the amino terminal end of $F_1$ just downstream of the fusion peptide, HR-B is at the opposite end of the protein subunit, just upstream of the transmembrane region. HR-A and HR-B are leucine zippers predicted to form α-helices that interact with one another during the conformational changes associated with membrane fusion induction (Buckland et al., 1992, Wild & Buckland, 1997, Zhu et al., 2003).

The disulfide bond that holds the two subunits together links a cysteine at position 68 immediately amino terminal to HR-C in $F_2$ with a cysteine at position 195 in HR-A in $F_1$. A hydrophobic fusion peptide-like sequence has been identified immediately downstream from HR-A. It has been proposed that this internal domain interacts with the target membrane during the F protein conformational changes prior to insertion of the amino terminal fusion peptide (Samuel & Shai, 2001). Additionally, there is a series of eight highly conserved cysteine residues that form a domain known as the C-rich region. The eight cysteines are predicted to form four disulfide-linked loops at the carboxyl end of the region between HR-A and HR-B (Wild et al., 1994). Recently, it has been discovered that the $F_1$ subunits of measles, mumps, and CDV undergo partial membrane-proximal cleavage following cleavage activation of $F_0$ (von Messling et al., 2004). This proteolytic processing requires a stretch of six residues adjacent to the transmembrane region and appears to be involved in the efficiency of fusion promotion.

The structure of the morbillivirus F protein is thought to be similar to those of the type I fusion proteins of the other paramyxoviruses. Currently, no MV F protein crystal structures are available, but a three-dimensional model of the extracellular domain has been generated using sequence alignment along with the crystal structure of the pre-fusion NDV $F_0$ protein as a template (Chen et al., 2001a, Chen et al., 2001b, Plemper et al., 2003).

The MV F structure is made up of three intertwined monomers that can be divided into three domains including a head and neck, formed from regions of $F_1$ and $F_2$, as well as a stalk composed of the three HR-A domains in a coiled-coil interaction (Fig. 10A). An axial channel appears to extend through the head and neck and split off into three radial channels located near the head-neck interface (Fig. 10B). It has been proposed that the three hydrophobic fusion peptides of the trimer are sequestered between the subunits prior to fusion initiation (Yin et al., 2006). The head of the molecule consists of a ß barrel domain with a hydrophobic core surrounded by an Ig-like domain partially formed by the cysteine-rich region in $F_1$. The neck and stalk are formed by the triple stranded coiled-coil of HR-A along with the HR-C helix and an additional ß barrel domain. As a result of the crystallization method, the transmembrane domain and cytoplasmic tail are absent from the NDV F structure and thus the MV F model. In addition, the cleavage site, fusion peptide, HR-B and part of HR-A are missing due to degradation of the purified NDV F protein. It has been suggested that the purified NDV F protein may have undergone spontaneous re-folding and that the

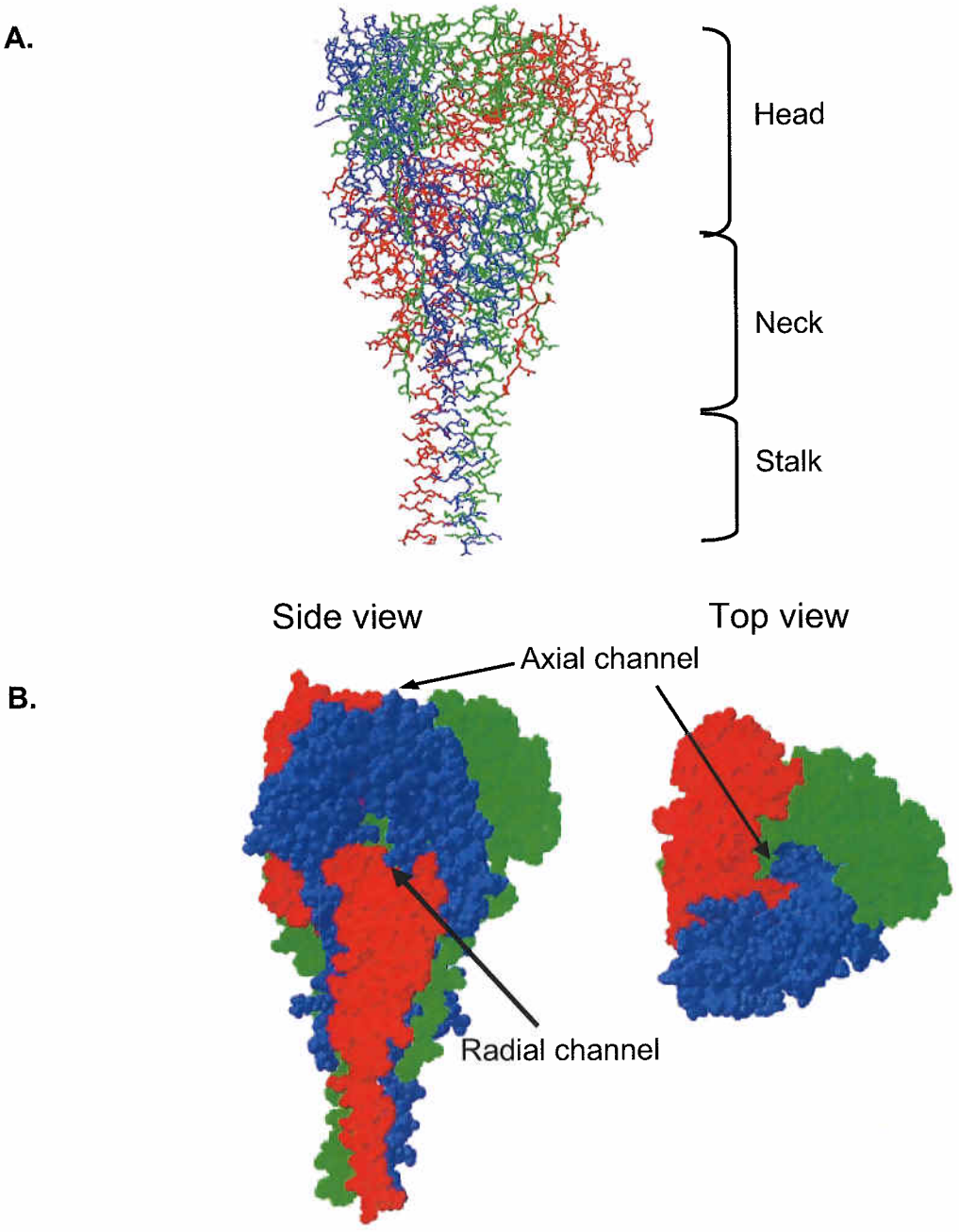



**Figure 10. Predicted structure of measles fusion (F) protein trimer based on the crystal structure of NDV F (adapted from Plemper et al., 2003).**

(A) The measles structure is made up of three intertwined monomers and can be divided into three main domains including the stalk, neck, and head. (B) An axial channel extends through the head and splits off into three radial channels at the head-neck interface.

crystal structure, as well as the measles F model, represents a post-fusion state (Lamb et al., 2006, Yin et al., 2006).

## 1.4 Mechanism of membrane fusion promotion by MV H and F

### 1.4.1 Requirement for MV H in membrane fusion

Paramyxoviruses, like other enveloped viruses, enter cells by fusing their lipid membranes with those of the target host cell. Paramyxovirus glycoprotein-promoted membrane fusion is thought to involve a series of conformational changes in the F protein structure from a metastable pre-fusion state to a highly stable post-fusion state. These changes occur at neutral pH at the surface of the target membrane and are triggered by MV H receptor recognition.

Expression of both MV H and F is required for membrane fusion (Wild et al., 1991). As the first step of the process, MV H mediates receptor binding in order to bring the two membranes into close proximity prior to F activation. In addition, there is evidence that MV H is involved in triggering F activation through a specific interaction between the two proteins. First, although the H glycoproteins of MV and CDV are to a certain extent interchangeable, fusion promotion is most efficient when the H and F proteins are derived from the same virus and strain (Bossart et al., 2002, Cattaneo & Rose, 1993, Stern et al., 1995, von Messling et al., 2001, Wild et al., 1994). Second, monoclonal antibodies to MV H that block infectivity and inhibit fusion without interfering with receptor recognition have been characterized (Fournier et al., 1997, Hu et al., 1993).

Together, these studies support the proposal that MV H plays a role in membrane fusion promotion beyond that of receptor recognition.

### 1.4.2 Conformational changes of paramyxovirus attachment proteins

Crystal structures have been solved for the ectodomains of the attachment proteins from three paramyxoviruses, including NDV, human parainfluenza virus-3 (hPIV3), and SV5, in both liganded and unliganded states (Crennell et al., 2000, Lawrence et al., 2004, Yuan et al., 2005, Zaitsev et al., 2004). Based on comparison of the NDV HN structures, it has been suggested that the globular head of the attachment proteins undergoes conformational changes after receptor binding. Ligand binding was found to dramatically alter the association of NDV HN dimers, indicating that changes in dimer association may be involved in the mechanism through which HN triggers fusion promotion. However, the low pH conditions required for crystallization of the unliganded form have led to uncertainty regarding the physiological relevance of the structure (Crennell et al., 2000). Crystallization of NDV under different conditions led to identification of an additional sialic acid binding site at the dimer interface (Zaitsev et al., 2004). Based on this structure, it was proposed that interaction of the neuraminidase active site with sialic acid leads to changes in the dimer interface that expose a second sialic acid-binding site (Zaitsev et al., 2004). Comparison of the various NDV HN crystal structures led to the suggestion that ligand binding by HN alters its oligomeric structure, leading to activation of fusion promotion by the F protein.

Crystal structures have also been solved for the hPIV3 and SV5 HN proteins in the presence and absence of ligands (Lawrence et al., 2004, Yuan et al., 2005). In contrast to the structures of NDV HN, the hPIV3 and SV5 structures did not reveal significant changes in the oligomeric assembly of the proteins upon ligand binding. However, it remains possible that receptor recognition could trigger a partial disassembly of the HN tetramer, or dimer, and, thus, an alteration in the HN-F interaction (Fig. 11) (Lamb et al., 2006). As it is thought that receptor recognition by the attachment protein triggers activation of the F protein, it is possible that these conformational changes are required for an interaction between the H and F proteins.

At this time, the relationship between receptor recognition by the attachment protein, the interaction between the attachment and fusion proteins, and the promotion of fusion has not been established. Co-immunoprecipitation assays have suggested that receptor recognition by NDV HN triggers an interaction with the F protein at the cell surface (Deng et al., 1999, Li et al., 2004, Melanson & Iorio, 2004). However, evidence has also been presented that suggests that NDV HN and F are associated at the cell surface prior to receptor recognition and that ligand binding by HN triggers dissociation of the complex (McGinnes & Morrison, 2006). In contrast to NDV, which binds to sialic acid, the morbilliviruses bind to specific cellular proteins to initiate membrane fusion with host cells. Because MV H binds to known proteins to initiate fusion, it is possible that this controversial aspect of the mechanism of membrane fusion

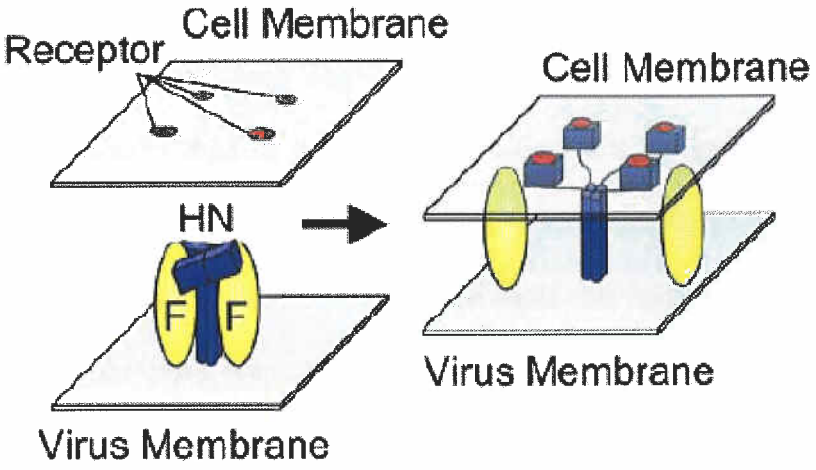



**Figure 11. Model for attachment protein (HN) conformation changes after receptor recognition (from Lamb et al., 2006).**

Receptor recognition triggers partial disassembly of the HN tetramer and an alteration in the HN-F interaction.

can be more clearly elucidated using the measles virus glycoproteins, facilitated by the ability to express the proteins in a receptor-free system.

### 1.4.3 Conformational changes of paramyxovirus F proteins

It is believed that the fusion process mediated by the paramyxovirus glycoproteins also requires conformational changes in the F protein from a metastable pre-fusion state to a highly stable post-fusion state. During the process, the hydrophobic fusion peptide is released and enters the target cell membrane. HR-A and HR-B are thought to undergo rearrangements that result in a stable six-helix bundle (6 HB) structure that pulls the lipid bilayers together in order to promote fusion pore formation.

Recently, crystal structures have been solved for the solubilized ectodomain of the $F_0$ protein of hPIV3 and a modified stabilized $F_0$ protein of SV5 (Yin et al., 2005, Yin et al., 2006). Within the ectodomain of hPIV3, in the absence of its cytoplasmic and transmembrane regions, HR-A and HR-B form a 6 HB similar to that predicted by the NDV F crystal structure. There is a strong similarity between the structures in terms of location of the axial and radial channels. However, there are slight differences in the arrangements of their sub-domains that may be a reflection of sequence differences. Both the NDV and hPIV3 F proteins were crystallized without native transmembrane and cytoplasmic domains and appear to represent the post-fusion structure (Lamb et al., 2006, Yin et al., 2006). This suggests that the transmembrane and/or cytoplasmic tail regions may be required for formation of the pre-fusion structure

and/or for its stability at the membrane surface. Additionally, the fusion peptide is missing from both structures, and thus, its post-fusion location remains unknown.

Comparison of the putative post-fusion crystal structures with that of a stabilized SV5 F protein reveals several conformation differences between the pre and post-fusion states of paramyxovirus F proteins (Fig. 12). In the stabilized SV5 F protein, which has been modified by the addition of a coiled coil domain that mimics the transmembrane domain, HR-B forms a helical stalk and the base of a head domain. Within the head domain, HR-A folds around a core that consists of three anti-parallel β-strands, an α-helix formed by HR-C, and a helical bundle. In this structure, HR-A is prevented from interacting with HR-B and the fusion peptide is buried within the interface between two monomers of the trimer. In contrast, in the hPIV3 structure, which is thought to represent the post-fusion conformation of F, HR-A and HR-B come together to form a 6 HB. Based on these differences, it has been proposed that fusion is initiated by the melting of the HR-B stalk and changes within the head domain (Lamb et al., 2006, Yin et al., 2006). HR-A could then form a trimeric coiled-coil structure leading to translocation of the fusion peptide into close proximity to the target membrane. Re-folding and compaction of the head domain could then lead to relocation of HR-B and formation of the 6 HB that is characteristic of the post-fusion state.

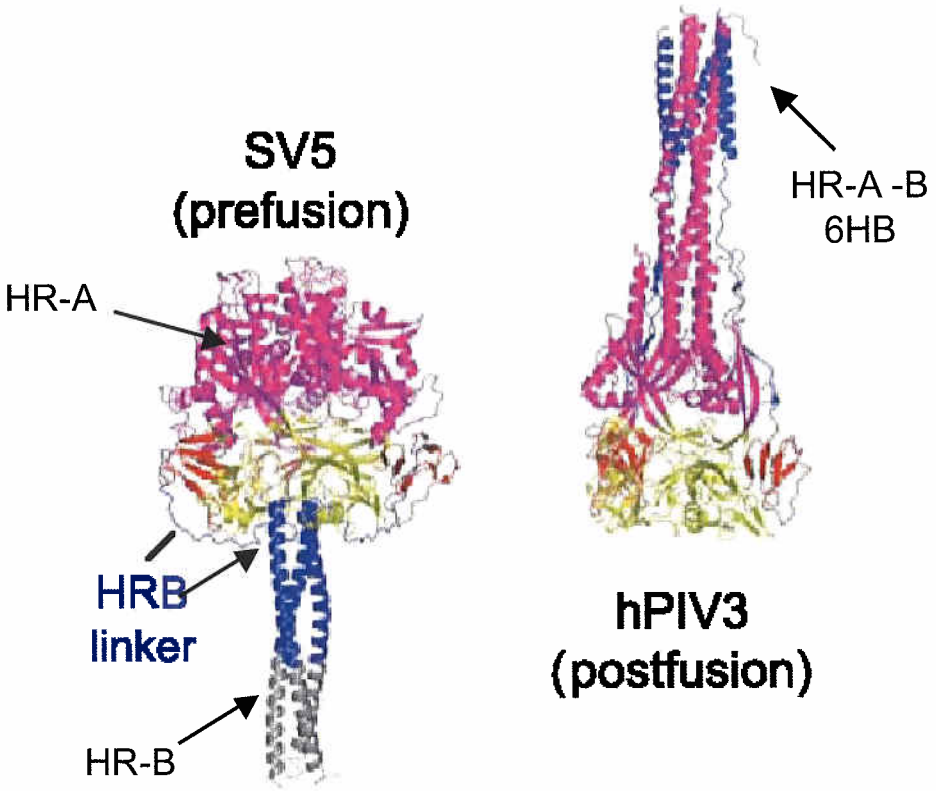



**Figure 12. Comparison of pre-fusion and post-fusion F structures (adapted from Yin et al., 2006).**

In the pre-fusion state, HR-B forms a helical stalk and the base of the head domain. HR-A folds around the core of the head domain and does not interact with HR-B. In the post-fusion state, HR-A and HR-B form a six-helix bundle (6 HB).

### 1.4.4 Interaction of MV H with F

Based on chemical crosslinking and co-immunoprecipitation of the NDV HN and F proteins independent of F cleavage, as well as a decrease in F immunoprecipitation upon receptor binding, it has also been argued that the NDV glycoproteins interact in the ER (Stone-Hulslander & Morrison, 1997). Additionally, expression of ER retention signal (KDEL) tagged F proteins from hPIV2 and hPIV3 results in down regulation of the surface expression of the corresponding HN protein (Tanaka et al., 1996, Tong & Compans, 1999). However, expression of the ER retention signal tagged proteins also decreased surface expression of the receptor binding proteins from other viruses, suggesting a non-specific interaction. In contrast, it has been suggested that the interaction of hPIV3 and SV5 HN and F proteins occurs at the cell surface rather than intracellularly (Paterson et al., 1997). Addition of ER retention signals to the cytoplasmic tails of the HN or F protein was not found to alter the intracellular transport of the partner protein for either virus.

Although measles H and F are efficiently transported to the cell surface when expressed independently, there is evidence of an intracellular interaction occurring between the two proteins. Addition of an ER retention signal to the cytoplasmic tail of MV H or F, RRRRR or KSKTH respectively, was concluded to result in efficient intracellular retention of both proteins (Plemper et al., 2001). Co-transfection of either ER retained protein with its wild type partner was

reported to result in a decrease in the kinetics of the processing of the non-tagged protein, suggesting the formation of an intracellular complex.

It is important to note that there are problems associated with the co-retention aspect of the measles glycoprotein ER retention study. First, it was not determined if co-expression of the ER-tagged proteins altered the cell surface expression and/or the function of the untagged partners. Evidence for co-retention was based solely on detection of a decrease in the kinetics of the processing of the non-tagged heterologous protein. Second, it was not determined if the putative co-retention was specifically associated with co-expression of the two measles glycoproteins. Expression of ER retention signal-tagged F proteins from hPIV2 and hPIV3 has been demonstrated to down-regulate the surface expression of not only the homologous HN protein, but also that of the attachment proteins of other paramyxoviruses, including measles (Tanaka et al., 1996, Tong & Compans, 1999). The non-specific nature of this phenomenon is inconsistent with a virus-specific glycoprotein interaction that is required for membrane fusion. Third, the experiments that were used to demonstrate the co-retention of heterologous proteins involved the use of FLAG epitope-tagged measles glycoproteins. While it was demonstrated that the FLAG epitope did not alter the processing of the proteins, it is possible that it could modulate the interaction between the H and F proteins. For example, addition of a FLAG epitope to the cytoplasmic tail of MV H has been shown to weaken an interaction between H and F (Plemper, 2002).

Although cell surface glycoprotein interactions in the absence of protein crosslinkers have been detected for other paramyxoviruses, chemical crosslinking was required to detect an association between MV H and F (Deng et al., 1999, Malvoisin & Wild, 1993, McGinnes & Morrison, 2006, Yao et al., 1997). More recently, an assay involving a Western blot to detect the co-immunoprecipitated measles glycoprotein has been used to study variations in the strength of the interaction between MV H and F (Plemper & Compans, 2003, Plemper et al., 2002, Plemper et al., 2003). The strength of the interaction between MV H and F has been shown to be inversely related to the extent of membrane fusion (Plemper et al., 2002). However, this assay does not distinguish between intracellular and surface interactions between the two proteins, and it remains unknown if there is a direct physical association of the MV H and F proteins at the cell surface.

Indirect evidence for a surface interaction is provided by an investigation of virus assembly in lipid raft microdomains (Vincent et al., 2000). In this study, it was demonstrated that MV F, in contrast to MV H, has the intrinsic ability to localize to lipid rafts within the cell membrane. When MV H is co-expressed with MV F, it also localizes to lipid rafts, indicating that there may be an interaction between the two proteins during the assembly process.

The exact mechanism by which MV H receptor recognition triggers conformational changes in F remains unknown. Based on the requirement for expression of MV and CDV glycoproteins from the same virus and strain for

maximum fusion efficiency, it has been suggested that a specific physical interaction between the proteins is required for fusion promotion (Cattaneo & Rose, 1993, von Messling et al., 2001). Additionally, it is thought that specific domains in each protein mediate the specific interaction. While multiple regions of both H and F have been shown to be involved in fusion promotion, it has not been conclusively determined which domains directly mediate the H-F interaction.

### 1.4.5 Regions of MV H required for fusion

In addition to the residues that mediate receptor recognition, there are three main regions of MV H that have been suggested to have a role in fusion promotion. First, a mutation at residue 98 in the stalk was found to be responsible for the lack of syncytium formation in a persistently infected cell line (Hummel & Bellini, 1995). However, this study did not examine the effect of this mutation on receptor recognition. Second, region 244 to 250 has been identified as a linear epitope in the globular region that binds to a monoclonal antibody (MAb) that inhibits syncytium formation, but not receptor recognition (Fournier et al., 1997). Based on this evidence, it has been suggested that this region constitutes either a functional or physical interface between MV H and F. However, this hypothesis was not directly tested and it remains possible that the antibody blocks fusion by preventing conformational changes in MV H or sterically blocking the H-F interaction. The third region of MV H shown to have a role in fusion is the cytoplasmic tail. Truncation and mutagenesis of the tail was

used to demonstrate that a minimum of 14 cytoplasmic residues is required for fusion promotion (Moll et al., 2002). In addition, when receptor recognition is normalized for expression, fusion promotion is disproportionately reduced, suggesting that alteration of the cytoplasmic tail of MV H reduces the efficiency of fusion promotion in a way other than interfering with receptor binding activity. It is important to note that each of these studies provides indirect evidence for involvement of a specific region of MV H in fusion promotion without directly determining if it mediates the physical H-F interaction.

### 1.4.6 Regions of MV F required for fusion

In addition to the fusion peptide that inserts into the target membrane, there are several other domains in the MV F protein that are believed to contribute to various aspects of membrane fusion. It has been demonstrated that the HRs in both $F_1$ and $F_2$ contribute to the fusion process. The C-rich region in $F_1$ is proposed to be a determinant of the specificity of the MV H-F interaction (Wild et al., 1994). Additionally, cysteine residues in the transmembrane region were found to modulate fusion promotion, possibly by acting as sites for palmitoylation (Caballero et al., 1998). In contrast to those of the hPIV3, NDV, and SV5 F proteins, as well as that of MV H, the cytoplasmic tail of MV F was found to be unnecessary for fusion promotion (Bagai & Lamb, 1996, Moll et al., 2002, Sergel & Morrison, 1995, Yao et al., 1997).

Mutagenesis of the four heptadic leucines in HR-B inhibits fusion promotion without altering oligomerization or processing, suggesting that the

region may be involved in fusion pore formation (Buckland et al., 1992). Recently, a residue in HR-B was found to provide conformational stability to MV F, as well as to modulate the affinity of HR-B for HR-A (Doyle et al., 2006). In contrast, residues in HR-C have been shown to be important for both protein folding and the strength of the H-F interaction (Plemper & Compans, 2003).

In addition to the three previously described HRs in MV F, a fourth HR-like region, HR-D, is located in $F_1$ between HR-A and the C-rich region. This domain was first identified in the F protein of SV and is conserved in several members of the paramyxovirus family, including MV (Ghosh et al., 1997, Ghosh & Shai, 1998). HR-D is not predicted to form an α-helical structure or to mediate coiled-coil interactions. It was demonstrated that a peptide identical to HR-D of SV F could self-assemble, associate with peptides identical to HR-A and -B, and disrupt packing of lipid membranes. This evidence suggests that HR-D may be involved in the oligomerization of the F protein, the conformational changes of F during fusion promotion, and/or the destabilization of the target membrane. Analysis of a similar peptide from SV5 found that HR-D is not involved in the final 6 HB structure (Dutch et al., 1999). Alanine-scanning mutagenesis of the heptadic residues in HR-D of NDV led to identification of a single mutant, L289A, which promoted fusion in the absence of HN (Sergel et al., 2000). Additionally, the L289A mutation enhanced fusion when co-expressed with HN. Mutation of the other heptadic leucines altered processing and/or expression of the F protein. Further, the enhanced fusion phenotype of the L289A mutant has been found to

result from decreased dependence on receptor recognition by NDV HN, possibly through destabilization of the pre-fusion conformation of NDV F (Li et al., 2005). Construction of chimeras and screening for fusion promotion led to identification of a single mutation, F278L, that was responsible for the reduced fusion phenotype of the AIK-C vaccine strain of MV (Nakayama et al., 2001). F278 is located immediately upstream of HR-D, suggesting that the region may be involved in fusion promotion by measles.

The primary domain of MV F thought to mediate the specificity of the interaction with MV H was mapped to the amino terminus of the C-rich region. This region was identified through the construction of chimeric proteins in which parts of CDV F were replaced with complementary portions of MV F, followed by screening for proteins that were able to promote fusion when co-expressed with MV H (Wild et al., 1994). Based on the results of this study, it was concluded that the first 44 residues of the C-rich region are responsible for the specificity of MV F for MV H. However, there are problems associated with evidence presented by this study that weaken the conclusions drawn regarding the role of the C-rich region in fusion promotion. First, not all chimeras containing the amino terminus of the C-rich region were able to promote fusion with MV H, suggesting that other regions could be involved in determining the specificity of the H-F interaction. Second, no actual quantitative data was presented; fusion promotion was reported as (+) or (–). Third, fusion with CDV H was not tested. This is an important point because, although fusion promotion is most efficient when the H

and F proteins are derived from the same virus and strain, the MV and CDV H proteins are to a certain extent interchangeable for fusion with MV F. Fourth, cleavage and processing of the chimeric proteins were not verified. Although some of the chimeras were detected by immunofluorescence, it remains possible that they were not properly cleaved or glycosylated. Finally, the investigators concluded that the C-rich region is the site of the MV H-F interaction without directly testing whether the F chimeras are able to physically interact with MV H. Mutation of the C-rich region could disrupt other aspects of fusion promotion, such as fusion pore formation, that were not tested.

## 1.5 Objectives of dissertation

The mechanism used by morbilliviruses to infect and spread among host cells differs from that of most other paramyxoviruses at the very first steps of the process. First, it has been asserted that, in contrast to some other paramyxoviruses, the MV H and F proteins interact intracellularly prior to receptor recognition. Second, while most of the paramyxoviruses bind to sialic acid in order to enter cells, the morbilliviruses engage specific protein receptors. Third, unlike those of most the other paramyxoviruses, the morbillivirus attachment glycoproteins have very low or undetectable levels of NA activity. Given these differences in the receptor binding proteins, it is likely that the mechanism of MV-induced membrane fusion differs from that of the other paramyxoviruses at other steps as well. A more complete understanding of the means by which measles

promotes membrane fusion may allow for development of specific anti-viral strategies that disrupt the early stages of infection. Therefore, the goal of this dissertation is to further investigate the mechanism of measles H and F glycoprotein-mediated membrane fusion. The **first aim** is to re-examine the conclusion that MV H and F form an intracellular complex. The **second aim** is to examine the H-F interaction at the cell surface and its relationship with receptor recognition by MV H. The **third aim** is to characterize the functions of two regions of H, including a heptad repeat domain in the stalk and the region 244 to 250 in the head, in the promotion of membrane fusion. The **fourth aim** is to characterize the roles of the HR-D domain and C-rich region in MV F in fusion promotion.

# CHAPTER II

## Materials and methods

### 2.1 Cell culture and transient transfection systems

#### 2.1.1 Cell lines

HeLa and CV-1 cells were maintained in Dulbecco's Modified Eagle medium (DMEM) with high glucose, L-glutamine, and pyridoxine hydrochloride supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mM MEM non-essential amino acids solution, 4 U/ ml penicillin and 4 µg/ ml streptomycin. CHO cell lines, including CHO 4.9 and CHO-CD46 4.5 (gifts of Dr. Kah-Whye Peng, Mayo Clinic Cancer Center), and Vero cells were maintained in the same medium with the exceptions of 5% FCS and 1 mM sodium pyruvate. BHK-21 cells were maintained in the same medium with the exceptions of 5% FCS and the absence of non-essential amino acids. All tissue culture reagents were obtained from Invitrogen (Carlsbad, CA).

#### 2.1.2 Plasmid constructs

The H and F genes of the Edmonston MV strain were a gift from Dr. Michael Oldstone (The Scripps Research Institute). MV H was released from the vector in which it was supplied by *Sac*II digestion and MV F was released by *Xho*I/ *Sac*II digestion. Both genes were ligated into pBluescript SK(+) (pBSK) (Stratagene, La Jolla, CA). The H and F genes of the Ondersterpoort CDV strain were a gift from Dr. Shmuel Rozenblatt (Tel Aviv University). Both genes were

released from the vectors in which they were provided by *Nco*I/ *Stu*I digestion and ligated into pBSK. MV H genes were excised from pBSK by *Xho*I/ *Sac*II digestion, blunt-ended by treatment with DNA polymerase I, large (Klenow) fragment (New England Biolabs, Beverly, MA) and T4 DNA polymerase (Roche Applied Science, Indianapolis, IN) and subcloned into pCAGGS (gift of Dr. Anne Moscona) at *Sma*I. The MV F gene was released from pBSK by *Kpn*I/ *Nhe*I digestion and ligated into pCAGGS using the corresponding sites. The CDV F gene was excised from pBSK by *Nco*I/ *Spe*I digestion, blunt-ended and subcloned into pCI (Promega, Madison, WI) at *EcoR*I. The MV genes were excised from pBSK with *Xho*I/ *Sac*II and subcloned into the corresponding sites of pCI.

### 2.1.3 Propagation of vaccinia viruses

CV-1 cells were grown to 60 to 70% confluence, inoculated with wt vaccinia virus or recombinant vaccinia virus carrying the T7 RNA polymerase gene (vTF7-3) (Fuerst et al., 1986) at a moi of 1 and incubated at 37°C in 5% $CO_2$ for 2 hours. After the inoculum was removed, the infected cell monolayers were washed with DMEM and incubated for 40 to 48 hours at 37°C in 5% $CO_2$ in complete medium until 50% CPE was reached. The infected cells were harvested by scraping followed by centrifugation at 2500 rpm for 5 min. The cells were resuspended in 1 ml DMEM per plate and the virus was released from the cells by three freeze/ thaw cycles followed by sonication on ice for three 10 sec

intervals. Finally, the cell debris was removed by centrifugation at 1000 rpm for 5 min and the supernatant was stored at -70°C until use. Viruses were titered in BHK-21 cells prior to use.

### 2.1.4 Vaccinia T7 RNA polymerase-driven transient expression

Infection by vTF7-3 was used to drive expression in HeLa cells transfected with genes under the control of the T7 promoter in pBSK or pCI. Cells were seeded a day prior to transfection at 3.5 x $10^5$ per well in 6-well plates. On the day of transfection, the monolayers were infected with vTF7-3 at a moi of 0.5 and incubated for 1 hour at 37°C. The cells were washed with DMEM and transfected with 0.5 µg of each DNA complexed with dimethyldioctadecylammonium bromide (DDAB) in OptiMEM for a total of 1 ml per well. After a five-hour incubation at 37°C, 1 ml of cell maintenance medium was added to each well and the cells were returned to 37°C for at least 20 hours. For co-immunoprecipitation assays, cells were seeded at 3 x $10^5$ per well and 1 ml DMEM was added immediately following addition of the DNA-DDAB complexes and incubated for a maximum of 16 hours at 37°C.

### 2.1.5 Transient expression with chicken β-actin and CMV promoters

Plasmid constructs were transfected into cells seeded in 6-well plates a day earlier at 2 x $10^5$ per well using PolyFect transfection reagent (Qiagen, Inc., Valencia, CA) or Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN) according to the protocols provided by the manufacturers. For

most experiments, 3 µg of each DNA were transfected per well and assays were performed at 18, 24, or 48 hours post-transfection.

## 2.2 Mutagenesis and chimera construction

### 2.2.1 Site-directed mutagenesis

pBSK or pCI-based templates, were transformed into *Escherichia coli* strain CJ236 (New England Biolabs, Beverly, MA) and single-stranded DNA (ssDNA) was rescued by R408 helper phage (Stratagene, La Jolla, CA). The DNA was precipitated with 20% polyethylene glycol and purified by phenol/ chloroform extraction. Mutagenesis primers (Integrated DNA Technologies, Coralville, IA) were phosphorylated with T4 polynucleotide kinase (New England Biolabs) and annealed to the ssDNA template. The primers were extended with T4 DNA polymerase and the ends ligated with T4 DNA ligase (Roche). The mutagenesis reactions were transformed into *E. coli* strain MV1190 (Bio-Rad, Hercules, CA) that were then selected for ampicillin resistance. Identification of colonies carrying mutant genes was facilitated by screening for the presence of a unique restriction site introduced by each mutagenic primer. Multiple clones were characterized for each mutant DNA and the introduction of the desired mutation was confirmed by DNA sequencing. Mutagenesis primer sequences with corresponding amino acid changes and restriction enzyme sites are listed in an appendix.

### 2.2.2 F chimera construction

Chimeras of the MV and CDV F proteins were created by either site-directed mutagenesis to introduce the desired changes or by restriction enzyme digestion and re-ligation to swap gene segments. Naturally-occurring restriction enzyme sites were used when possible, but sites were added by mutagenesis as needed. The primers used for mutagenesis are listed in an appendix. Multiple clones were characterized for each chimera. In order to swap gene segments, the plasmids were digested with the appropriate restriction enzymes. The resulting fragments were run on 0.8% agarose gels and purified with a QIAquick gel extraction kit (Qiagen). The purified DNA fragments were ligated with T4 DNA ligase (Roche), transformed into *E. coli* strain MV1190 (Bio-Rad) and the colonies were selected for ampicillin resistance. Chimeric DNA constructs were screened by sequencing for the correct restriction enzyme site junctions.

## 2.3 Antibody production

### 2.3.1 Monoclonal anti-measles H

B2 hybridoma cells (gift from Dr. Paul Rota) were cultured in 150 $mm^2$ tissue culture plates in DMEM supplemented with 20% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 U/ml penicillin and streptomycin at 37°C in 5% $CO_2$. Once the culture was exhausted, the cells were pelleted by centrifugation at 3000 rpm for 10 min and the supernatant was aliquoted and stored at -20°C until use.

### 2.3.2 Polyclonal anti-measles F

Polyclonal rabbit anti-peptide serum (Fcyt) directed against the 14 carboxyl-terminal amino acids of the cytoplasmic tails of MV F and CDV F ($NH_2$-(C)PDLTGTSKSYVRSL-COOH), as described by Cathomen et al. (1998b), was generated by Proteintech Group Inc. (Chicago, IL). A cysteine was added to the amino terminus of the peptide sequence to allow coupling to keyhole limpet hemocyanin carrier protein prior to rabbit immunization. Each rabbit was boosted three times at approximately one-week intervals after the initial immunization.

## 2.4 Assays for measles virus glycoprotein expression and function

### 2.4.1 Flow cytometry

Flow cytometric analysis was used to quantitate cell surface expression. Cells in six well plates were washed twice with PBS-FCS (phosphate buffered saline (PBS) containing 5% FCS) and then incubated at room temperature for 30 min with either 1 ml of hybridoma supernatant or a 1:1000 dilution of serum in PBS-FC. The cells were washed twice with 1.5 ml PBS-FCS and then incubated with a 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated to either goat anti-mouse or anti-rabbit antibody in PBS-FCS for 30 min. After two additional washes with PBS-FCS, the monolayers were detached with 0.0625 mM EDTA in PBS, pelleted by centrifugation at 4000 rpm for 1 min and washed once with PBS-FCS. The cells were fixed in 0.5 ml PBS with 1% FCS plus 1%

paraformaldehyde and incubated at 4°C for 7 min and then washed twice with PBS-FCS. Finally, the cells were resuspended in 400 µl of PBS and then analyzed for FITC - labeling. Expression level is presented as mean fluorescent intensity minus background labeling of control cells.

### 2.4.2 Hemadsorption

Hemadsorption activity of transfected cells was determined by the ability of the expressed MV H protein to adsorb African green monkey (AGM) erythrocytes (Three Springs Scientific Inc., Perkasie, PA). These assays were performed at 37°C to reduce non-specific binding of the erythrocytes to the monolayers. MV H expressing monolayers were incubated for 30 min with a 2% suspension of erythrocytes in pre-warmed PBS supplemented with 1% $CaCl_2$ and 1% $MgCl_2$ (PBS+). Monolayers were washed 3 times with pre-warmed PBS+ and then incubated an additional 10 min at 37°C to allow release of non-specifically bound erythrocytes. The cells were washed twice more with warm PBS+ and then adsorbed erythrocytes were lysed in 250 µl 50 mM $NH_4Cl$. Lysates were cleared by centrifugation at 13000 rpm for 5 min and then absorbance was quantified at 540 nm with a Spectra Max 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

### 2.4.3 Fusion

Initially, fusion promotion was assessed by microscopy. Transfected monolayers were fixed with methanol for 2.5 min and then allowed to dry for 5 min. The fixed monolayers were then incubated with Giemsa Accustain (Sigma

Diagnostics, St. Louis, MO) for at least 20 min and washed twice with water prior to microscopic examination for syncytium formation.

The ability of H and F proteins to promote fusion was quantitated using a content mixing assay. Monolayers of cells were infected with vTF7 and co-transfected with H and a trypsin activated cleavage site mutant form of F (F-CSM). A second set of monolayers was infected with wt vaccinia at a moi of 10 and transfected with 1 µg per well of plasmid pGINT7β-gal, which carries a β-galactosidase gene under the control of a T7 promoter. At twenty to twenty-two hours post-transfection, cells were removed from the wells by treatment with 0.05% trypsin and 0.53 mM EDTA (Gibco) and washed with DMEM. Effector cells (vTF7-3 infected) were resuspended in 0.4 ml medium. Target cells (wt vaccinia infected) were resuspended in 0.8 ml medium. The two cell populations were combined in replicate wells of a 96-well microtiter plate at an effector to target ratio of 2:1. Content mixing assays using BHK-21 cells were performed with a 1:1 ratio of effector to target cells. Cells were incubated at 37°C for 5 hours and then lysed for at least 30 min with 10 µl of 10% IGEPAL CA-630 (Sigma). Then, 50 µl of the lysate was mixed with 20 µl of the β-galactosidase substrate 16 mM chlorophenol red-β-D-galactopyranoside and incubated briefly at room temperature. The extent of fusion was quantitated by determination of the absorbance at 590 nm with a Spectra Max 250 microplate spectrophotometer.

### 2.4.4 Hemifusion

The ability of MV H or F mutants to promote hemifusion, or lipid mixing, was assessed by the transfer of octadecyl rhodamine b chloride (R18; Invitrogen) from AGM erythrocytes to cells co-transfected with MV H and F genes. Freshly labeled erythrocytes were prepared just prior to each experiment. To label the erythrocytes, 20 ml of a 2% suspension were incubated with 30 µl of 100 µM R18 in ethanol in cold PBS+ for 15 min in the dark at room temperature. Unbound R18 was removed by addition of 30 ml DMEM with 5% FCS and an additional 15 min incubation. The erythrocytes were washed three times with DMEM with 5% FCS and two times with PBS+ followed by resuspension in 20 ml PBS+.

Hemifusion assays were performed using HeLa cells at 18 hours post-transfection with the MV H and F genes. For each assay, 1 ml of the R18-labeled erythrocytes was added to each monolayer of transfected HeLa cells. After incubation for 30 min on ice in the dark to allow erythrocyte binding by MV H, fusion between the cell monolayers and erythrocytes was initiated by transferring cells to 37°C. After the desired amount of time, the cell monolayers were washed three times with warm PBS+ to remove unbound erythrocytes. Images were immediately acquired with a 20x objective using fluorescent microscopy and OPEN Lab software (Improvision Inc., Cambridge, MA).

### 2.4.5 Immunoprecipitation

At twenty hours post-transfection, cells were starved for 1 hour at 37°C in DMEM lacking cysteine and methionine. The cells were then labeled for 3 hours

at 37°C with 100 µCi/ ml of Expre$^{35}$S$^{35}$S-cysteine-methionine labeling mix (Dupont-New England Nuclear, Boston, MA) and chased for 4 hours with medium. The cells were lysed with 0.5 ml IP lysis buffer [PBS; 1% Triton X-100; 0.5% Deoxycholate; 1 mM phenylmethylsulfonylfluoride (PMSF)] for 45 min on ice. Lysates were collected and added to 100 µl of IP lysis buffer mixed with 1 µl of the appropriate antibody. MV H proteins were immunoprecipitated with a commercially available mixture of two antibodies (Chemicon, Temecula, CA). F proteins were immunoprecipitated with polyclonal serum directed against the F cytoplasmic tail (Fcyt). The immunoprecipitation mixtures were rotated at 4°C overnight and then cleared by centrifugation at 13000 rpm for 5 min. Antigen-antibody complexes were collected from the supernatants with 10 µl of BSA-blocked Immunopure Immobilized Protein G beads (Pierce, Rockford, IL) in the presence of 0.8% SDS, 10 mM Tris (pH 8) and 25 mM NaCl at room temperature for 1 hour. The Protein G bound proteins were washed six times with 0.8% SDS, 0.5% IGEPAL CA-630, 50 mM Tris (pH 8) and 150 mM NaCl at room temperature. Finally, the beads were resuspended in 15 µl reducing buffer containing 10% v/v β-mercaptoethanol and 5% SDS, boiled 5 min, and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels.

### 2.4.6 Sucrose gradient sedimentation

Transfected cells were lysed for 45 min on ice with 250 µl 1% octylglucoside (Sigma) in PBS containing 1 mM PMSF and then rotated for at

least 3 hours at 4°C. Lysates were cleared by centrifugation at 13000 rpm for 5 min and 600 µl of each supernatant was loaded onto 5 to 22% sucrose in 0.1% octylglucoside in PBS with 1 mM PMSF with a 60% sucrose base. Gradients were centrifuged at 37000 rpm for 17 hours at 8°C in a model SW41 Beckman Coulter rotor. Twenty-four 500 µl fractions were collected from each gradient. Alternate fractions were precipitated with 25% trichloroacetic acid (TCA). The precipitates were washed three times with acetone, and resuspended in 30 µl of reducing buffer. Molecular markers, including bovine albumin, aldolase, catalase, and ferritin (Crescent Chemical Company, Inc., Islandia, NY) were run on a separate gradient and TCA purified for analysis by SDS-PAGE with Coomassie Blue staining. The samples were boiled for 5 minutes and then analyzed by SDS-PAGE on 10% acrylamide gels. The proteins were transferred to Immobilon P membranes (Millipore Corp., Billerica, MA) overnight at 100 mA.

For detection of MV H, the Western blots were blocked with 0.2% Detector Block (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) for 1 hour, washed twice with 0.5% Tween-20 in PBS, and then incubated with a 1:3000 dilution of rabbit anti-measles H serum (gift from Dr. Paul Rota) in Detector Block. Membranes were washed once for 20 min, four times for 10 min each with 0.5% Tween-20 in PBS and then incubated for 1 hour with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody. Finally, the 0.5% Tween-20 in PBS washes were repeated and antibody binding was detected with the ECL Western Blotting reagents (Amersham Biosciences, Piscataway, NJ).

### 2.4.7 Co-immunoprecipitation

At 16 hours post-transfection, cells were starved for 1 hour at 37°C in DMEM lacking cysteine and methionine. The cells were then labeled for 5 hours at 37°C with 100 µCi/ ml of Expre$^{35}$S$^{35}$S-cysteine-methionine labeling mix (Dupont-New England Nuclear, Boston, MA). Then, the cells were washed three times and incubated for 30 min on ice with cold PBS-CM (PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$). Cell surface proteins were biotinylated with sulfo-NHS-SS-biotin (Pierce, Rockford, IL) dissolved in cold PBS-CM for 30 min on ice with gentle agitation. Excess biotinylating reagent was removed by two washes with PBS-CM and then the labeled cells were lysed with 0.4 ml DH lysis buffer [50 mM HEPES, pH 7.3 (United States Biochemical (USB), Cleveland, OH); 10 mM lauryl maltoside (USB); 150 mM NaCl; 1 mM PMSF] for 45 min on ice. Lysates from three wells of a six well plate were combined and split into two equal aliquots into tubes containing 100 µl DH lysis buffer with 750 µl of the appropriate antibody. The immunoprecipitation reactions were incubated for 90 min on a rotator at 4°C and then cleared by centrifugation at 13000 rpm for 5 min. Antigen-antibody complexes were collected from the supernatants with 10 µl of BSA-blocked Immunopure Immobilized Protein G beads for 1 hour at 4°C and then washed 6 times with 0.75 ml DH lysis buffer. The protein-bead complexes were boiled for 5 min in 10 µl of 10% SDS and the released proteins were resuspended in 0.5 ml DH lysis buffer. The Protein G beads were removed by centrifugation at 13000 rpm for 5 min and then the

supernatants were incubated with 10 µl Immobilized Streptavidin beads (Pierce) overnight at 4°C. The beads were washed twice with DH lysis buffer and then resuspended in 15 µl reducing buffer for analysis by SDS-PAGE.

# Chapter III

## Examination of the formation of an intracellular complex between MV H and F

### Introduction

The cellular site(s) of interaction between the paramyxovirus attachment and fusion proteins remains a controversial topic. Studies of different members of the paramyxovirus family have produced evidence both supporting and opposing the hypothesis that an intracellular interaction between the two glycoproteins is required to maintain the fusion protein in its pre-fusion metastable state. Previous investigators have suggested that there is an intracellular interaction between the measles glycoproteins (Plemper et al., 2001). Addition of an endoplasmic reticulum (ER) retention signal to the cytoplasmic tail of either MV H or F, RRRRR or KSKTH respectively, was concluded to result in the efficient intracellular retention of both proteins (Plemper et al., 2001). Co-transfection of either ER retained protein with its wild type partner was reported to result in a decrease in the kinetics of the processing of the non-tagged protein, consistent with the formation of an intracellular complex between the two proteins.

However, there are problems with the co-retention aspect of these studies. First, it was not determined if co-expression of the ER-tagged proteins altered the cell surface expression and/or the function of the untagged partners. Second, it

was not determined if the putative co-retention was specific for co-expression of the two measles glycoproteins. In another study, expression of ER retention signal-tagged F proteins from hPIV2 and hPIV3 resulted in down regulation of the surface expression of not only the homologous HN protein, but also that of the attachment proteins of heterologous viruses, including measles (Tanaka et al., 1996, Tong & Compans, 1999). Clearly the non-specific nature of this phenomenon is inconsistent with the demonstrated virus-specific fusion relevant glycoprotein interaction. Third, the experiments that were used to demonstrate the co-retention of heterologous proteins characterized the processing of FLAG epitope-tagged measles glycoproteins. While it was demonstrated that the FLAG epitope did not directly alter the processing of the proteins, it is possible that it could modulate the interaction between the H and F proteins. For example, a study by the same group demonstrated that the addition of a FLAG epitope to the cytoplasmic tail of MV H weakened an interaction between H and F (Plemper, 2002).

The **aim** of the research discussed in this chapter is to re-examine the conclusion that MV H and F form an intracellular complex. The **hypothesis** to be tested is that if MV H and F form an intracellular complex that is required for fusion promotion, the ER retention of one of the proteins should diminish the cell surface expression (CSE) and functional activities of the other protein in a virus-specific manner. The **rationale** for this aim is that the apparent ER co-retention of the measles glycoproteins by their ER-tagged heterologous partners has not

been shown to be virus-specific. In order to definitively establish the existence of an intracellular interaction between MV H and F, as well as its relevance to the fusion process, it is critical to fully characterize the specific nature of the interaction. It remains possible that the reported formation of an intracellular complex between the two proteins is an artifact of a non-specific process and/or the introduction of epitope tags. The **first approach** is to test the specificity of the detection of an intracellular complex by characterizing the effects of ER retention of MV F on the surface expression and function of MV H, as well as NDV HN. The **second approach** is to determine whether a decrease in processing can be detected utilizing measles glycoproteins lacking epitope tags.

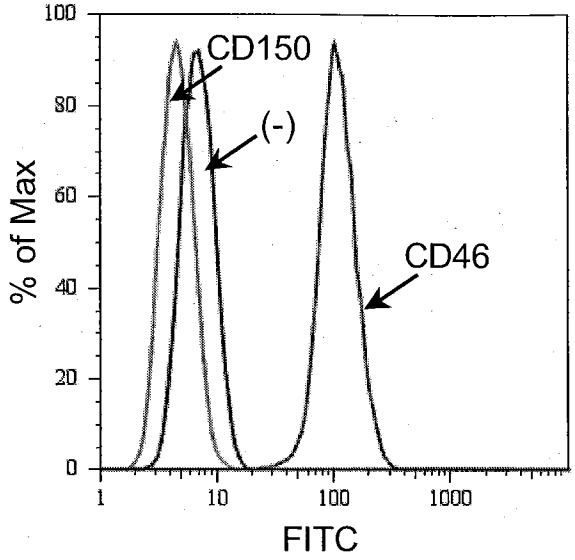
## Results

### 3.1 MV glycoproteins are efficiently expressed and functional using the HeLa/ vTF7-3 system.

#### 3.1.1 Surface expression and function of MV H and F proteins.

HeLa cells are human epithelial cells derived from a cervical carcinoma. As demonstrated by immunofluorescent labeling and flow cytometry, they express CD46, but not CD150 (Fig. 13A). Expression of MV H and F genes derived from an Edmonston vaccine strain of measles using the vTF7-3 transfection system in HeLa cells can be demonstrated by several methods. As seen in Figure 13B, MV H protein expression on the surface of HeLa cells can be detected by flow cytometry using an antibody that binds to the ectodomain.

A.




B.

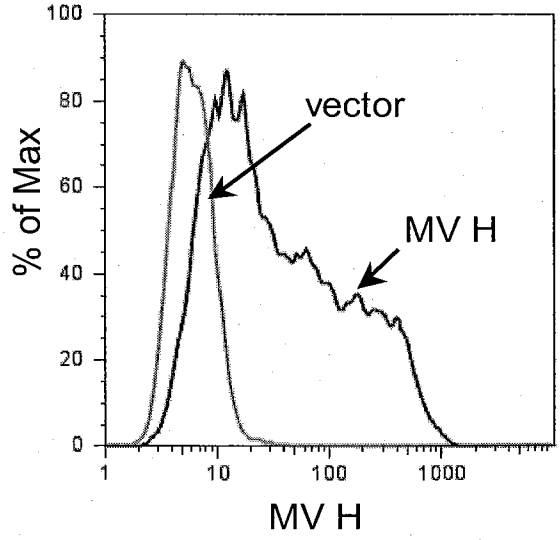



**Figure 13. CSE of measles receptors and H by HeLa cells.**

(A) CSE of CD46 and CD150 by untransfected HeLa cells was determined by flow cytometry using an anti-CD46 or anti-CD150 monoclonal antibody. A negative control labeled with the secondary antibody alone is shown for comparison. (B) CSE of MV H by HeLa cells transiently transfected using the vTF7-3 expression system was determined by flow cytometry using a monoclonal antibody against the ectodomain of MV H. A negative control transfected with pBSK is shown for comparison.

Additionally, receptor binding by MV H can be assessed by hemadsorption of AGM erythrocytes (Fig. 14A). Both glycosylation isoforms of MV H can be detected by immunoprecipitation (IP) (Fig. 14B). MV F cleavage and expression also can be detected by IP (Fig. 14C).

As shown in Figure 15, transient co-transfection of the H and F genes leads to membrane fusion and robust syncytium formation. While fusion is not visible when either MV H or F is expressed alone, large multinucleate cells can be seen when the proteins are co-expressed. As seen in Figure 15A, MV H does not promote fusion when co-expressed with CDV F in HeLa cells. This is consistent with other studies that have found fusion promotion to be the most efficient for MV and CDV when the H and F proteins are derived from the same virus and strain (Cattaneo & Rose, 1993, von Messling et al., 2001).

### 3.1.2 MV F fusion activity can be controlled by mutation of the cleavage site.

In order to make control of the initiation of the fusion process possible, a form of F, Fcsm, which is inactive until treatment with exogenous trypsin, was created by mutation of the cleavage site from RRHKR to RNHNR, as described by (Maisner et al., 2000). This mutated protein can be detected at the cell surface in an uncleaved form (Figure 14C). Fcsm is unable to promote fusion when co-expressed with MV H in the absence of exogenous proteases. However, as shown in Figure 15B, large multinucleate cells can be detected a few hours after incubation with trypsin.

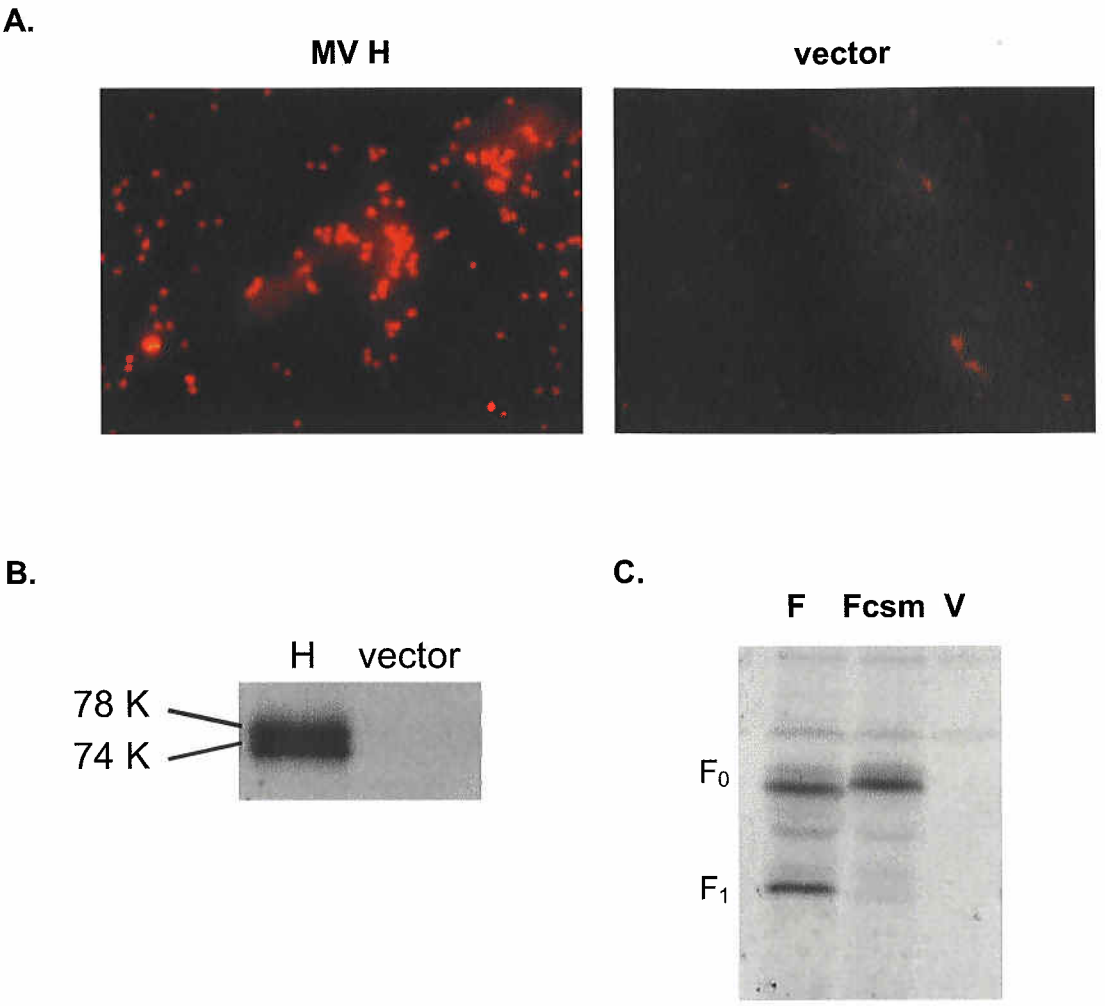



**Figure 14. Detection of expression of MV H and F in transfected HeLa cells.**

(A) Hemadsorption of R18-labeled AGM erythrocytes by cells transfected with either MV H or pBSK vector. (B) IP of MV H. Cells transfected with either MV H or vector (V) were labeled for 3 hours and incubated in chase medium for 4 hours prior to lysis of the cells and IP with a pair of anti-H antibodies against the ectodomain. (C) IP of MV F and Fcsm with an anti-F antiserum against the cytoplasmic tail. IP from cells transfected with vector alone is shown for comparison. Cells transfected with MV F, Fcsm or vector were labeled for 3 hours and incubated in chase medium for 4 hours prior to lysis of the cells.

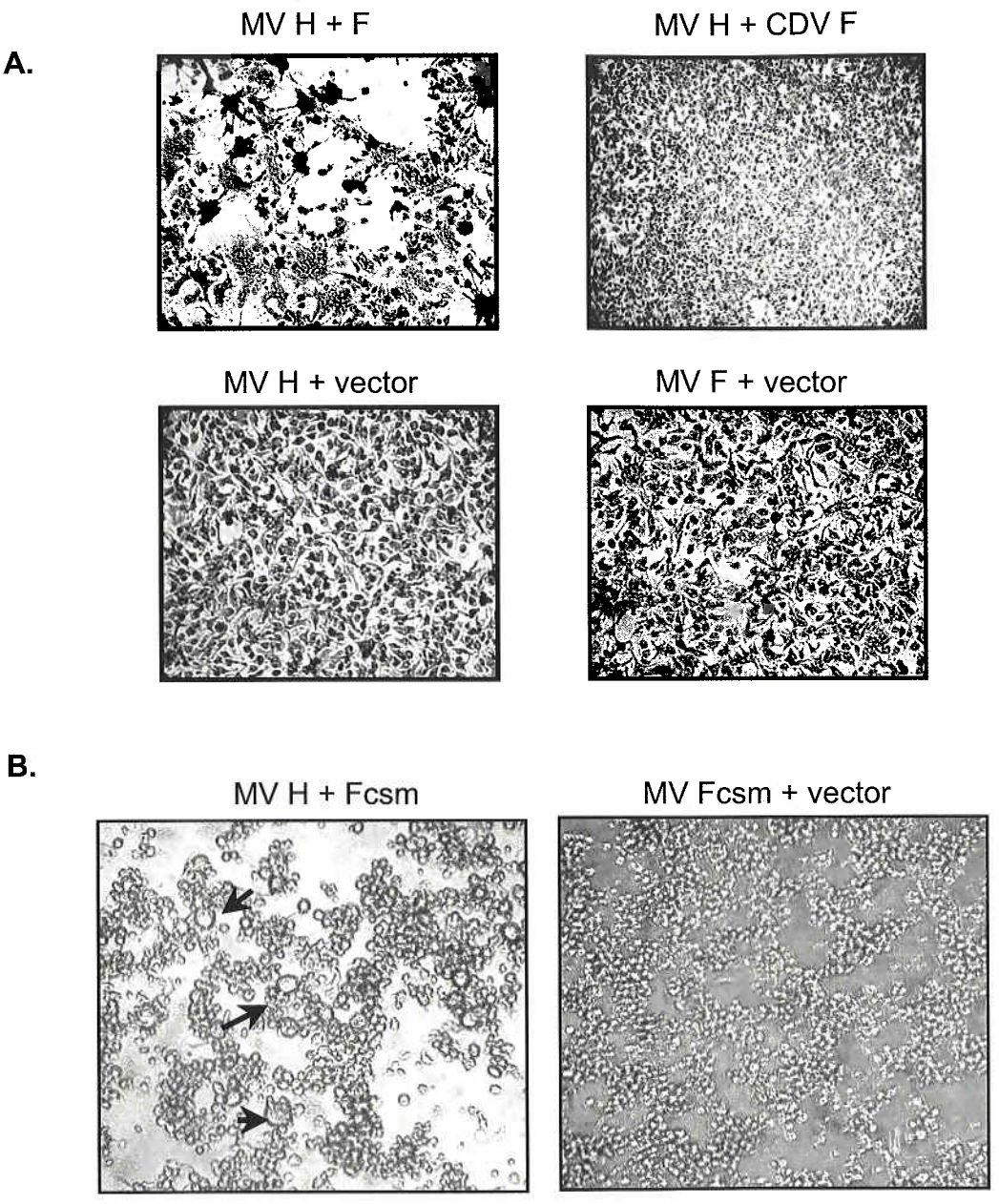



**Figure 15. Membrane fusion promotion by virus glycoproteins in transiently transfected HeLa cells using the vTF7-3 expression system.**

(A) At 20 hours post-transfection, the monolayers were fixed and stained with Giemsa stain. (B) At 20 hours post-transfection, the monolayers were treated with trypsin for 5 min, and then, the cells were washed and resuspended in HeLa cell medium. After 5 hours incubation, the cells were visually examined for membrane fusion. Arrows indicate fused cells.

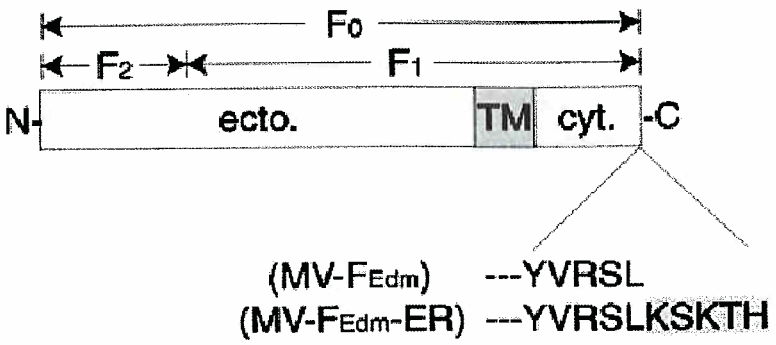
## 3.2 Characterization of the effects of ER retention of MV H and F expressed using the HeLa/ vTF7-3 system.

### 3.2.1 ER-tagged MV F is expressed intracellularly.

An ER retention signal, consisting of KSKTH, which is identical to that used by Plemper et al. (2001), was added to the cytoplasmic tail of MV F by site-directed mutagenesis (Fig. 16A). The efficiency of intracellular retention was examined by IP of both the intracellular and surface proteins at different time points. The MV proteins were expressed with the vTF7-3 RNA polymerase system in HeLa cells. As seen in Figure 16B, while $F_0$ is the primary form of wild type (wt) MV F detected at time 0, $F_1$ is also present after 180 minutes. In contrast, at both time points, only an uncleaved form of F-ER can be detected. Since cleavage occurs in the trans-Golgi, this is consistent with retention of F-ER in the ER.

MV F-ER was tested for its ability to co-retain untagged MV F by examining the efficiency of syncytium formation when both proteins were co-expressed with MV H. Figure 17 shows the extent of membrane fusion when different combinations of MV H, F, and F-ER are expressed together. In contrast to the strong fusion seen in the monolayers co-expressing MV H and F, no fusion can be detected in the cells co-expressing MV H and F-ER. The efficiency of fusion promotion is only slightly decreased when all three proteins are expressed at a 1:1:1 ratio of H: F: F-ER. However, there is a more apparent dominant negative effect on fusion promotion when the proteins are expressed at a ratio of

A.

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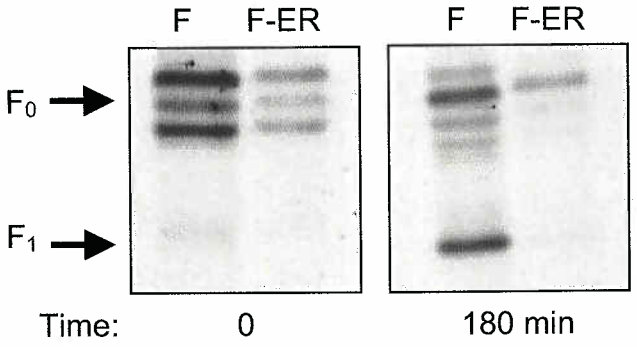
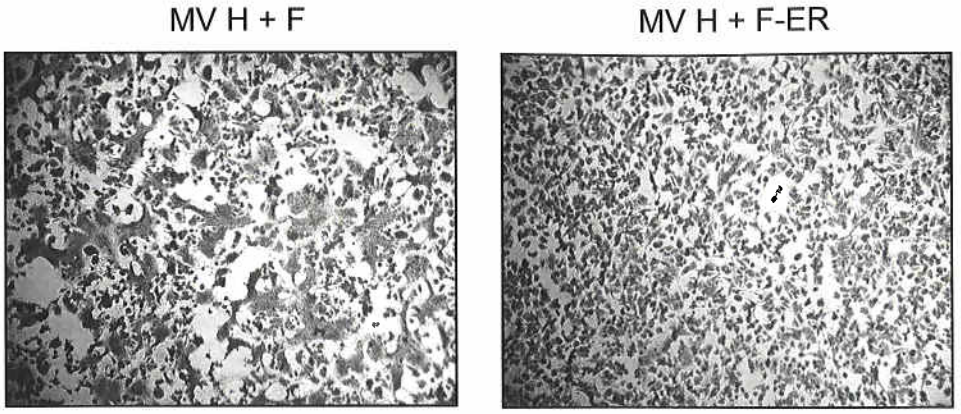



**Figure 16. ER retention of MV F.**

(A) Diagram of ER retention signal added to the cytoplasmic tail of MV F. Highlighted residues were added by site-directed mutagenesis immediately upstream of the termination codon (from Plemper et al., 2001). (B) IP of MV F and F-ER from transiently transfected HeLa cells using the vTF7-3 expression system. At 18 hours post-transfection, cells were labeled for 45 minutes, and then, they were either lysed immediately or incubated with chase medium for 180 minutes prior to lysis. F proteins were immunoprecipitated with an antibody against the MV F cytoplasmic tail

A.

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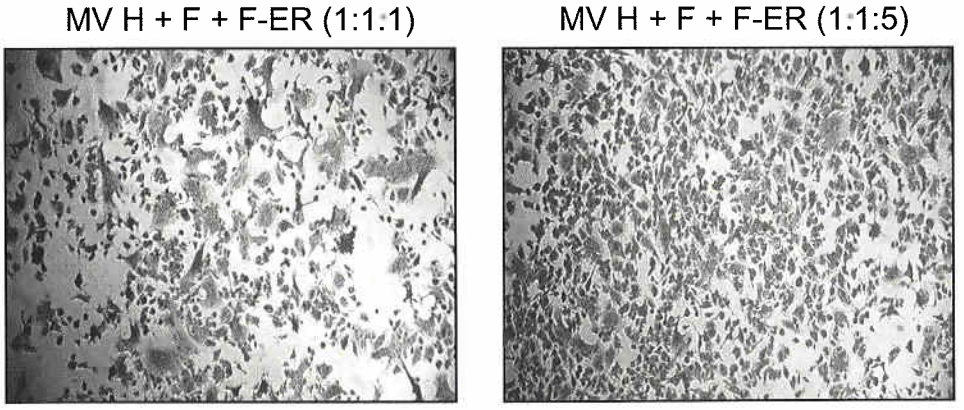



**Figure 17. Syncytium formation in monolayers expressing ER-retention tagged MV F.**

(A) The extent of membrane fusion in cells transiently transfected with MV H and F or F-ER. (B) The extent of membrane fusion by MV H and F when co-transfected with increasing amounts of F-ER. At 20 hours post-transfection, the monolayers were fixed and stained with Giemsa stain.

1:1:5. These results are consistent with the intracellular retention of wt F by MV F-ER through homo-oligomerization of the two forms of the protein.

### 3.2.2 ER retention of MV F does not significantly decrease cell surface expression or receptor binding by MV H.

It is possible that that the loss of fusion when MV H and F are expressed with an excess of F-ER is due to the co-retention of H by the ER retained form of F. Indeed, such a conclusion was drawn in the ER co-retention study by Plemper, et al. (2001). To test this hypothesis, flow cytometry was used to assess the effect of the co-expression of the F-ER protein on the CSE of MV H. If ER retention-tagged MV F co-retains H in the ER, then a detectable decrease in the CSE of MV H would be expected when it is co-expressed with an excess of F-ER.

Initially, expression was assayed when the MV H and F genes were transfected at a ratio of 1:1. In order to determine the amount of CSE of MV H in the absence of membrane fusion, a cleavage site mutant of MV F, Fcsm, which is non-fusogenic in the absence of trypsin, was co-expressed with H. As seen in Figure 18, co-expression of MV H with MV F-ER at a ratio of 1:1 does not result in a decrease in CSE of MV H as compared to co-expression with MV Fcsm.

Since there was no detectable difference in the level of CSE of MV H when it was co-expressed with F-ER compared to Fcsm, MV H was co-transfected with increasing amounts of the MV F mutants. The data obtained for expression of MV H when co-expressed with MV Fcsm at a ratio of 1:1 was set at

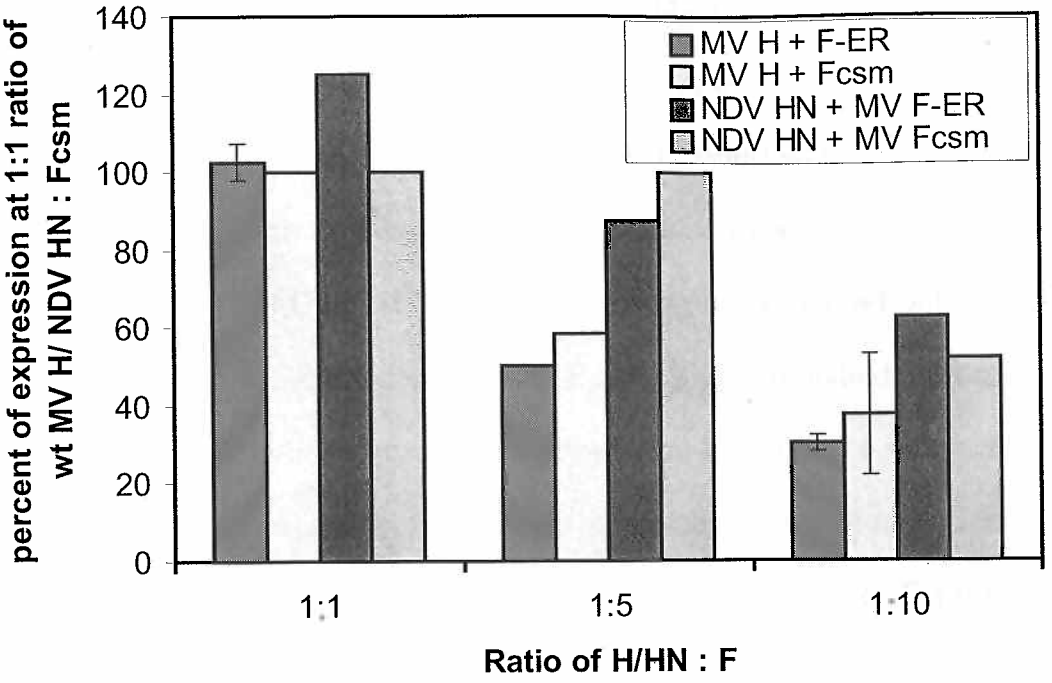



**Figure 18. CSE of MV H/ NDV HN co-expressed with MV Fcsm or F-ER.**

CSE was determined by flow cytometry. Cells expressing MV H were labeled with an antibody against the ectodomain at 16 to 20 hours post-transfection. Cells expressing NDV HN were labeled with a cocktail of at least four anti-HN monoclonal antibodies. For each assay, background detected in cells expressing vector alone is subtracted. All data are expressed relative to the amount for the wt H protein transfected at a 1:1 ratio with MV Fcsm, which has been set as 100% CSE. MV H data at ratios of 1:1 and 1:5 with MV F represent the mean of at least two experiments. Remaining data points represent the results of a single experiment.

100%. While CSE of MV H co-transfected with either form of MV F at a ratio of 1:3 is similar to that at a ratio of 1:1, it decreases when the proteins are expressed at a ratio of 1:5 to levels of 58% with MV Fcsm and 50% with F-ER. An additional decrease is detected when the proteins are expressed at a ratio of 1:10 with levels of 38% and 30% for MV H co-expressed with MV Fcsm and F-ER, respectively. Taken together, the results of these experiments reveal only a slight decrease in the CSE of MV H when it is co-expressed with F-ER in comparison to when it is co-expressed with Fcsm. These results do not correlate with the significant decrease in fusion promotion by MV H and F when they are co-expressed with an excess of F-ER. This indicates that the reduction in fusion is the result of homo-oligomerization between the two forms of F rather than hetero-oligomerization between H and F-ER.

To examine the specificity of the phenomenon, the effects of co-expression of the MV F proteins on the CSE of the attachment protein of NDV were also assessed. NDV was chosen for use as a control because its glycoproteins lack significant sequence homology with those of MV, and heterologous pairs of the glycoproteins from the viruses are unable to promote membrane fusion. The CSE of NDV HN when co-expressed with MV Fcsm at a ratio of 1:1 was set at 100%. Expression of the MV F proteins with NDV HN at increasing ratios results in decreases in the CSE of HN (Fig.18). This suggests that the decrease in CSE of MV H by co-expression of MV F is not the result of a specific H-F interaction.

The amount of receptor binding by MV H was assessed by hemadsorption of AGM red blood cells. Based on the previous analysis of CSE, these assays were performed using increasing MV F to H ratios. The data obtained at a MV H:Fcsm ratio of 1:1 was set at 100%, and then, MV H was co-transfected with increasing amounts of the MV F genes. At a ratio of 1:1, the amount of hemadsorption by MV H when it is co-expressed with F-ER is comparable to the amount of CSE detected by flow cytometry (109% of wt activity). As seen in Figure 19, the reduction in hemadsorption when H is co-expressed with Fcsm and F-ER increases with increasing ratios of MV F to H. At the highest ratio of 1:10, it is 46% and 32% for MV H with Fcsm and F-ER, respectively.

To determine whether the decrease in MV H hemadsorption that results from co-transfection with increasing amounts of F is specific, the effects of co-expression of the F protein from NDV on MV H receptor binding were also assessed. For these experiments, MV H was expressed with increasing amounts of either NDV Fcsm or NDV F-ER (Deng, 1999; Melanson and Iorio, unpublished). The data obtained for expression of MV H when co-expressed with MV Fcsm at a ratio of 1:1 was set at 100%, and then, MV H was co-transfected with increasing amounts of the NDV F mutants. As seen in Figure 19, there is a significantly stronger decrease in hemadsorption by MV H when it is co-expressed with either NDV F mutant, even at a ratio of 1:1, as compared to co-expression with the MV F proteins. Again, these results indicate that the decrease in hemadsorption by MV H that results from co-expression with F-ER is

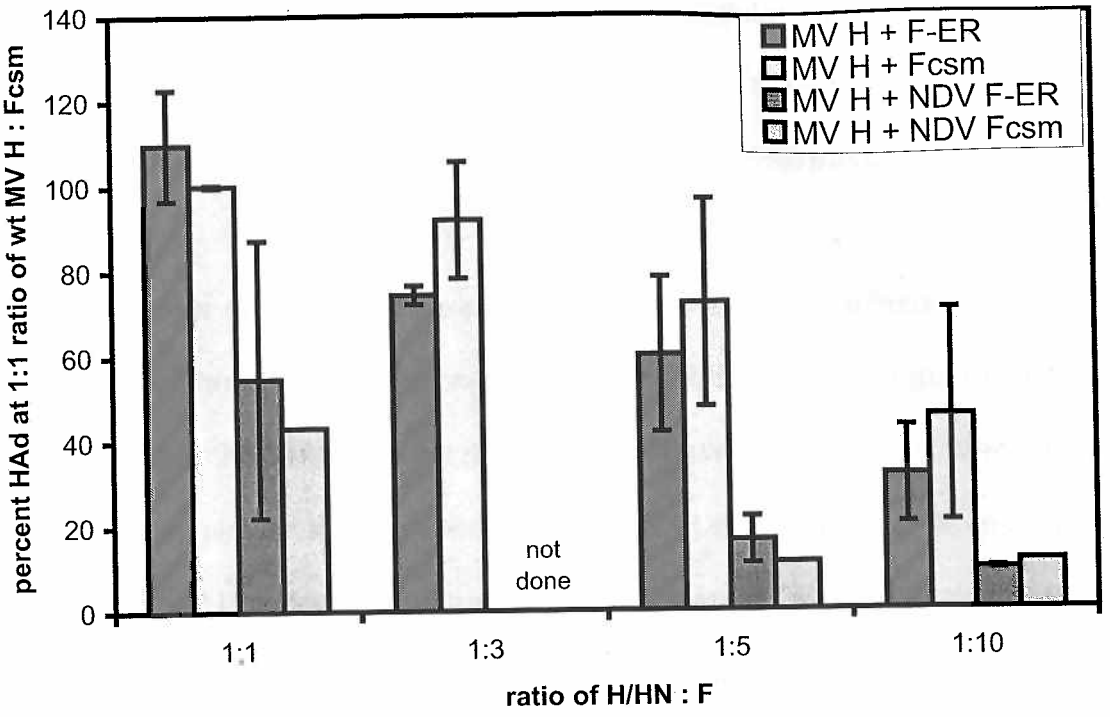



**Figure 19. Hemadsorption (HAd) by MV H co-expressed with MV/ NDV Fcsm or F-ER.**

HAd was determined by the ability of the transfected cells to adsorb AGM red blood cells. For each assay, background detected in cells expressing vector alone is subtracted. All data are expressed relative to the amount for the wt H protein transfected at a 1:1 ratio with MV Fcsm, which has been set as 100% HAd. Each data point represents the mean from at least two experiments, with the exception of the data for co-expression with NDV Fcsm for which only one experiment was performed. Each experiment was performed between 16 and 20 hours post-transfection.

not the result of a specific H-F interaction. Additionally, the CSE and hemadsorption data are inconsistent with an intracellular interaction between MV H and F of sufficient strength to result in co-retention of MV H by MV F-ER.

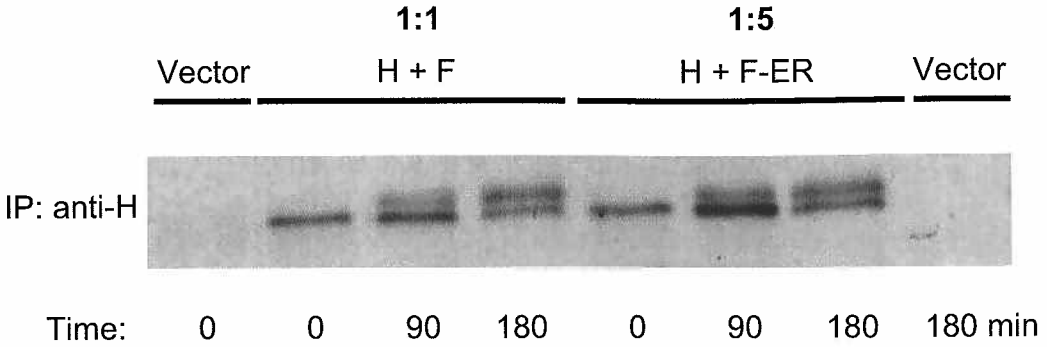
### 3.2.3 ER retention of MV F does not significantly decrease the rate of MV H processing.

Because the previous experiments examining the effects of MV F-ER expression on surface expression and receptor binding failed to demonstrate co-retention of wt MV H, a more direct analysis was performed. Although the approach was similar to that used by Plemper et al. (2001), the assay involved the IP of wt MV H lacking an epitope tag. As shown in Figure 20A, IP of wt MV H co-expressed with either MV F or F-ER was used to assess the amount of conversion from the 74 kDa form to the 78 kDa form at three time points, including 0, 90 min, and 180 min. Consistent with the CSE and hemadsorption data, there is no significant difference in the rate of processing of MV H when expressed with either F protein. Additionally, Figure 20B shows that no reduction in processing is detected even when H is expressed with a five-fold excess of MV F-ER.

### 3.2.4 ER retention of MV H does not significantly decrease the rate of MV F processing.

Because a decrease in the processing kinetics of MV H could not be detected when it was co-expressed with MV F-ER, the reciprocal effect of ER retention of MV H on the processing kinetics of MV F was also assessed. For

A.

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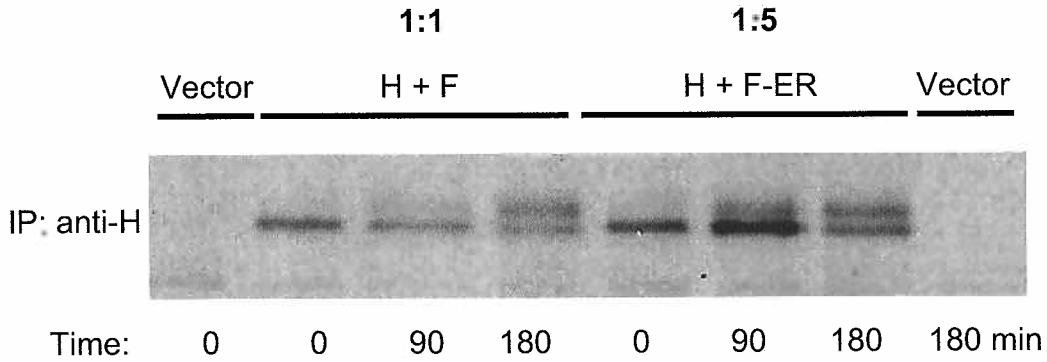



**Figure 20. Intracellular processing of MV H is not altered by co-expression with MV Fcsm or F-ER.**

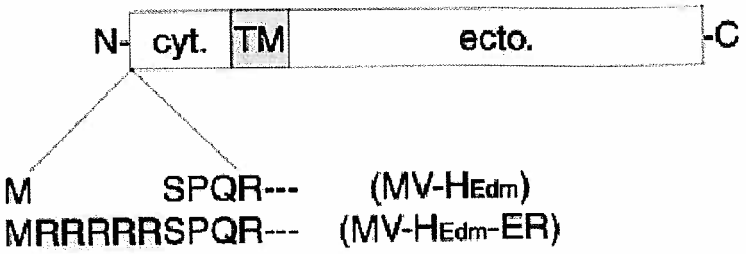
HeLa cells expressing MV H and Fcsm or F-ER were labeled for 45 min, and then, the cells were incubated with chase medium for the times indicated prior to lysis and immunoprecipitation with an anti-H antibody. IP from cells transfected with vector alone are shown for comparison. (A) IP from cells transfected with the H and F genes at a 1:1 ratio. (B) IP from cells transfected with the H and F genes at a 1:5 ratio.

these experiments, an ER retention signal consisting of RRRRR, which is identical to that used by Plemper et al. (2001), was added to the cytoplasmic tail of MV H by site-directed mutagenesis (Fig. 21A).

To demonstrate the intracellular retention of the ER-tagged H protein, IP of MV H was used to compare the extent of conversion from the 74 kDa form to the 78 kDa form at two time points for H and H-ER. As seen in Figure 21B, at time 0, the 78 kDa form of MV H is not present in either the wt or H-ER sample. In contrast, both the 74 and 78 kDa glycosylation isoforms of MV H are present after 4 hours. However, only a 74 kDa form of H can be detected in cells expressing H-ER. At both time points, the 74 kDa form of MV H migrates slightly faster than H-ER. Since *N*-linked oligosaccharides are modified by both trimming and the addition of sialic acid residues after proteins have entered the Golgi apparatus, these results are consistent with the retention of H-ER in the ER.

Additionally, MV H-ER was tested for the ability to co-retain MV H by determining the level of hemadsorption activity when the two forms of H are co-expressed. Consistent with its ER retention, receptor binding is not detectable when MV H-ER is expressed alone. As shown in Figure 22, co-expression of MV H and H-ER at a ratio of 1:1 reduces hemadsorption by MV H to 30% of the level achieved when MV H is expressed alone. Expression of the MV H in the presence of a three-fold excess of H-ER results in a further decrease to 7% of the activity of wt H alone. This down-regulation of MV H activity is consistent with intracellular homo-oligomerization between the two forms of H.

A.

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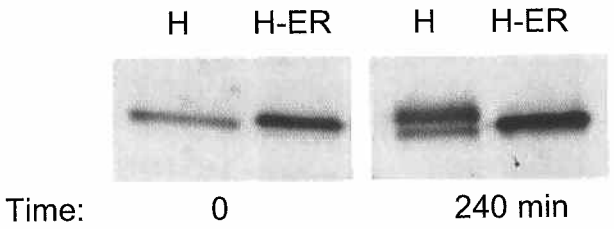



**Figure 21. ER retention of MV H.**

(A) Diagram of ER retention signal added to the cytoplasmic tail of MV H. Highlighted residues were added by site-directed mutagenesis immediately downstream of the methionine start codon (from Plemper et al., 2001). (B) IP of MV H and H-ER from transiently transfected HeLa cells using the vTF7-3 expression system. At 18 hours post-transfection, cells were labeled for 45 minutes and then they were either lysed immediately or incubated with chase medium for four hours prior to lysis. H proteins were immunoprecipitated with a pair of monoclonal antibodies against the ectodomain.

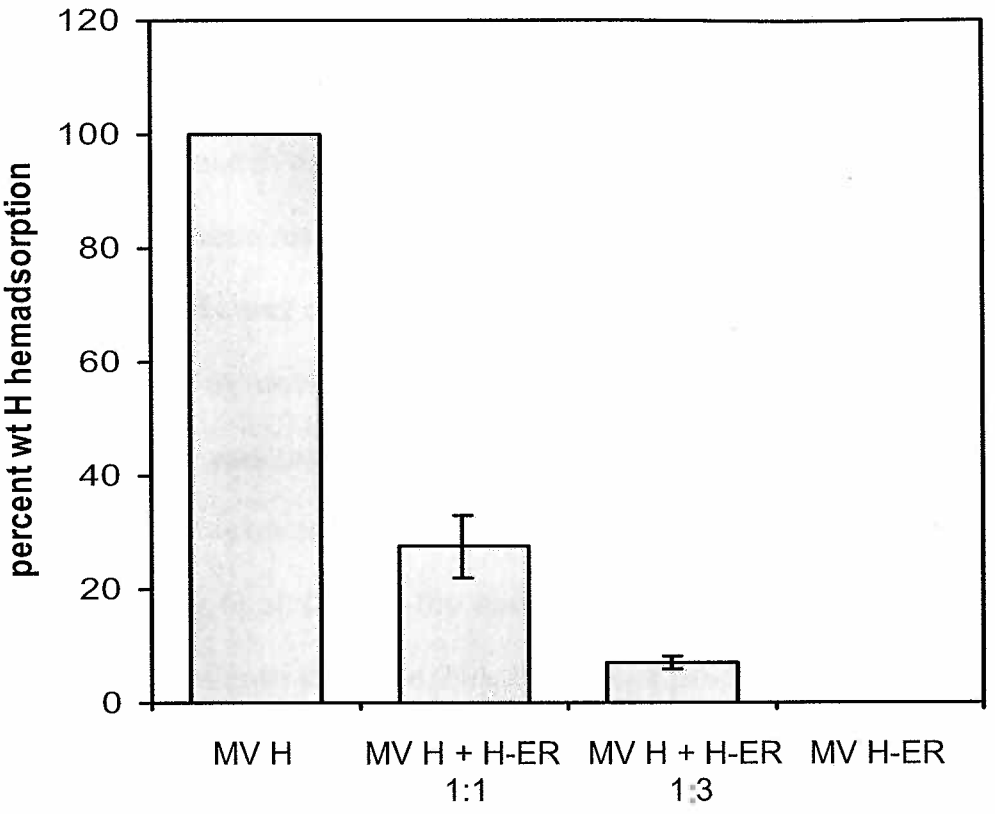


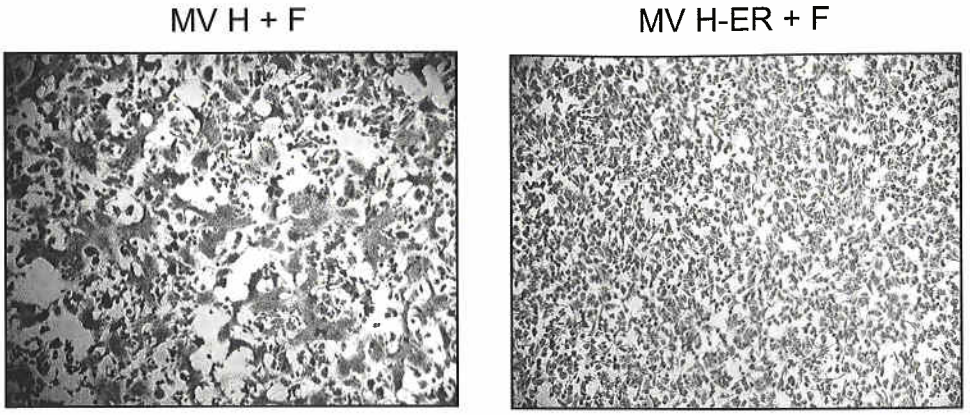

**Figure 22. Hemadsorption (HAd) by MV H is decreased by co-expression with H-ER.**

At 18 hours post-transfection, HAd was determined by the ability of the transfected cells to adsorb AGM red blood cells. HAd by MV H expressed alone is set as 100% activity. These data represent the results of a single experiment.

MV H-ER was also tested for its ability to co-retain untagged MV H by examining the efficiency of syncytia formation when both proteins were co-expressed with MV F. In contrast to wt MV H and F, H-ER co-expression with MV F does not promote membrane fusion (Fig. 23). As shown in Figure 23, expression of MV H-ER with MV H and F at ratios of 1:1:1 and 5:1:1 results in a significant decrease in fusion promotion in comparison to co-expression of MV H and F alone. These results could be due to homo-oligomerization between the two forms of MV H and/ or co-retention of MV F by H-ER.

In order to determine if MV H-ER hetero-oligomerizes with MV F intracellularly, IP was used to assess whether co-expression of MV H-ER with F delayed its processing and cleavage. Although the approach was similar to that used by Plemper et al. (2001), the assay involved the IP of wt MV F lacking an epitope tag. As shown in Figure 24A, IP of MV F co-expressed with either MV H or H-ER was used to assess the extent of conversion from $F_0$ to $F_1$ at three time points including 0, 2, and 4 hours. Consistent with the failure to detect an intracellular interaction between MV F-ER and MV H, no significant difference in the extent of processing of MV F occurs when it is expressed with either H protein. Additionally, Figure 24B shows that there is no significant reduction in the extent of processing when the proteins are expressed at a ratio of MV H-ER:F of 5:1. The efficiency of cleavage of F is very similar in the presence of both wt H and H-ER.

A.




B.

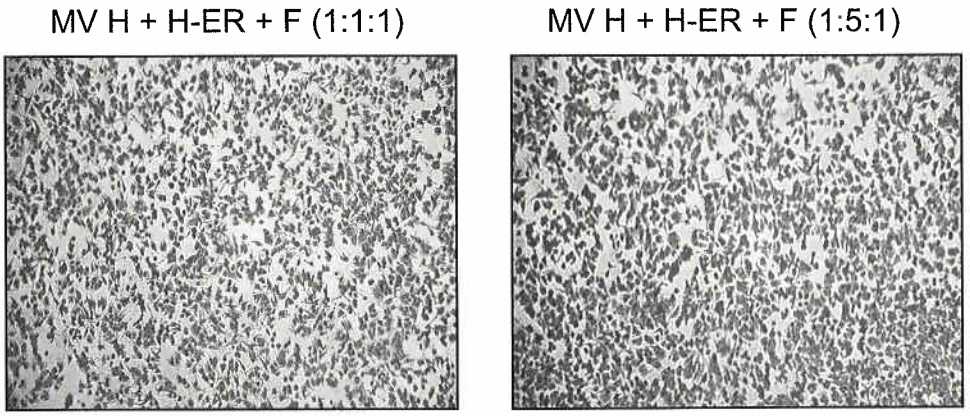



**Figure 23. Syncytium formation in monolayers expressing ER-retention tagged MV H.**

(A) The extent of membrane fusion in cells transiently transfected with MV F and H or H-ER. (B) The extent of membrane fusion by MV H and F when co-transfected with increasing amounts of H-ER. At 20 hours post-transfection, the monolayers were fixed and stained with Giemsa stain.

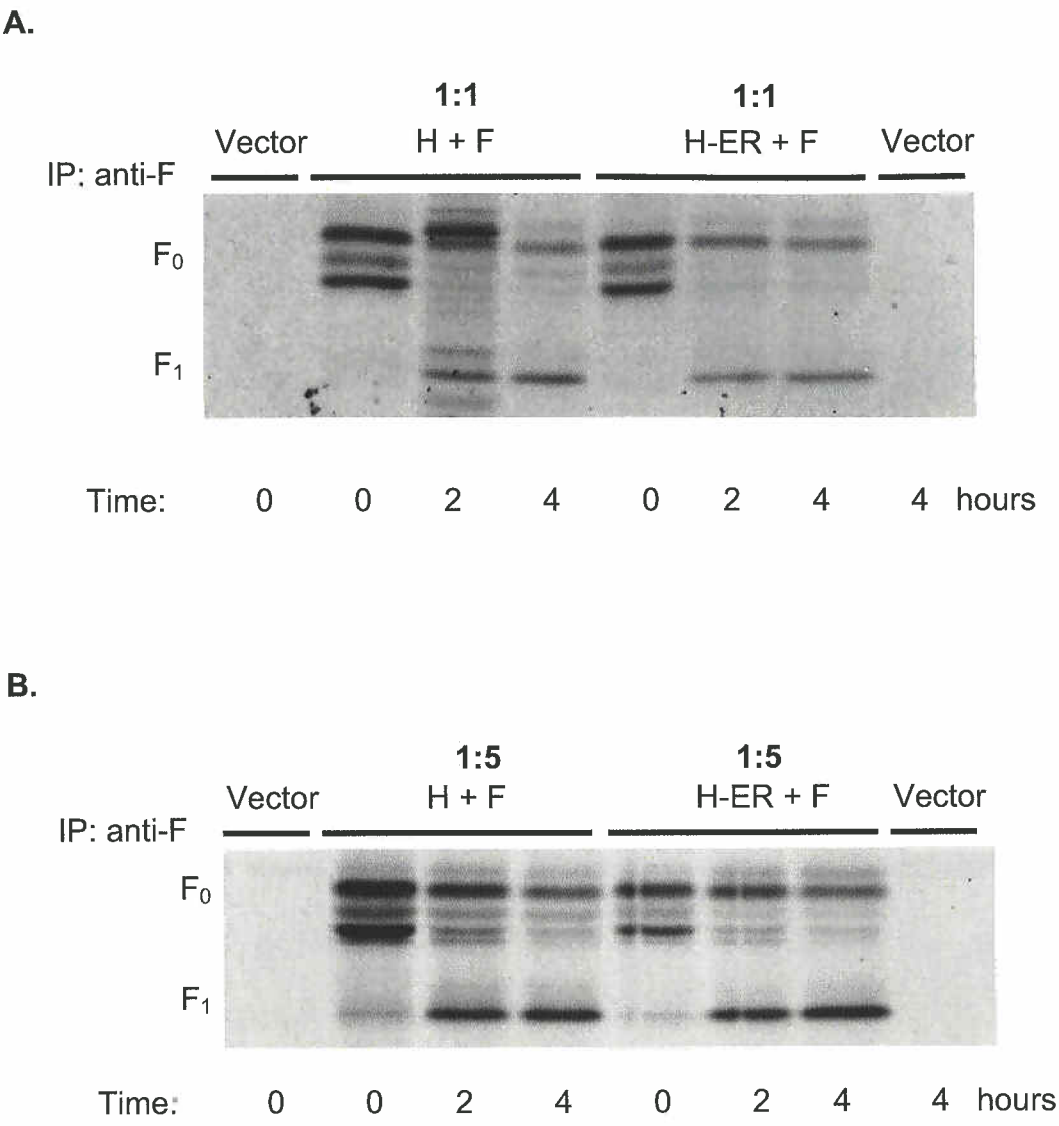



**Figure 24. Intracellular processing of MV F is not altered by co-expression with MV H or H-ER.**

HeLa cells expressing MV F and H or H-ER were labeled for 45 min and then the cells were incubated with chase medium for the times indicated prior to lysis and immunoprecipitation with an anti-F antibody against the cytoplasmic tail. IP from cells transfected with vector alone are shown for comparison. (A) IP from cells transfected with the H and F genes at a 1:1 ratio. (B) IP from cells transfected with the H and F genes at a 5:1 ratio.

## Summary

In this chapter, evidence is presented that disputes the hypothesis that MV H and F form an intracellular complex of sufficient strength to result in co-retention of one protein in the ER when the heterologous protein is tagged with an ER retention signal. While cell surface expression and function of untagged wt MV H can be slightly reduced by co-expression of an excess of ER-tagged MV F compared to Fcsm, the decrease is non-specific in that it can also be induced by co-expression of NDV F. Additionally, cell surface expression of NDV HN is reduced by co-expression of an excess of MV F. Finally, no difference can be detected in the extent of intracellular processing of either wt untagged glycoprotein when co-expressed with a five-fold excess of its ER-tagged partner. Although this approach did not detect a specific intracellular interaction between MV H and F, it cannot be ruled out that there is a weak association of the proteins that is undetectable by this method, suggesting the need for an alternative approach to address this issue.

# CHAPTER IV

## A MV H-F complex can be detected at the cell surface, independent of receptor binding

### Introduction

Despite the considerable amount of effort put forth for the various members of the paramyxovirus family, the relationship between receptor recognition by the attachment protein, the interaction between the attachment and fusion proteins, and the promotion of fusion has not been clearly elucidated. It may, in part, differ among various members of the group. On one hand, co-immunoprecipitation assays have suggested that receptor recognition by NDV HN triggers an interaction with the F protein at the cell surface (Deng et al., 1999, Li et al., 2004, Melanson & Iorio, 2004). On the other hand, recent evidence has been presented that suggests that NDV HN and F are associated at the cell surface prior to receptor recognition and that ligand binding by HN triggers dissociation of the complex (McGinnes & Morrison, 2006).

In the case of measles, it has been concluded that the H and F proteins interact in the ER prior to receptor recognition (Plemper et al., 2001). However, the evidence presented in Chapter III does not support this hypothesis. It is possible that the co-retention approach is not sensitive enough to detect such an interaction, suggesting that a different approach is needed to address this issue.

The **aim** of the research discussed in this chapter is to examine the H-F interaction at the cell surface and its relationship with receptor recognition by MV H. The **hypotheses** to be tested are that an H-F complex can be detected at the cell surface and that its relationship to receptor binding can be determined through the use of receptor-deficient cells and receptor binding deficient H proteins. The **rationale** for this aim is that if H and F are associated in a complex prior to their arrival at the cell surface, this interaction should be detectable in the absence of receptor binding. The **first approach** is to determine if a H-F complex can be detected at the surface of cells expressing CD46. **The second approach** is to determine if a H-F complex can be detected in cells lacking measles receptors. The **third approach** is to assess the ability of MV H proteins carrying mutations that diminish receptor recognition activity to interact with F at the cell surface.

## Results

### 4.1 Interaction of MV H and F can be detected at the cell surface.

As the MV H and F proteins are expressed and functional in HeLa cells, these cells were used to develop a co-immunoprecipitation assay to try to detect an interaction between the proteins can be detected at the cell surface. For this assay, a cleavage site mutant of MV F, which requires the addition of exogenous trypsin for activation, was used in order to prevent comparison of fusing and non-fusing monolayers. The method used for co-immunoprecipitation is analogous to

that used in our laboratory for studies of NDV glycoprotein interactions (Deng et al., 1999). Figure 25A shows that both glycosylation isoforms of MV H are co-immunoprecipitated with Fcsm, demonstrating the formation of a complex between MV H and F at the cell surface. Approximately, 21% (± 9%) of the total amount of MV H can be co-immunoprecipitated with Fcsm, using an anti-F antibody against the cytoplasmic tail.

Figure 25A also shows critical controls. The first lane for each pair shows the maximum amounts of the two proteins that can be immunoprecipitated from the cell surface for each sample. The first pair of lanes shows that neither protein is present in control cells transfected with an empty vector. The second pair of lanes demonstrates that MV H does not IP with the F antibody in the absence of the F protein, and the third pair shows that the protein that co-immunoprecipitates with F is not present in cells that are not transfected with the MV H gene. Additional controls are shown in Figure 25B, which demonstrates that a complex of MV H and wt F can also be detected.

It is interesting to note that a third band similar in size to $F_1$ is present in the co-immunoprecipitation of MV H with MV F-CSM. The protein can be detected only when the H and F proteins are co-expressed. At present, the identity of this protein and its connection to the fusion process have not been investigated. It is possible that it is a cellular protein that interacts with MV H and/or F as part of the fusion process or that it is a degradation product of H or F

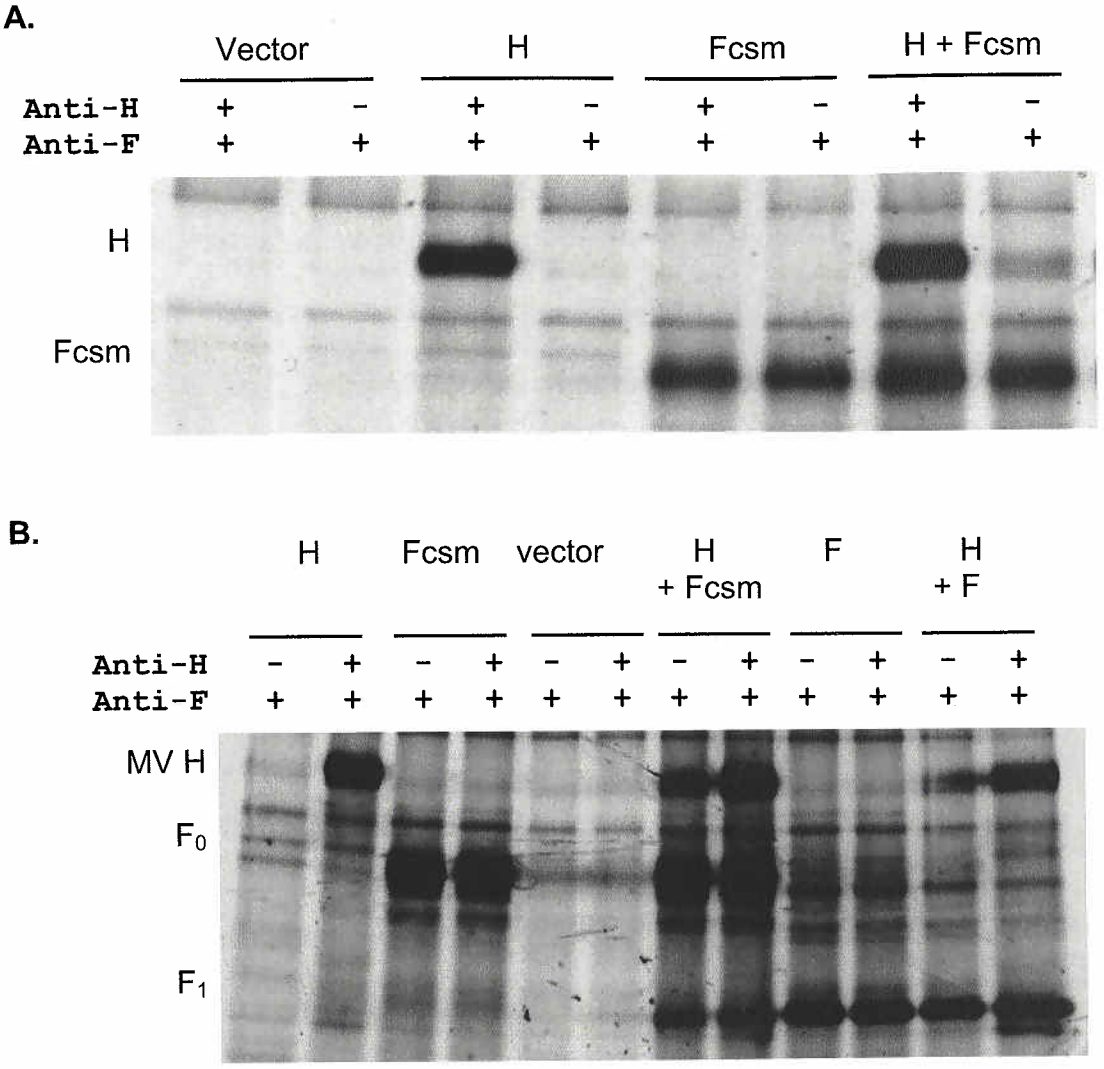



**Figure 25. Detection of an interaction between MV H and F at the cell surface of HeLa cells using co-immunoprecipitation assays.**

At 16 hours post-transfection cells were radiolabeled for 3 hours, and then, they were incubated for five hours with chase medium. The cell surface proteins were biotinylated and lysed. The lysates were equally split into two aliquots and then the proteins were immunoprecipitated with a combination of an antibody against the cytoplasmic tail of MV F and a pair of antibodies against the H ectodomain or the anti-F antibody alone. The immunoprecipitates were collected with Protein G beads and then the captured proteins were re-precipitated with streptavidin beads prior to analysis by SDS-PAGE under reducing conditions. (A) Co-immunoprecipitation of MV H with Fcsm. (B) Co-immunoprecipitation of MV H with wt F.

that results from exposure of a protease cleavage site as a consequence of conformational changes in the proteins when they are expressed together.

As shown in Figure 26, MV H and F do not co-immunoprecipitate when they are expressed in separate monolayers and mixed together after the cell lysis. Additionally, consistent with the inability of MV H to promote fusion when co-expressed with CDV F, MV H does not co-immunoprecipitate with CDV Fcsm at the cell surface. These results provide support for the physiological relevance of the interaction between H and F detected in these assays. All together, the results of these experiments are consistent with a specific interaction between MV H and F at the surface of HeLa cells.

## 4.2 MV H and F interact at the cell surface in the absence of receptors.

### 4.2.1 Characterization of surface expression and function of MV H and F proteins in CHO and CHO-CD46 cell lines.

Chinese hamster ovary (CHO) cells express neither CD46 nor CD150, and thus, are not permissive to MV binding or infection (Dorig et al., 1993). CHO cell lines that stably express human CD46 (CD46) at moderate levels have been shown to be susceptible to MV infection and fusion promotion (Anderson et al., 2004). One of these CHO-CD46 cell lines was generously donated by Dr. Kah-Whye Peng (Mayo Clinic Cancer Center). Initially, its MV receptor expression pattern was compared to the parental CHO cells by flow cytometry with anti-

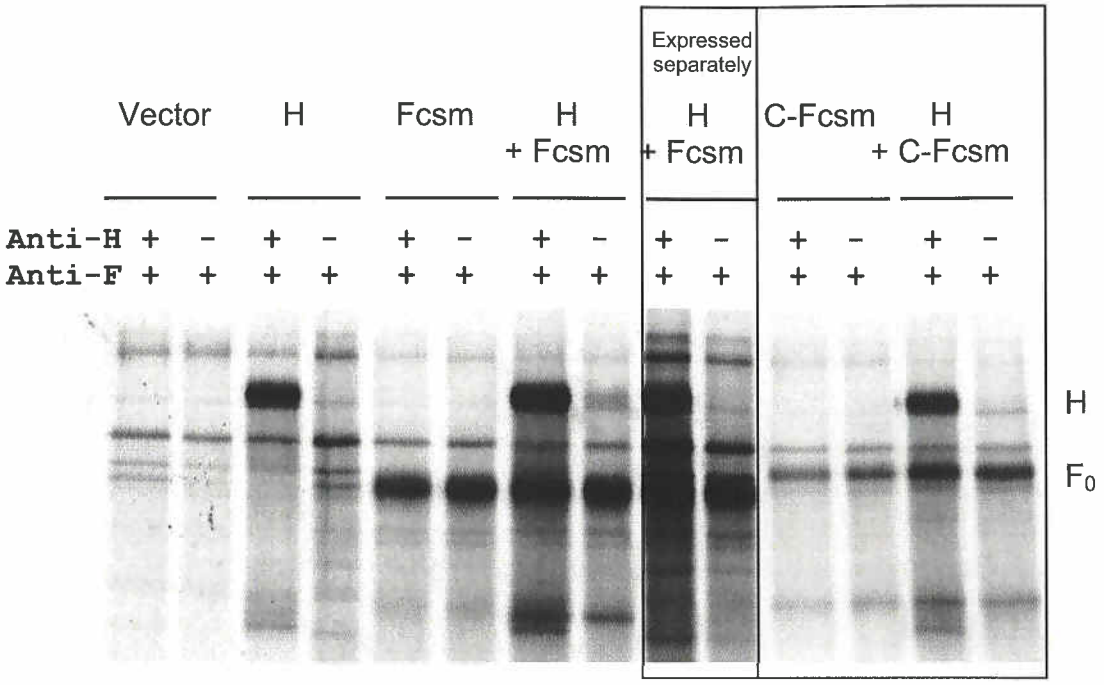



**Figure 26. MV H does not co-immunoprecipitate with MV Fcsm when the proteins are expressed in separate monolayers, and it does not co-immunoprecipitate with CDV Fcsm.**

The experiment was performed as described in the legend to Figure 25, except that in the fifth pair of lanes, MV H and Fcsm were expressed in separate monolayers and not combined until after cell lysis. Additionally, CDV Fcsm (C-Fcsm) was immunoprecipitated with an antibody against the MV F cytoplasmic tail as the epitope recognized by the antibody is conserved between the proteins. A band similar in size to MV H is present in the co-immunoprecipitation lanes of H + Fcsm expressed separately and H + C-Fcsm, but it is also detected when H is expressed alone and immunoprecipitated with the anti-F antibody, suggesting that it is a non-specific precipitate. It is also detectable in the vector control lanes, suggesting that it is unrelated to measles glycoprotein expression.

CD46 and anti-CD150 antibodies. Figure 27A shows that CD46 is present only on the CHO-CD46 cells, as well as that neither the CHO nor the CHO-CD46 cells express CD150.

CHO cell lines undergo apoptosis as a result of vaccinia virus infection (Ramsey-Ewing & Moss, 1995), precluding the use of the vTF7-3 expression system with these cells. Thus, a chicken β-actin promoter was used to drive MV H and F expression. Susceptibility to membrane fusion promotion by the measles glycoproteins was tested by co-expression of the H and F genes. As shown in Figure 28A, while membrane fusion can be induced in the CHO-CD46 cells, it is not detectable in CHO cells. MV H and F expression at the cell surface of both the CHO and the CHO-CD46 cells can be detected by IP, as shown in Figure 28B. This gel also shows that the F protein is properly cleaved in CHO cells. MV H is also detectable by flow cytometry and hemadsorption of AGM erythrocytes (not shown). MV H CSE can also be detected by using an anti-MV H antibody and flow cytometry (Fig. 27B). These results confirm that the lack of syncytia in the CHO cells is not the result of inefficient expression or processing of the MV glycoproteins.

### 4.2.2 MV H and F proteins form a complex at the cell surface of CHO-hCD46 cells.

Initially, because the use of a different expression system is required for expression of MV H and F in CHO cells compared to HeLa cells, the ability to

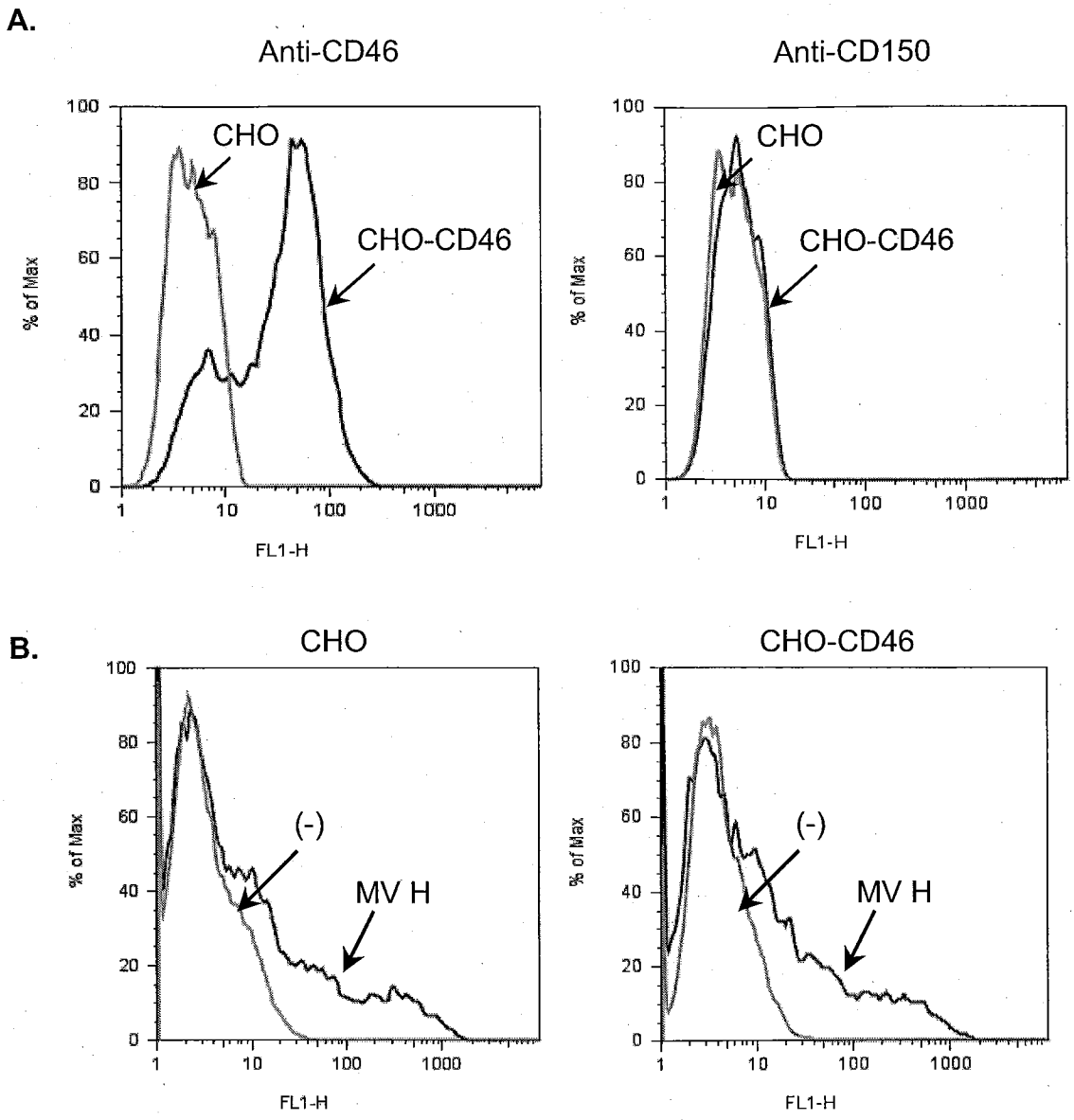



**Figure 27. CSE of receptors and MV H by CHO and CHO-CD46 cells.**

(A) CSE of CD46 and CD150 by the CHO cell lines was determined by flow cytometry using an anti-CD46 or anti-CD150 monoclonal antibody. (B) CSE of MV H by transfected cells was determined by flow cytometry using an anti-MV H antibody. CSE of cells transfected with vector (-) are shown for comparison.

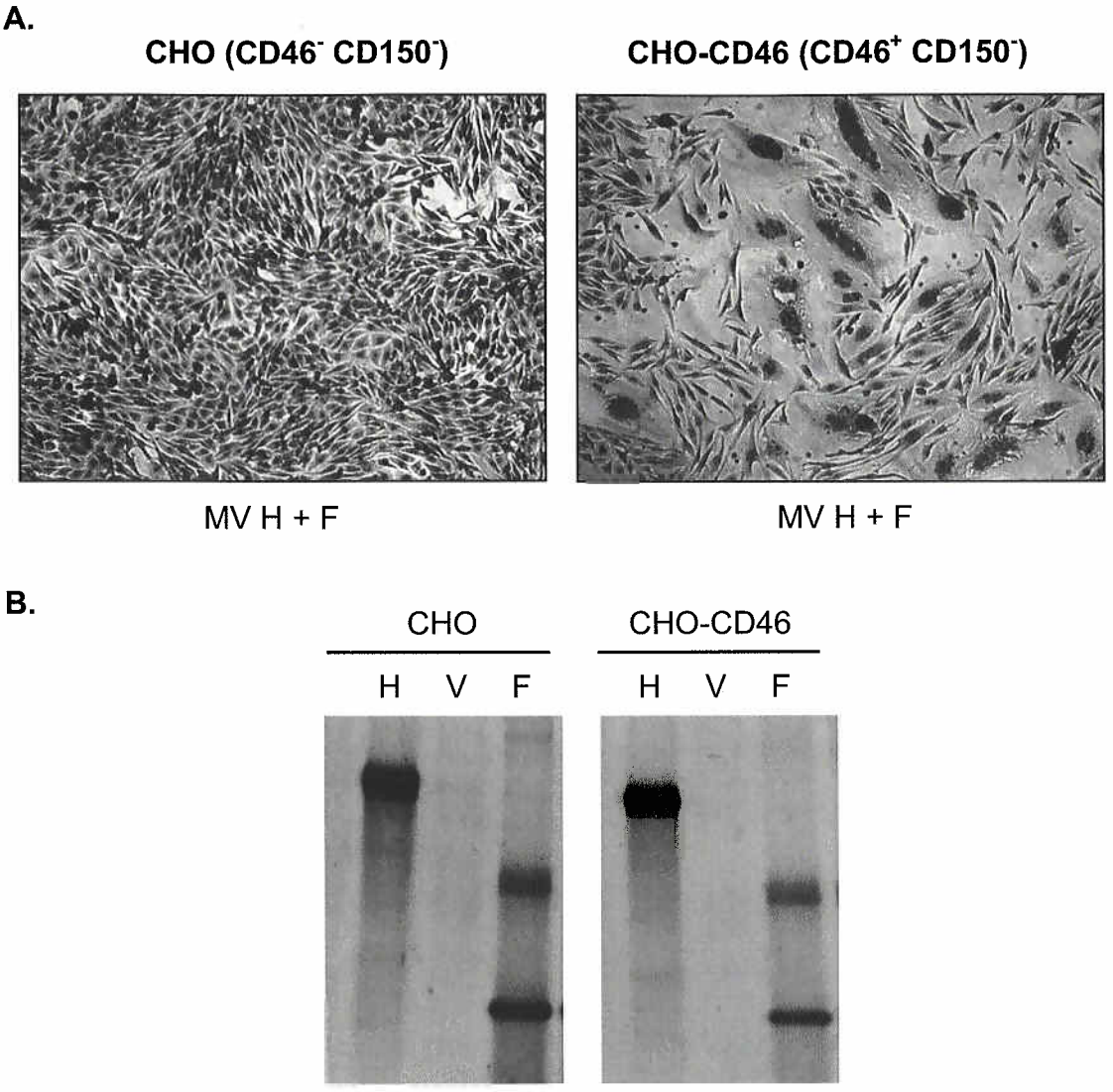



**Figure 28. Measles protein expression in CHO and CHO-CD46 cells.**

(A) Membrane fusion promotion by measles glycoproteins in transiently transfected CHO cell lines using the pCAGGS expression system. At 40 hours post-transfection, the monolayers were fixed and stained with Giemsa stain. (B) IP of MV H and F from CHO cell lines. Cells transfected with MV H, F, or vector were labeled for 3 hours and incubated in chase medium for 5 hours prior to biotinylation of cell surface proteins. Immunoprecipitated proteins were collected with Protein G beads and then re-precipitated with streptavidin beads.

detect a MV H and F complex at the cell surface of CHO cells in the presence of receptor expression was tested by co-immunoprecipitation of the proteins from CHO-CD46 cells. As shown in Figure 29A, a surface interaction between MV H and F can be detected in these cells. There is a slight reduction in the amount of MV $F_0$ that can be immunoprecipitated from the cell surface when MV H and F are co-transfected compared to when it is transfected alone. It is possible that this results from more efficient processing of MV F when it is co-expressed with H or from a slight non-specific decrease in the total expression of MV F due to co-transfection with the H gene.

Figure 29A also shows critical controls for co-immunoprecipitation in CHO-CD46 cells. The first lane for each pair shows the maximum amounts of the two proteins that can be immunoprecipitated from the cell surface for each sample. The first pair of lanes shows that neither protein is present in control cells transfected with an empty vector. The second pair of lanes demonstrates that MV H is not immunoprecipitated by the F antibody in the absence of the F protein, and the third pair shows that the protein that co-immunoprecipitates with F is not present in cells that are not transfected with the MV H gene.

Overall, the results of these experiments are consistent with an interaction between the MV H and F glycoproteins at the surface of CHO-CD46 cells. This interaction has also been shown when the proteins are expressed in HeLa cells that naturally express high levels of CD46. Importantly, these results also indicate that the cell surface interaction between MV H and F that can be

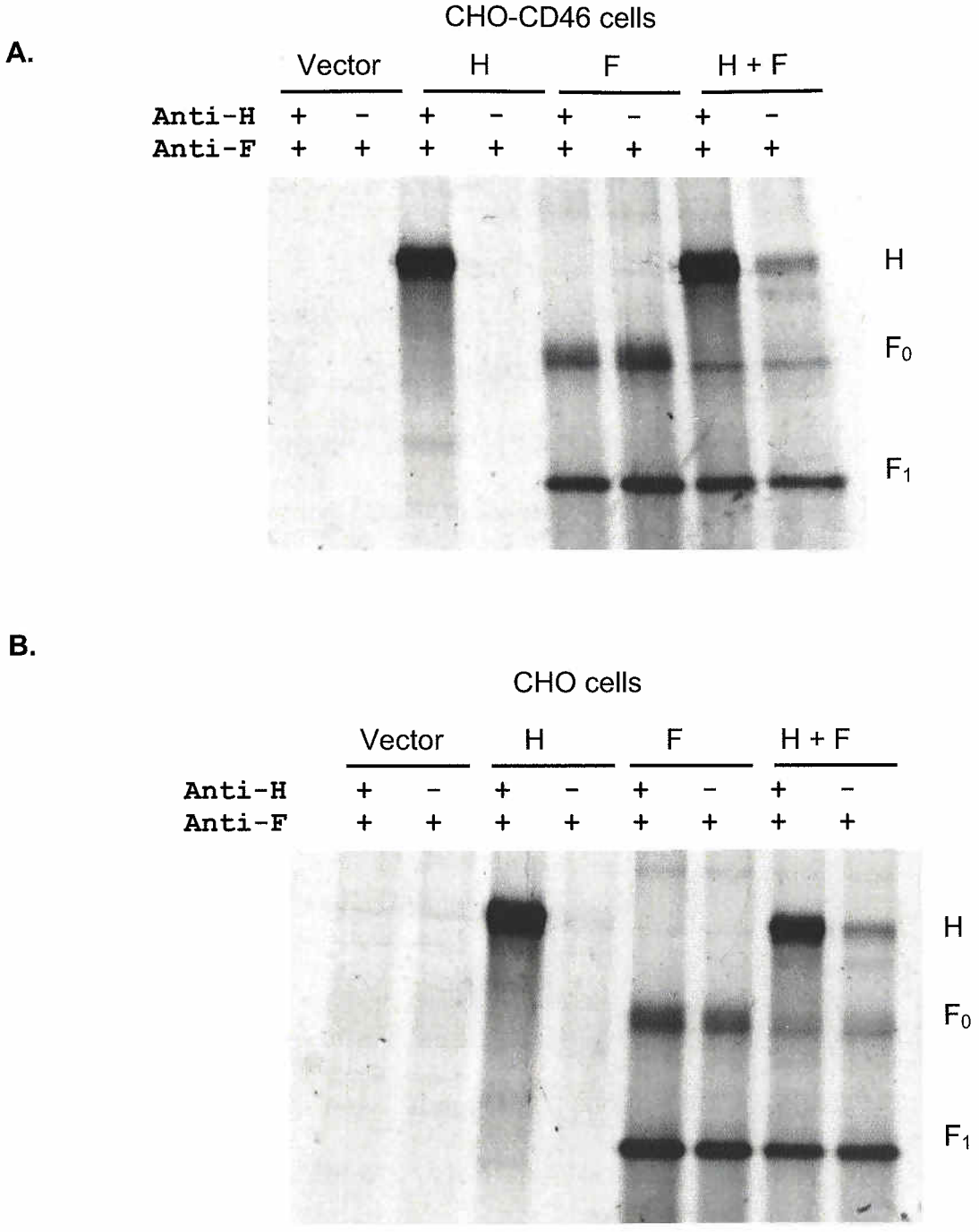



**Figure 29. Detection of a MV H and F complex at the cell surface of CHO cell lines using co-immunoprecipitation assays.**

At 40 hours post-transfection using the pCAGGS expression system, the experiments were performed as described in the legend to Figure 25. (A) Co-immunoprecipitation of MV H with F in CHO-CD46 cells. (B) Co-immunoprecipitation of MV H with F in CHO cells.

demonstrated using the vTF7-3 expression system in HeLa cells is not an artifact of the use of the vaccinia virus expression system. This provides additional support for the physiological relevance of the complex between H and F detected using this approach.

### 4.2.3 MV H and F proteins form a complex at the cell surface of CHO cells in the absence of receptors.

Based on the ability to detect an MV H-F interaction at the cell surface of CHO-CD46 cells using the pCAGGS expression system, the ability of H and F to interact at the cell surface in the absence of receptor expression was assayed by co-immunoprecipitation of the proteins from CHO cells. As seen in Figure 29b, the efficiency of the MV H-F interaction at the cell surface is not significantly altered in receptor-negative CHO cells.

Quantitation of the amount of MV H present in each sample of the co-immunoprecipitation assays reveals that approximately, 17% (average of two experiments resulting in 17.7% and 16.2%) and 19% (average of two experiments resulting in 25.5% and 12.5%) of the total amount of MV H is co-immunoprecipitated with MV F from the cell surface of the CHO-CD46 and CHO cells, respectively (Fig. 29A and -B). These results demonstrate that MV H and F interact at the cell surface both in the presence and absence of receptor binding by H. The results of these experiments are significant in that they are consistent with an interaction occurring between the MV H and F proteins at the cell surface

prior to receptor binding, despite our inability to confirm an intracellular interaction between the two proteins using the ER co-retention approach.

## 4.3 MV H lacking receptor binding interacts with MV F.

### 4.3.1 Mutation of residues involved in CD46 recognition by MV H.

Following the identification of CD46 as a cellular receptor for MV, several groups have mapped multiple regions of MV H that may be involved in receptor recognition. Mutations at MV H residues 451, 481, and 473-477 have been shown to reduce CD46 recognition by MV H (Lecouturier et al., 1999, Patterson et al., 1999). It has also been demonstrated that mutation of residues 431 and 527, along with 451 and 481, results in a CD46-blind MV H protein, suggesting a role for these residues in CD46 recognition (Vongpunsawad et al., 2004). Additionally, reduced fusion promotion and CD46 down-regulation resulted from mutation of the adjacent residues, S548 and F549 (Masse et al., 2002). Based on these studies, several of these residues were mutated individually, or in groups, to identify mutated MV H proteins completely deficient in receptor recognition (Fig. 30). Proteins carrying the following substitutions were prepared and characterized: F431S, V451E, Y481N, S544G, S546G, S548L/F549S, I473A, P474A, R475A, F476A, K477A, 473-477A, and 473-477A/Y481N.

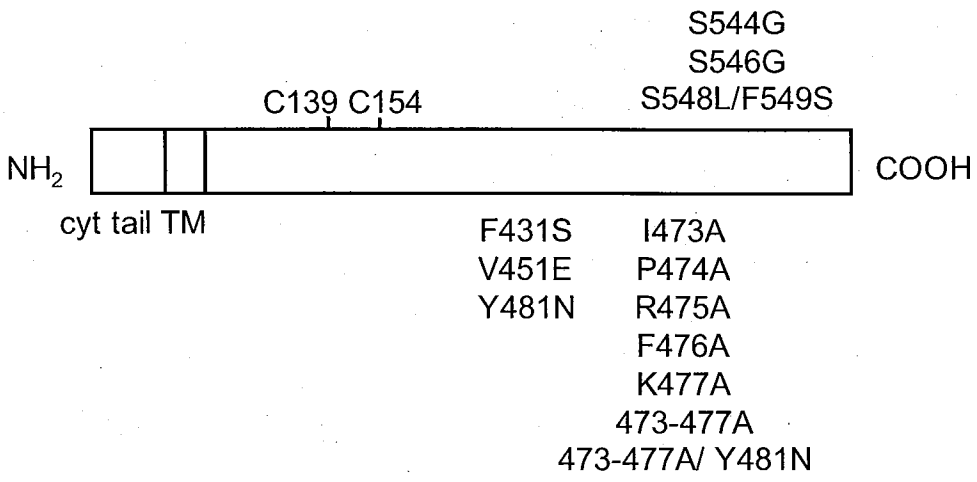



**Figure 30. Diagram showing the locations of mutations created in regions of MV H thought to be involved in recognition of CD46.**

### 4.3.2 Amino acid substitutions F431S, V451E, and Y481N result in moderate to strong reductions in CD46 recognition.

The ability of MV H proteins carrying individual mutations of F431S, V451E, and Y481N to interact with CD46 was assessed by hemadsorption of AGM erythrocytes at 37°C. All three amino acid substitutions resulted in a strong reduction in receptor recognition, which cannot be accounted for by reduced CSE (Fig. 31). The MV H proteins F431S, V451E, and Y481N promote receptor recognition at levels of only 26.8%, 65.5%, and 17.2% of wt H activity, though they are expressed at levels of 45.5%, 95.3%, 56.9% of wt H, respectively. Consistent with defects in receptor recognition, all three mutated proteins also exhibit significantly reduced fusion promotion (Fig. 31). However, the protein carrying the V451E mutation has a much stronger deficiency in fusion promotion activity than can be attributed to its decrease in receptor binding. This suggests that mutation of residue 451 also affects the fusion helper function of MV H.

Because mutations in the globular head domain could alter the conformation of the epitope recognized by the antibody used for immunofluorecent labeling, expression of the mutants was also assessed by IP with an antibody that binds to the cytoplasmic tail after a four-hour chase. As shown in Figure 32, both glycosylation forms of all three mutant proteins are present in the samples. However, in comparison to that present in the wt H sample, the F431S and V451E mutations appear to reduce the amount of the 78 kDa form.

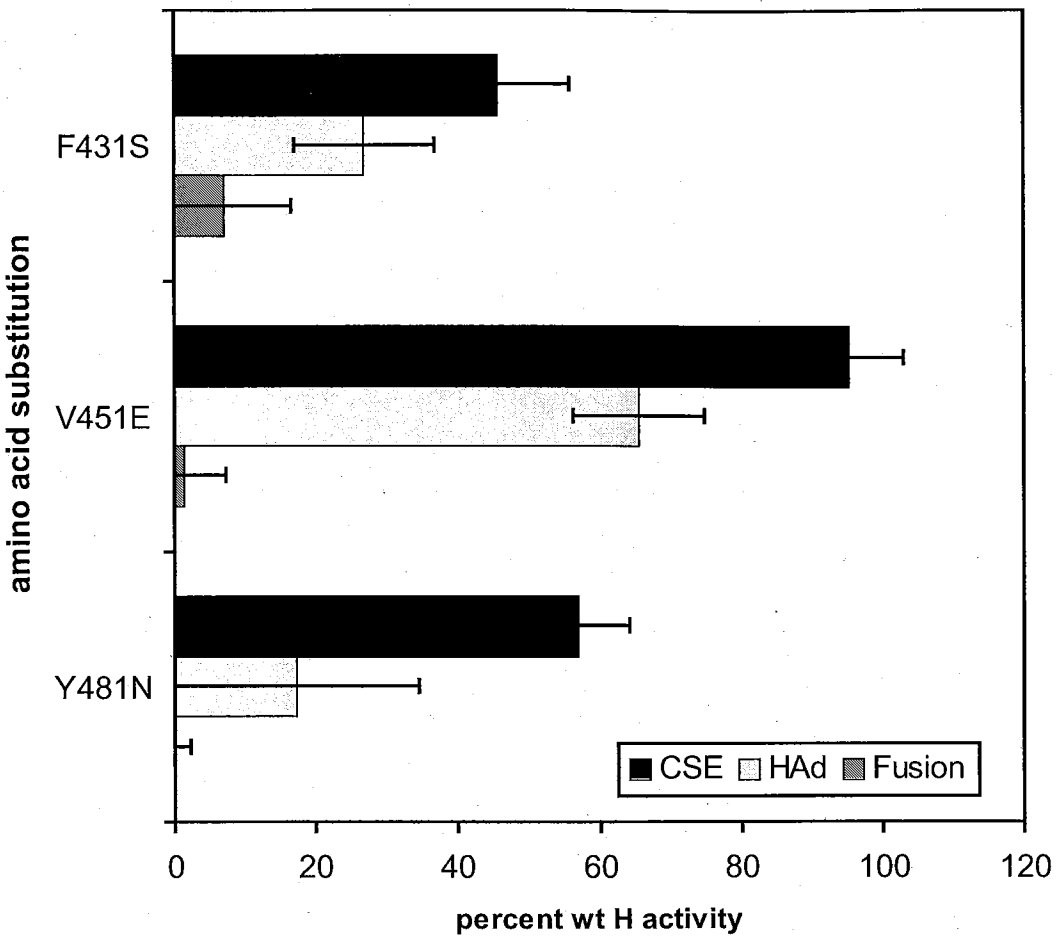



**Figure 31. CSE and functional characteristics of MV H proteins carrying mutations at F431, V451, and Y481.**

CSE was determined by flow cytometry using an anti-H antibody that recognizes the ectodomain. The HAd activity was determined by the ability of monolayers transfected with the H genes to adsorb AGM erythrocytes at 37°C. The ability of the H proteins to complement MV Fcsm in the promotion of membrane fusion was determined by a content mixing assay. For each assay the background detected in cells transfected with vector has been subtracted. All data points represent the mean of at least three independent experiments and are expressed relative to the activity of the wt proteins.

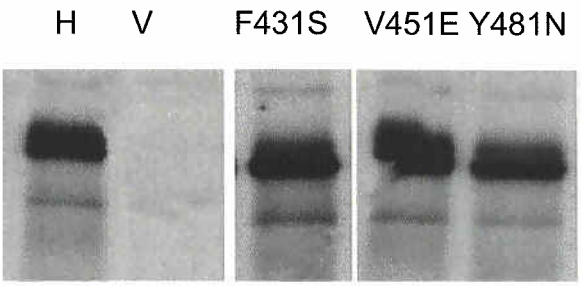



**Figure 32. IP of MV H proteins carrying mutations at F431, V451, and Y481.**

Cells transfected with MV H, vector (V), or a mutated H gene were labeled for 3 hours and incubated in chase medium for 4 hours prior to lysis. The proteins were immunoprecipitated with an antibody against the cytoplasmic tail of H. The immunoprecipitates proteins were collected with Protein G beads and then analyzed by SDS-PAGE under reducing conditions.

### 4.3.3 Amino acid substitutions S544G, S546G, and S548L/F549S result in moderate reductions in CD46 recognition and fusion.

MV H mutant proteins carrying the amino acid substitutions S544G, S546G, and S548L/F549S have moderately decreased receptor-binding levels of 87.8%, 81.7%, and 60.9% of wt H activity, respectively. As seen in Figure 33, the decreased receptor recognition activity of these mutants correlates with reductions in their levels of CSE. While membrane fusion promoted by the S544G mutant is slightly greater than the activity of wt H, the S546G and S548L/F549S mutations result in moderate decreases in fusion that correspond to the reductions in receptor recognition and CSE. IP of these mutated proteins after a four-hour chase revealed the expression of both glycosylation isoforms of the proteins in ratios similar to that of wt H (Fig. 34).

### 4.3.4 Alanine substitutions in the region 473-477 completely abolish CD46 binding by MV H.

Based on the findings of Patterson et al. (1999), all five residues in the region 473-477 were initially mutated to alanine. As shown in Figure 35, the 473-477A mutations do not decrease CSE, as detected by flow cytometry. However, the hemadsorption and fusion activities of the protein are abolished. The mutated protein can also be detected by IP with an antibody against the MV H tail after a four-hour chase (Fig. 36).

To determine if one specific residue in this region was responsible for the phenotype, each residue was mutated individually to alanine. Each of the

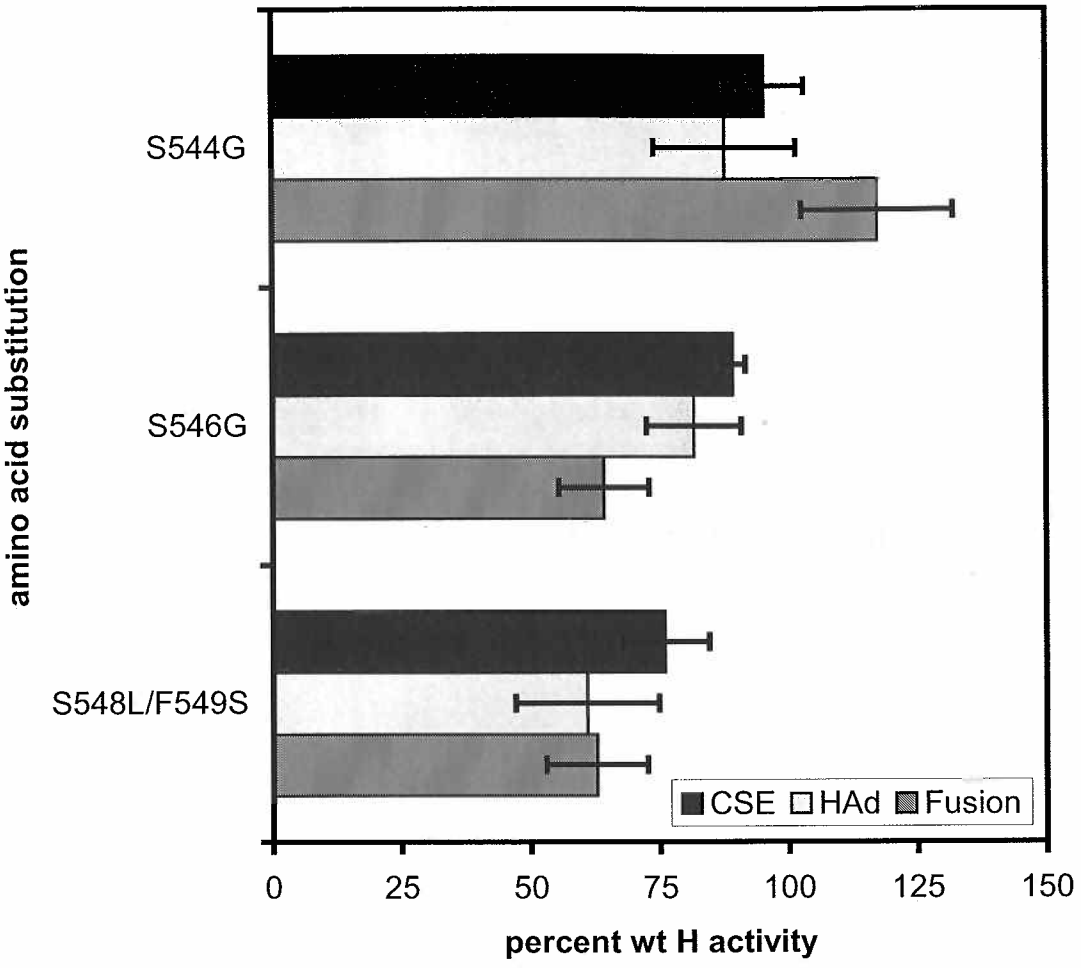



**Figure 33. CSE and functional characteristics of MV H proteins carrying the mutations S544G, S546G, and S548L/F549S.**

The experiments were performed as described in the legend to Figure 34. All data points represent the mean of at least three independent experiments and are expressed relative to the activity of the wt proteins.

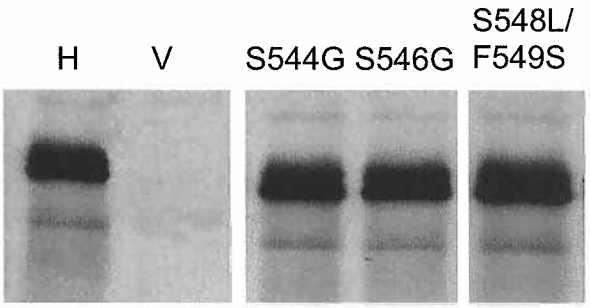



**Figure 34. IP of MV H proteins with the mutations S544G, S546G, and S548L/F549S.**

Cells transfected with MV H, vector (V), or a mutated H gene were labeled for 3 hours and incubated in chase medium for 4 hours prior to lysis. The proteins were immunoprecipitated with an antibody against the cytoplasmic tail of H. The immunoprecipitated proteins were collected with Protein G beads and then analyzed by SDS-PAGE under reducing conditions.

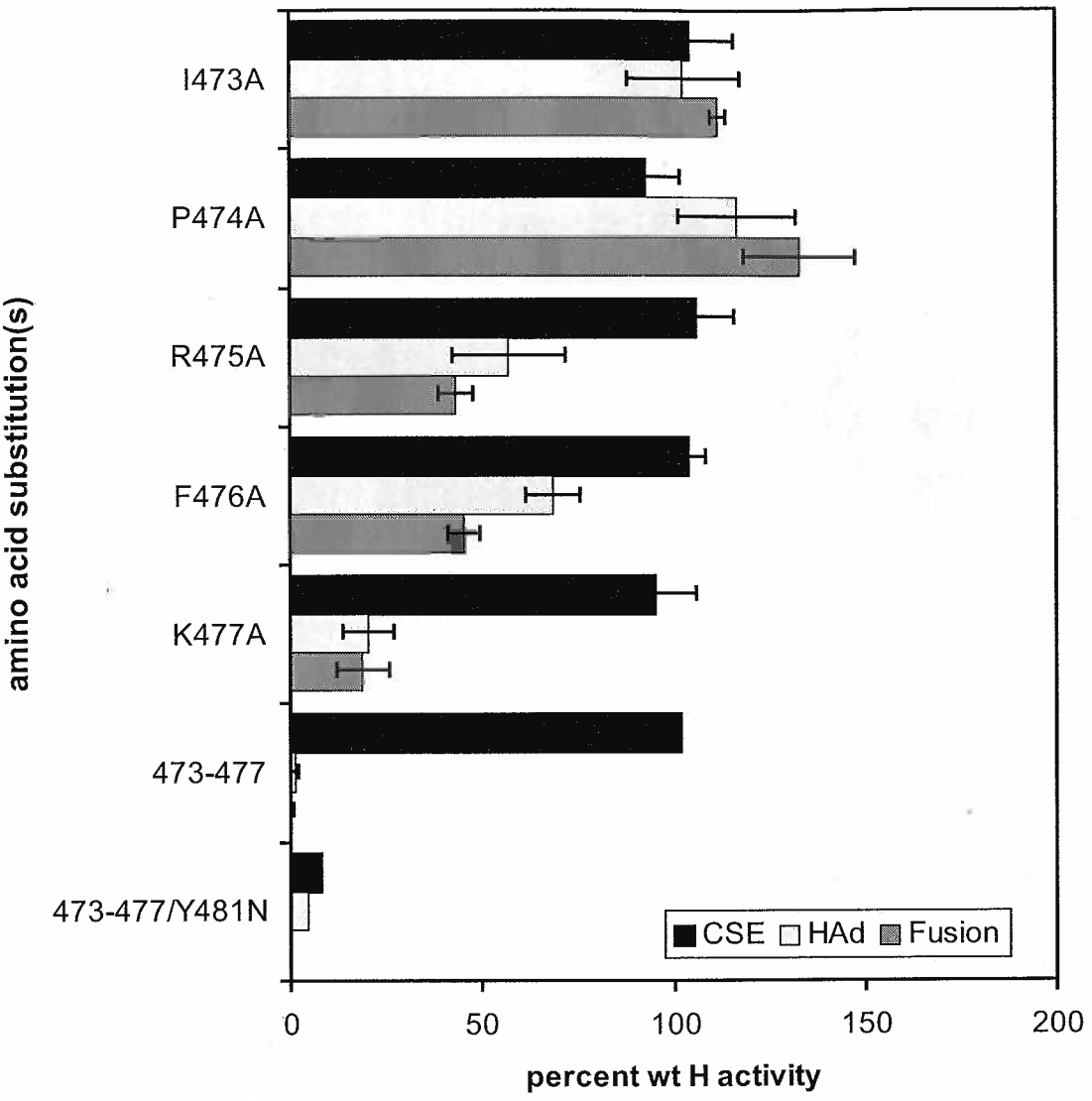



**Figure 35. CSE and functional characteristics of MV H proteins with mutations in the region 473-477.**

The experiments were performed as described in the legend to Figure 33. All data points represent the mean of at least three independent experiments and are expressed relative to the activity of the wt proteins.

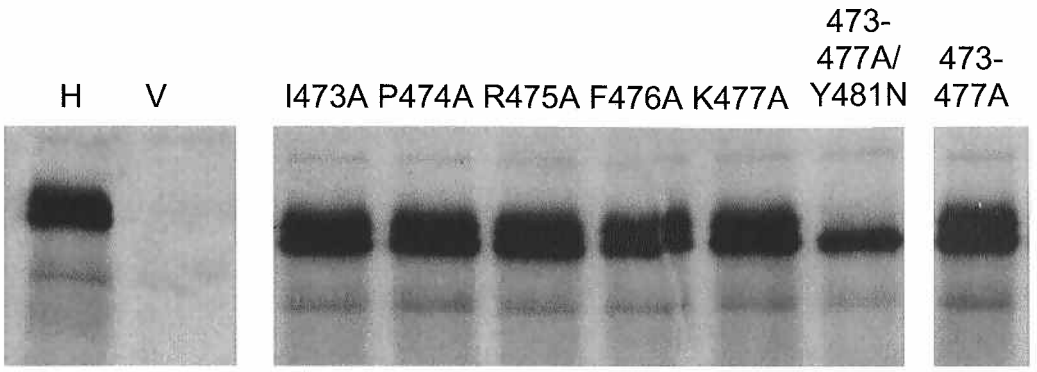



**Figure 36. IP of MV H proteins with mutations in the region 473-477.**

Cells transfected with MV H, vector (V), or a mutated H gene were labeled for 3 hours and incubated in chase medium for 4 hours prior to lysis. The proteins were immunoprecipitated with an antibody against the cytoplasmic tail of H. The immunoprecipitated proteins were collected with Protein G beads and then analyzed by SDS-PAGE under reducing conditions.

mutated proteins is expressed at the cell surface at a level similar to that of wt MV H (Fig. 35). Mutation of either of the first two residues, 473 or 474, does not cause a decrease in either receptor recognition or fusion promotion. It is interesting to note that P474A actually slightly increases CD46 binding and membrane fusion to levels of 116.7% and 133% of wt H activity, respectively. In contrast, mutation of each of the three remaining residues decreases receptor binding to levels of 57.2%, 68.6%, and 20.4% of wt for R475A, F476A, and K477A, respectively. This suggests that the last three residues in this region may have additive, or possibly synergistic, effects that result in the phenotype of 473-477A. Consistent with defects in receptor recognition, all three mutations also exhibit significantly reduced fusion promotion. For each of the substitutions, both glycosylation isoforms of the mutated MV H protein can be detected by IP after a four-hour chase in a ratio similar to that of wt expression (Fig. 36).

**4.3.5 Combined mutation of 473-477A and Y481N results in elimination of both the 78 kDa glycosylation isoform of MV H and receptor recognition.**

In order to attempt to maximize the disruption of receptor recognition of MV H, the substitutions that resulted in the strongest reductions in CD46 binding, 473-477A and Y481N, were introduced together. As shown in Figure 35, the resulting protein exhibits almost complete absence of receptor binding and no detectable fusion promotion activity. While the CSE of this protein, as detected by flow cytometry, was dramatically decreased, the 74 kDa glycosylation isoform of the protein is detectable by IP with an antibody against the MV H cytoplasmic

tail after a four hour chase (Fig. 36). These results suggest that the epitope recognized by the antibody that was used for immunofluorescent labeling and flow cytometry has been altered by the combined mutation of residues 473-477 and Y481.

### 4.3.6 Measles H proteins with defects in receptor recognition interact with MV F at the cell surface.

In order to assess the ability of MV H proteins lacking CD46 binding activity to interact with the F protein at the cell surface, the mutants exhibiting the strongest defects in receptor recognition were assayed for the ability to co-immunoprecipitate with F using an anti-F antibody. For this assay, four mutants were chosen, including K477A, Y481N, 473-477A, and 473-477A/Y481N. As demonstrated, all four of the mutated proteins have significantly reduced receptor recognition and fusion promotion activities (Fig. 37).

Figure 38A shows the co-immunoprecipitation results for the K477A, Y481N, 473-477A, and 473-477A/Y481N mutated MV H proteins with MV Fcsm from the cell surface of HeLa cells. Despite dramatic defects in receptor binding, each of the mutated proteins can be co-immunoprecipitated efficiently with F. Interestingly, despite the lack of the 78 kDa form of H, an interaction between MV F and the 74 kDa form of the MV H mutant 473-477A/Y481N can be detected. Additionally, although the MV H protein carrying the V451E mutation has defects in both receptor binding and fusion helper activities, it also co-immunoprecipitates with MV Fcsm (Fig. 38B). These results are consistent with

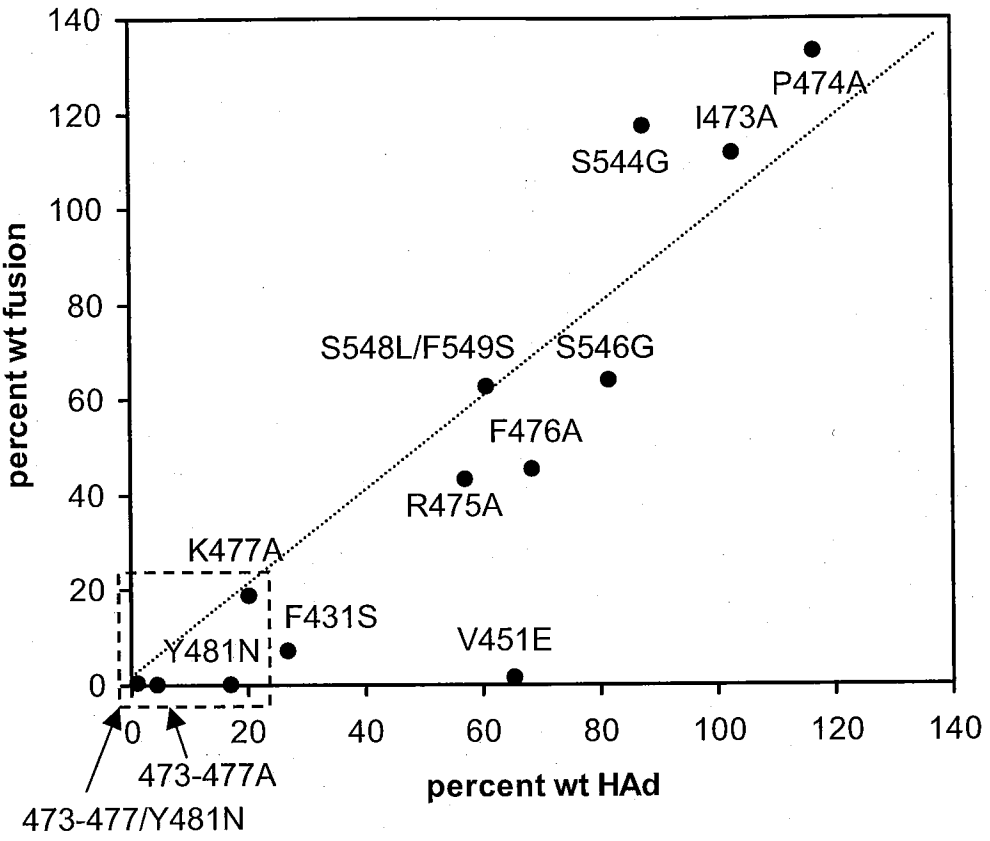



**Figure 37. Graph of hemadsorption versus fusion for MV H proteins with mutations in regions thought to be involved in CD46 binding.**

Each data point represents the average of at least three independent experiments. Mutated proteins enclosed in the dashed box were assayed for the ability to interact with MV F.

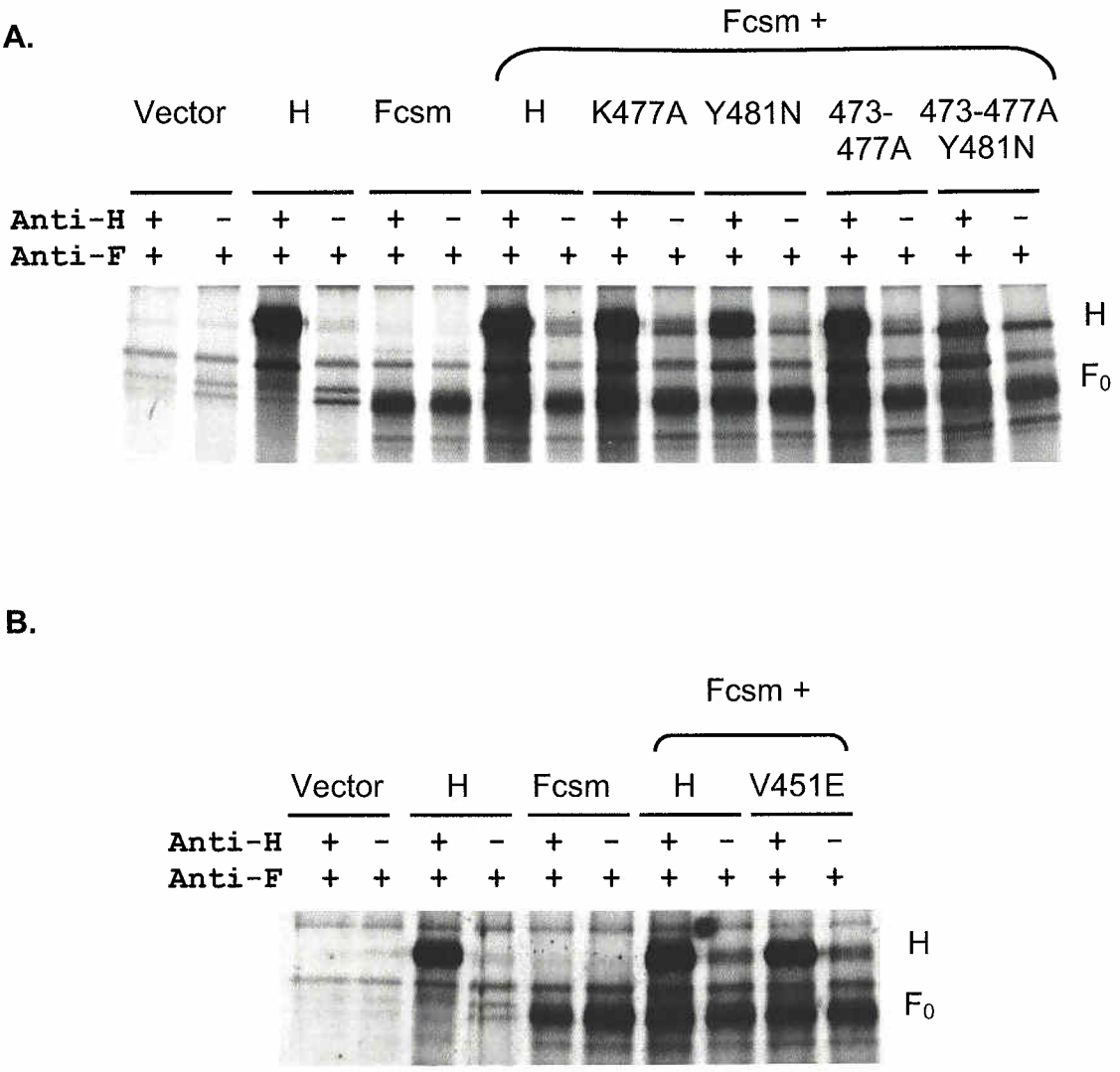



**Figure 38. Co-immunoprecipitation of MV H proteins with defects in receptor recognition.**

(A) Co-immunoprecipitation of MV H, K477A, Y481N, 473-477A, and 473-477A/Y481N mutated H proteins with MV Fcsm. (B) Co-immunoprecipitation of MV H V451E with MV Fcsm. These experiments were performed as described in the legend to Figure 25.

the formation of a complex of the MV H and F glycoproteins at the cell surface independent of receptor recognition by H.

**Summary**

In this chapter, evidence is presented that supports the hypothesis that the MV H and F glycoproteins interact at the cell surface. While an intracellular interaction between the MV H and F proteins expressed in HeLa cells using the vTF7-3 expression system could not be detected, a complex of the two proteins was identified at the cell surface. Additionally, two lines of evidence are presented that indicate that the H-F complex forms independent of receptor recognition. First, co-immunoprecipitation assays were used to demonstrate that an interaction between MV H and F could be detected at the cell surface of CHO cells, which lack both CD46 and CD150. Second, MV H mutants lacking CD46-binding were expressed at the cell surface and shown to interact with MV F in HeLa cells, which express CD46. This is in direct contrast to NDV HN proteins lacking receptor-binding activity, for which an interaction with F at the cell surface cannot be detected.

# CHAPTER V

## Heptad repeat in stalk of MV H is critical to its fusion helper function

### Introduction

Chimera studies and mutation of individual residues in the attachment proteins of other paramyxoviruses have identified domains responsible for the virus-specific communication between the two glycoproteins, as well as specific amino acids involved in triggering of F and regions of HN-F interaction (Deng et al., 1995, Melanson & Iorio, 2004, Melanson & Iorio, 2006, Porotto et al., 2003, Tsurudome et al., 1995). MV H is thought to be involved in multiple aspects of membrane fusion promotion, including receptor recognition and triggering of F activation. It has been suggested that fusion promotion depends on a specific physical interaction between the MV H and F proteins at the cell surface, which as demonstrated in Chapter III, can be detected at the cell surface by a co-immunoprecipitation assay. Residues located both in the stalk (I98) and in the globular head (244 to 250) have been proposed to mediate the fusion helper activity of MV H, but their roles in this function have not been fully examined.

The **aim** of the research discussed in this chapter is to characterize the roles of two regions of H. The **hypothesis** to be tested is that mutations in these regions, which have been proposed to mediate the fusion helper function of H, will modulate fusion without affecting receptor-binding activity. The **rationale** for studying residues 244-250 is the putative identification as the F-interactive site of MV H (Fournier et al., 1997). The **rationale** for studying a heptad repeat in the

stalk is that mutation at one of the heptadic residues, I98T, was found to be responsible for the lack of syncytium formation in a persistently infected cell line (Hummel & Bellini, 1995). Additionally, data from our lab suggest that a corresponding domain in the NDV HN protein may mediate the virus-specific interaction with the homologous F protein (Hummel & Bellini, 1995, Melanson & Iorio, 2004, Melanson & Iorio, 2006). The **approach** used to test the hypothesis is to perform a site-directed mutational analysis of these two regions of MV H and to determine the effects on all aspects of the fusion process.

## Results

### 5.1 Residues in the region between 244 and 250 in the globular head domain of MV H are not involved in mediating the interaction with F that is necessary for fusion promotion.

#### 5.1.1 Alanine-scanning mutagenesis of residues 244-250 of MV H.

Residues 244 to 250 are located in the globular head domain of MV H. In the hypothetical structural model of the MV H protein (Masse et al., 2004), this domain is located at the top of the predicted dimer interface (Fig. 39A). Residues 236-256 form a loop that connects two strands of the first beta-sheet in the globular domain of MV H and has been proposed to form a helical structure (Deroo et al., 1998). As shown in Figure 39B, the amino acid sequence in this

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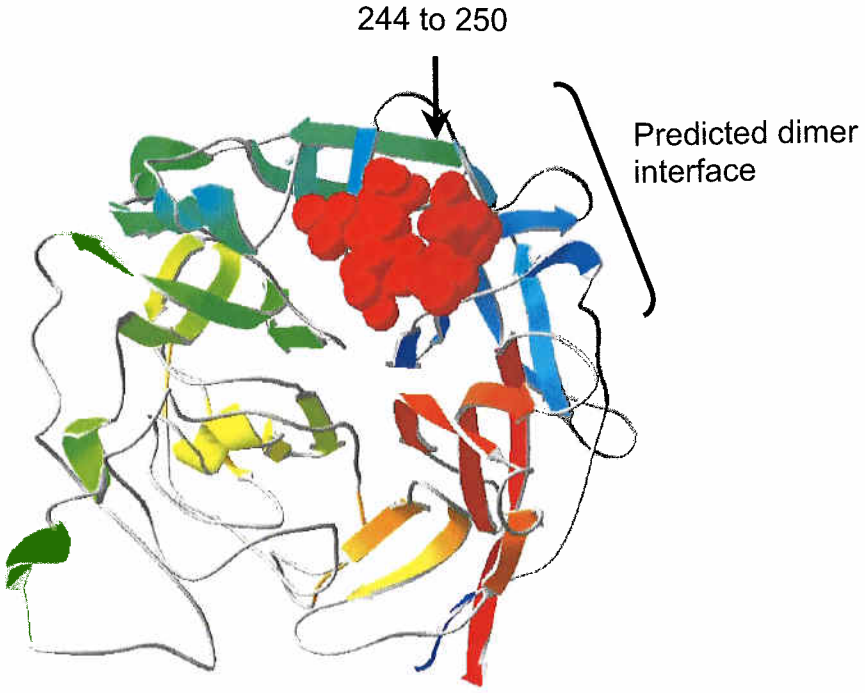



B.

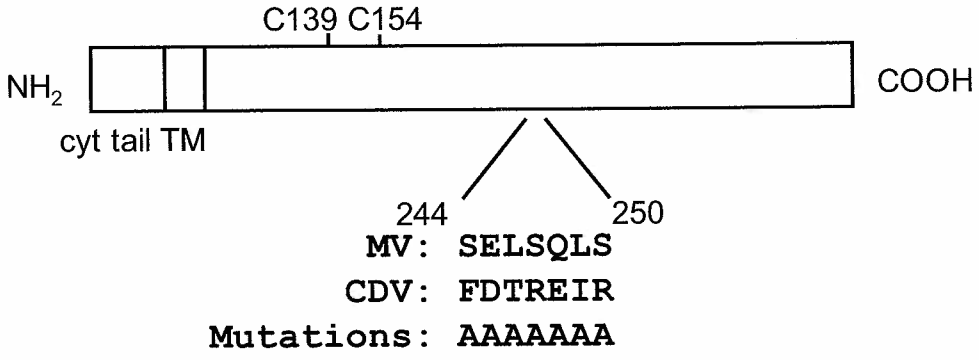



**Figure 39. Location of region 244-250 in the MV H ectodomain.**

(A) Hypothetical structure of MV H with residues 244-250 highlighted in red (adapted from Vongpunsawad et al., 2004). (B) Diagram of MV H showing the sequence of region 244 to 250, as well as an alignment with the corresponding region of CDV H.

region is not conserved between the MV and CDV H proteins. This region has been proposed to form the "topographical or functional interface" between MV H and F (Fournier et al., 1997). However, the roles of residues 244 to 250 in the fusion promotion functions of MV H, including the receptor recognition and fusion helper activities, have not been fully examined. Thus, proteins carrying the following alanine substitutions were prepared and characterized: S241A, K242A, R243A, S244A, E245A, L246A, S247A, Q248A, L249A, S250A, S244A/ L246A, and S244A/ E245A (Figure 39B).

### 5.1.2 Alanine substitutions for residues 244 to 250 do not significantly alter the biological characteristics of MV H.

The ability of MV H proteins carrying alanine substitutions at residues 244 to 250 to promote membrane fusion was quantitated using a content mixing assay. All of the mutated proteins promote cell-to-cell fusion at levels similar to that of wt MV H, ranging from 80-95% of the wt level (Fig. 40). CSE of the mutated proteins was determined by flow cytometry, and receptor-binding activity of the proteins was evaluated by assaying their ability to adsorb AGM erythrocytes. As would be expected, based on their ability to efficiently promote membrane fusion, the CSE and receptor binding levels of these proteins are similar to wt H. CSE of the proteins ranges from 92% for Q248A to 111% for L246A. Hemadsorption levels range from 88% for L249A to 99% for E245A. A plot of hemadsorption versus fusion shows that the slight reductions in membrane fusion for some of the mutants, including S244A, L246A, and L249A,

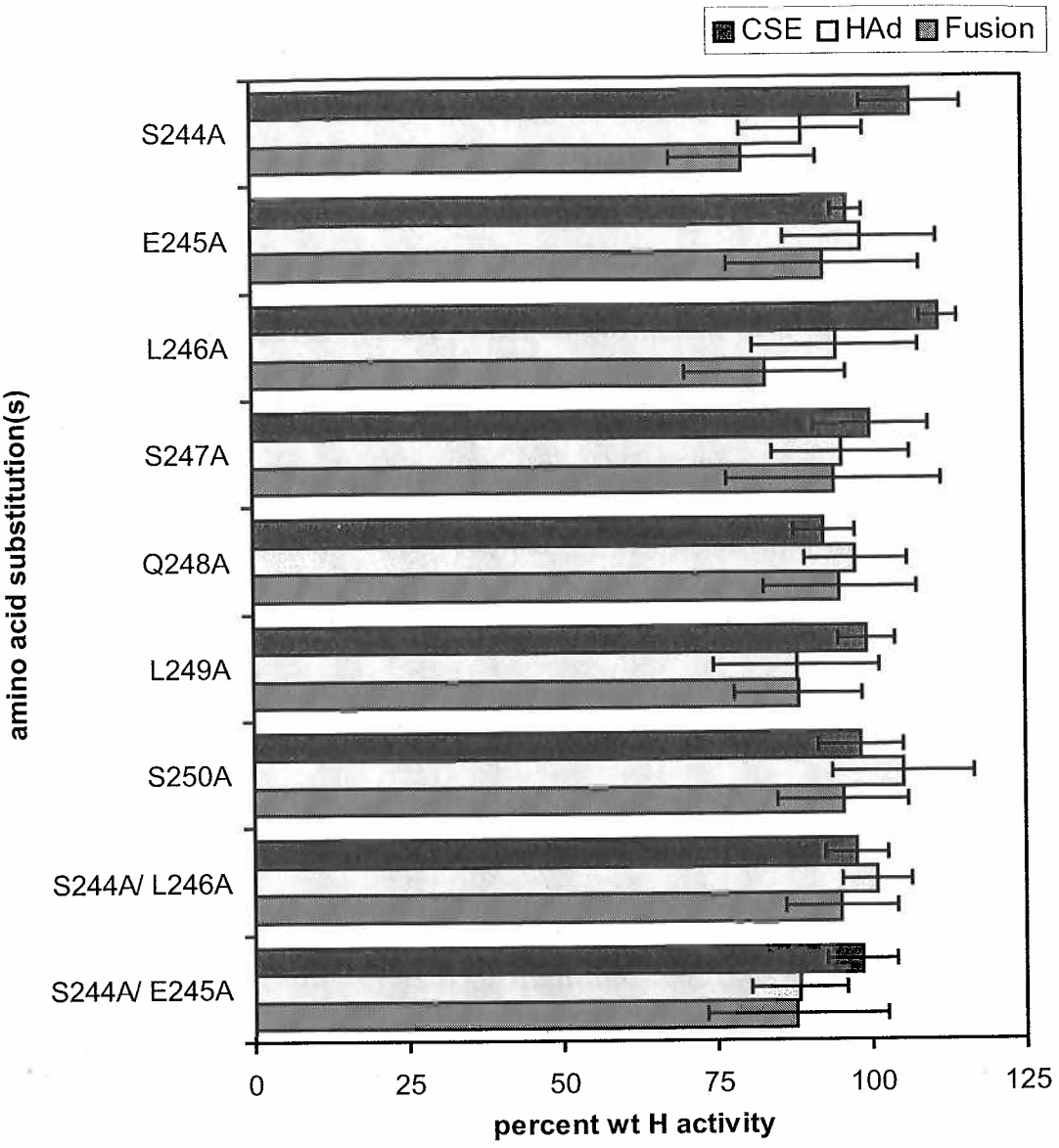



**Figure 40. CSE and functional characteristics of MV H proteins carrying mutations in residues 244-250.**

CSE was determined by flow cytometry using an anti-H antibody that recognizes the ectodomain. The HAd activity was determined by the ability of monolayers transfected with the H genes to adsorb AGM erythrocytes at 37°C. The ability of the H proteins to complement MV Fcsm in the promotion of membrane fusion was determined by a content mixing assay. For each assay, the background detected in cells transfected with vector has been subtracted. All data points represent the mean of at least three independent experiments and are expressed relative to the activities of the wt proteins.

correlate with reductions in receptor recognition (Fig. 41). As it is possible that a single alanine mutation in this region could be insufficient to disrupt the functions of the H protein, two additional proteins carrying double alanine substitutions were prepared and characterized. Both double mutations, including S244A/ L246A and S244A/ E245A, result in fusion promotion at a level similar to wt H protein activity (95% and 88% of wt activity, respectively) and greater than that of any of the three individual mutations. Corresponding results were obtained for CSE and receptor binding. These results are not consistent with the region 244 to 250 acting as the functional interface between MV H and F. On the basis of these findings, this region was not investigated further.

## 5.2 Mutations in a heptad repeat (HR) in the stalk of MV H modulate the fusion helper function.

### 5.2.1 Site-directed mutagenesis of heptadic residues in the HR domain of the MV H stalk.

Examination of the sequence of the MV H stalk reveals a HR of hydrophobic residues spanning the region I84 to L105 (Fig. 42). A mutation at one of the heptadic residues, I98T, was found to be responsible for the lack of syncytium formation in a persistently infected cell line (Hummel & Bellini, 1995). Computer models of this region predict that it has an alpha-helical structure followed by a region of random coil (Fig. 42). The roles of the heptadic residues in the fusion process were examined by the introduction of an alanine

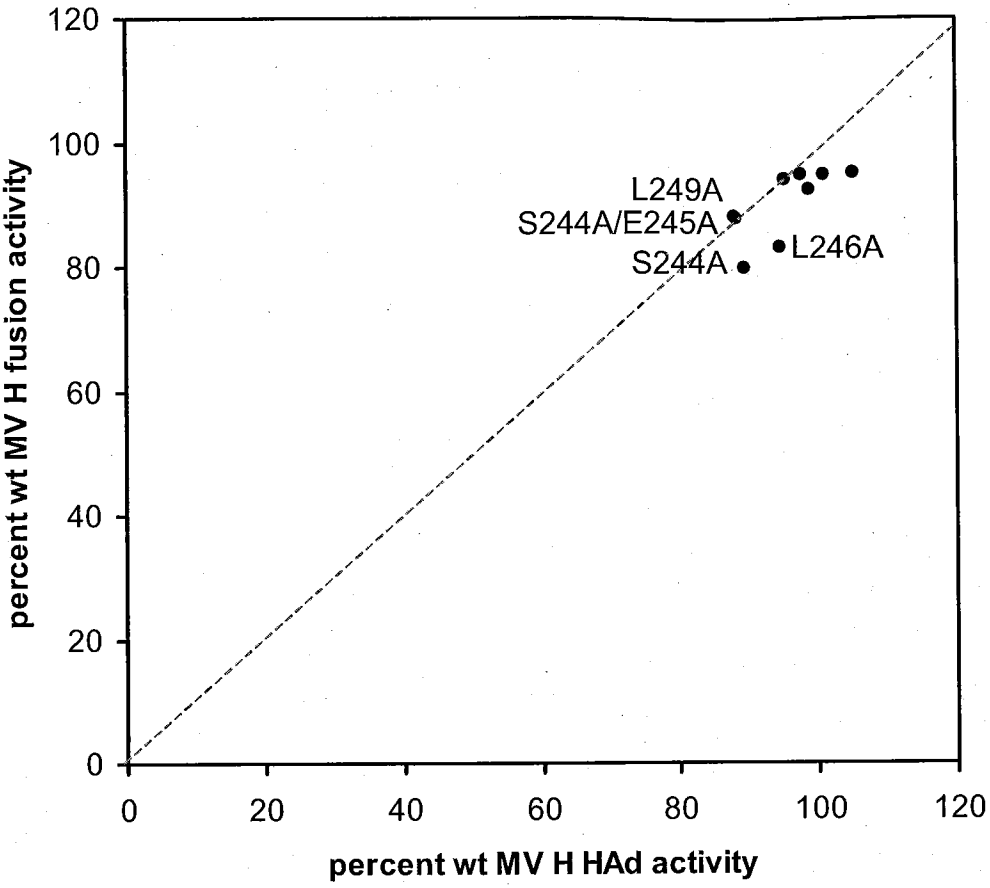
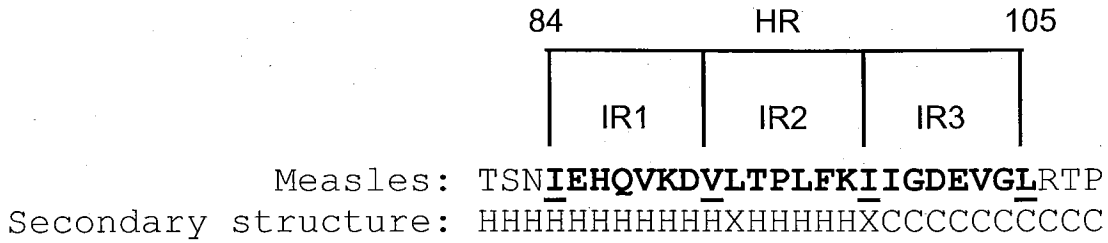



**Figure 41. Plot of hemadsorption versus fusion activity of MV H proteins carrying mutations in residues 244-250.**




H = alpha helix, X = ambiguous state, C = random coil

**Figure 42. Amino acid sequence of heptad repeat (HR) in the stalk of MV H.**

Heptadic hydrophobic residues are underlined. There are four heptadic residues (underlined) with three intervening regions (IRs). The secondary structure prediction was performed on Network Sequence Analysis (Combet et al., 2000). Eight secondary structure prediction programs (DPM, DSC, GOR4, HNC, PHD, Predator, SIMPA96, and SOPM) were used, and the final prediction represents the consensus of their results.

substitution at each position in the MV H protein, and the resulting proteins were characterized (Fig. 43). Based on the results of Hummel and Bellini (1995), an additional protein with an I98T substitution was prepared and characterized.

### 5.2.2 Amino acid substitutions at the heptadic residues of the HR domain modulate fusion promotion by MV H.

The ability of MV H proteins with amino acid substitutions at I84, V91, I98, and L105 to complement MV F in fusion promotion was quantitated using a content mixing assay. H proteins carrying the mutations I84A, I98A, I98T, and L105A exhibit reduced fusion promotion activities of 0%, 0.7%, 5%, and 47% of wt H, respectively (Fig. 44). The mutation V91A results in only a very slight reduction in fusion promotion (94% of wt H). CSE of the mutated proteins was determined by flow cytometry. The reduction in fusion promotion by the mutated proteins cannot be attributed to reduced CSE, which ranges from 90 to 108% of wt H expression.

To determine if the basis for the reduced fusion promotion by the mutated proteins results from decreased receptor recognition, hemadsorption of AGM erythrocytes was used to quantitate CD46 binding activity. Only one of the four mutated proteins with deficiencies in membrane fusion promotion, I84A, also has significantly reduced hemadsorption activity (34% of wt H). This suggests that the reduced fusion promotion exhibited by the I84A-mutated protein correlates with an alteration in the structure of the globular head domain. The other

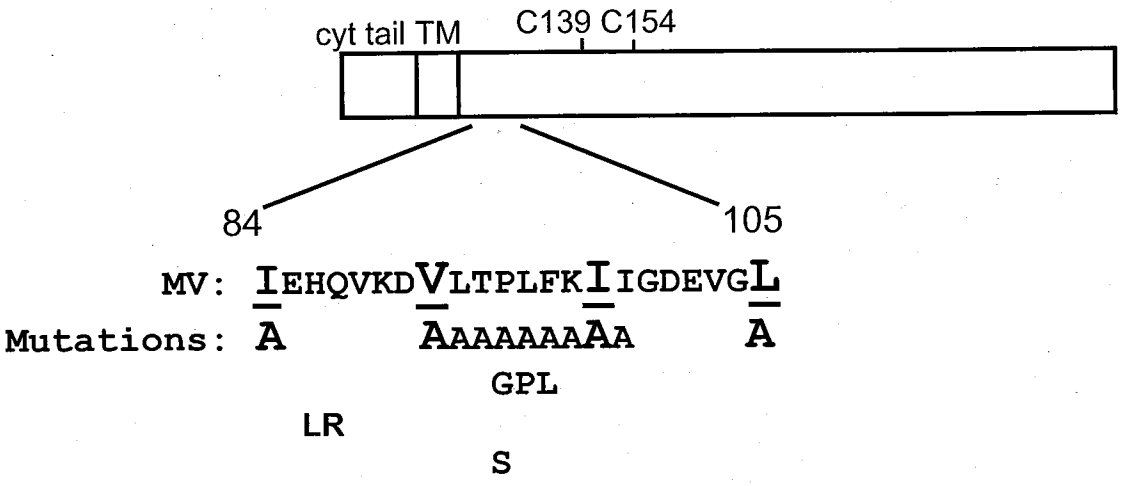



**Figure 43. Diagram of MV H showing the location and sequence of the heptad repeat domain in the putative stalk.**

The heptadic residues are underlined, and the amino acid substitutions introduced into the region are listed below the sequence.

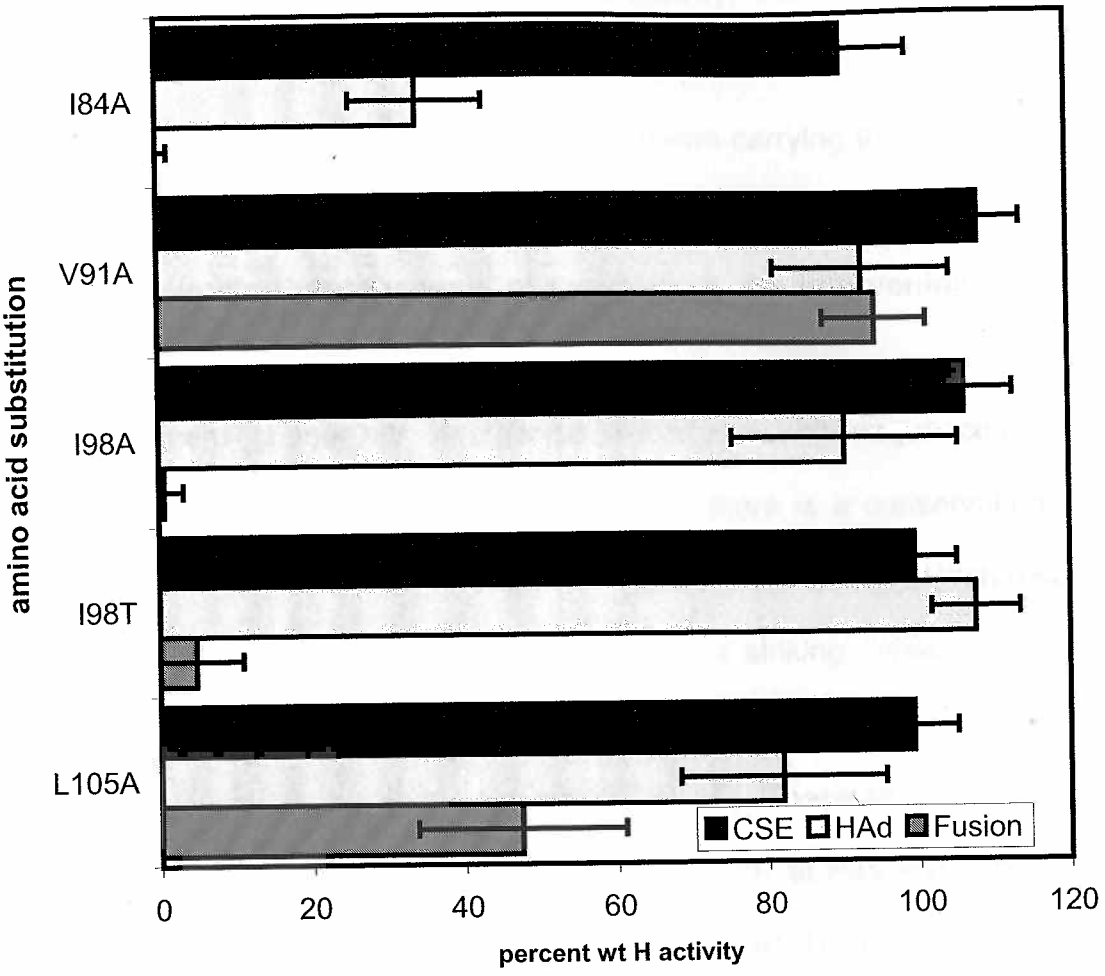



**Figure 44. CSE and functional characteristics of MV H proteins carrying mutations in heptadic residues of the HR domain.**

The experiments were performed as described in the legend to Figure 40. All data points represent the mean of at least three independent experiments and are expressed relative to the activities of the wt proteins.

proteins, carrying the mutations I98A, I98T, and L105A, have hemadsorption activities ranging from 82 to 108% wt H activity, indicating that the fusion-deficiency of these mutated proteins is not a result of decreased recognition of CD46. Importantly, the phenotype of the H protein carrying the I98T mutation is consistent with the results of the study by Hummel and Bellini (1995).

### 5.2.3 Site-directed mutagenesis of residues in the intervening regions of the HR domain of the MV H stalk.

Alignment of the MV H stalk sequence with the sequences of other paramyxoviruses reveals that, within the family, there is a conservation of HR domains in similar locations. Although the HRs are not conserved with respect to location, length, or number of repeats, there is a striking conservation of two residues near the middle of each region: a proline that is completely conserved, as well as a leucine that is semi-conserved (Fig. 45). These residues are located in the second intervening region (IR) of the MV H HR at P94 and L95. Initially, their roles in CD46 binding and fusion promotion by MV H were examined by the introduction of alanine residues at each position. Subsequently, the following MV H mutant proteins were prepared and characterized: P94G, -L, and –S, as well as L95P and – R. The remaining residues in the second IR between the heptadic hydrophobic residues were also each individually replaced with alanine residues (Fig. 43). Mutated proteins, carrying L92A, T93A, F96A, and K97A substitutions, were constructed. Two additional mutations, including F96L and I99A, were also tested.

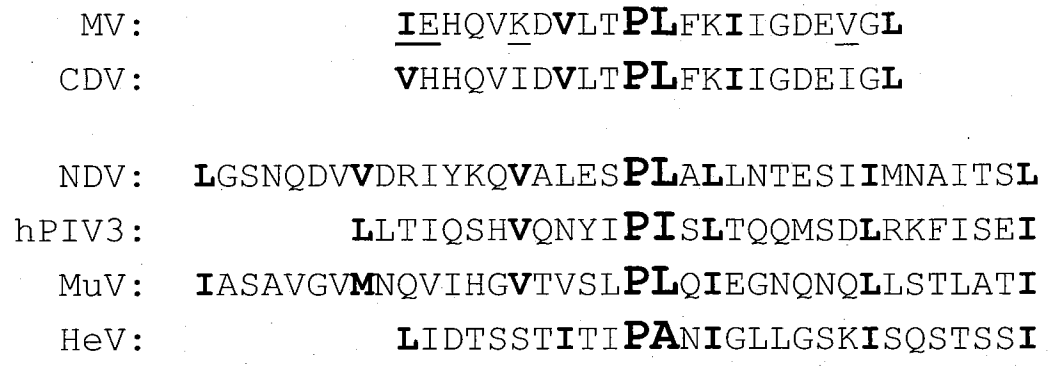



**Figure 45. Comparison of the amino acid sequences in the HR domains in the stalks of paramyxovirus attachment glycoproteins.**

The completely conserved proline and highly conserved leucine residues are highlighted in bold and enlarged. Residues that differ between the MV and CDV H domains are underlined.

### 5.2.4 Amino acid substitutions for intervening residues in the HR domain modulate receptor binding and/or fusion promotion by MV H.

Initially, the MV H proteins with amino acid substitutions at P94 were tested for the ability to complement MV F in fusion promotion. As shown in Figure 46, H proteins carrying the mutations P94A, -G, -L, and- S exhibit fusion promotion activities of 3%, 0%, 2.8%, and 7.9%, respectively, of the level of wt H activity. While each of the mutated proteins has a CSE level similar to wt H, ranging from 95% to 102%, the receptor binding by the proteins is significantly reduced to levels of 36%, 36%, 44%, and 48% of wt H, respectively (Fig. 46). These results suggest that amino acid substitutions at P94 alter the structure and/or orientation of the globular head domain, in which the receptor-binding site resides.

MV H proteins with amino acid substitutions at L95 were also tested for the ability to complement MV F in fusion promotion. As shown in Figure 46, each of the mutated proteins exhibits a significant deficiency in the ability to promote membrane fusion, with activities ranging from 0.5% to 32% of wt H level. Each of the proteins carrying a substitution at L95 is expressed at the cell surface at a level similar to wt H (95% to 109%). While L95A and –R mutations do not significantly decrease CD46 recognition by MV H, mutation of L95 to proline strongly reduces receptor binding to a level of 34% of wt H (Fig. 46). These results suggest that the identity of the amino acid at position 95, not only influences the structure of the globular domain of MV H, but may also play a role

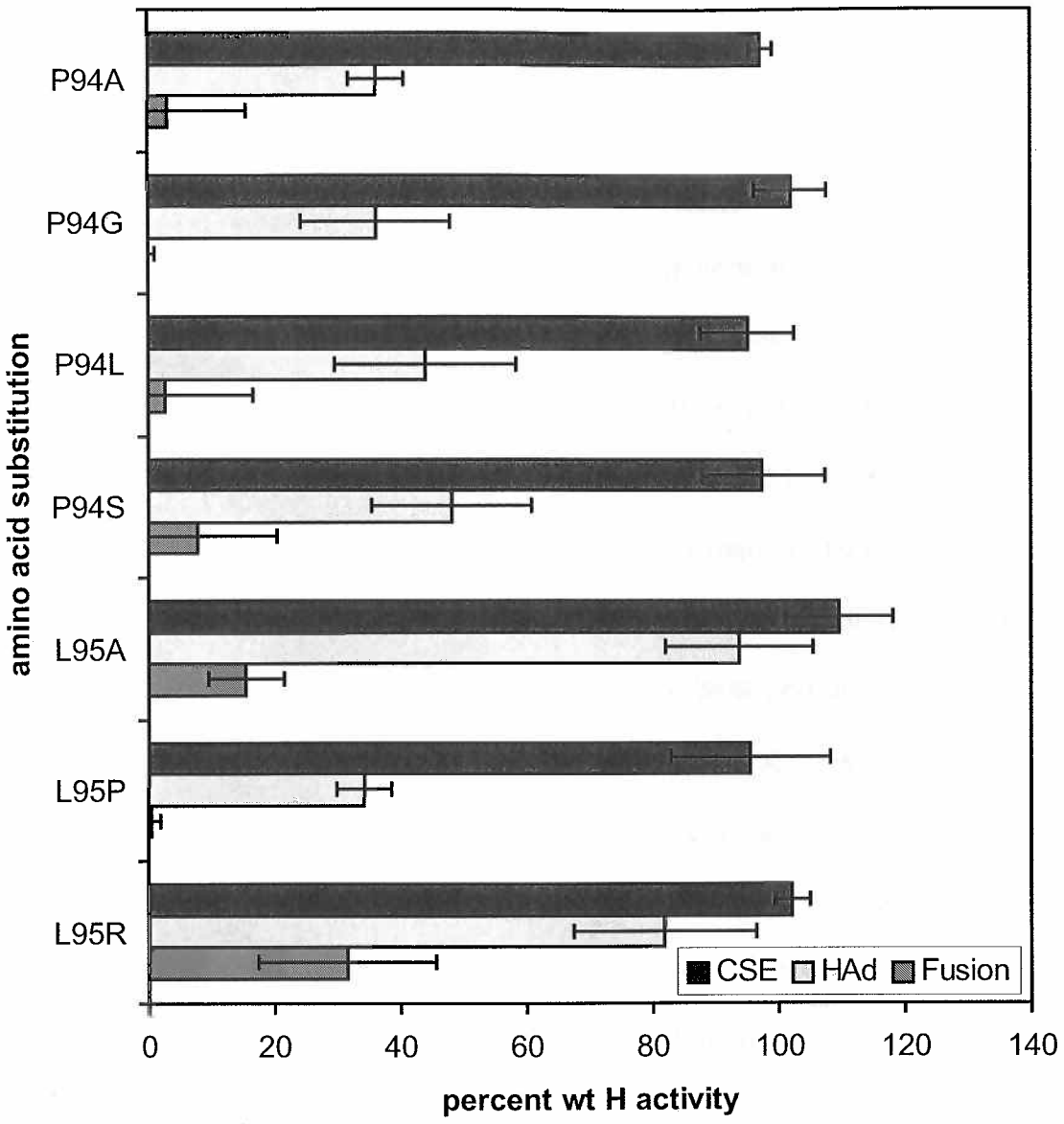



**Figure 46. CSE and functional characteristics of MV H proteins carrying mutations in P94 and L95 in the HR domain.**

The experiments were performed as described in the legend to Figure 40. All data points represent the mean of at least three independent experiments and are expressed relative to the activities of the wt proteins.

in fusion promotion.

Alanine substitutions at the remaining residues in the second IR of the HR domain, including L92, T93, F96, and K97, were tested for their ability to modulate the functions of MV H. Subsequently, F96 was also mutated to leucine. One residue, I99, in the third intervening sequence was also tested. Each of the mutated proteins is expressed at the cell surface at a level similar to wt H, ranging from 86% to 100% of the level of wt H (Fig. 47). The ability of the mutated MV H proteins to promote membrane fusion with MV F was quantitated, and, as Figure 47 shows, all except one of the mutations, T93A, significantly decreases fusion promotion. Two of the amino acid substitutions, L92A and I99A, also decrease receptor recognition to levels of 34% and 38%, respectively. Substitutions at the two remaining residues, F96 and K97, result in significant deficiencies in fusion promotion not attributable to corresponding reductions in CD46 recognition. Together with the data obtained for the conserved P94 and L95 residues, these results suggest that the intervening sequences of the HR domain in the stalk of MV H are important for both the structure and functions of MV H.

### 5.2.5 Comparison of hemadsorption versus fusion promotion activity segregates amino acid substitutions in the MV H HR into three groups.

Examination of a plot of hemadsorption versus fusion promotion activity of MV H proteins carrying amino acid substitutions in the HR domain reveals that the mutated proteins can be divided into three groups based on their functional

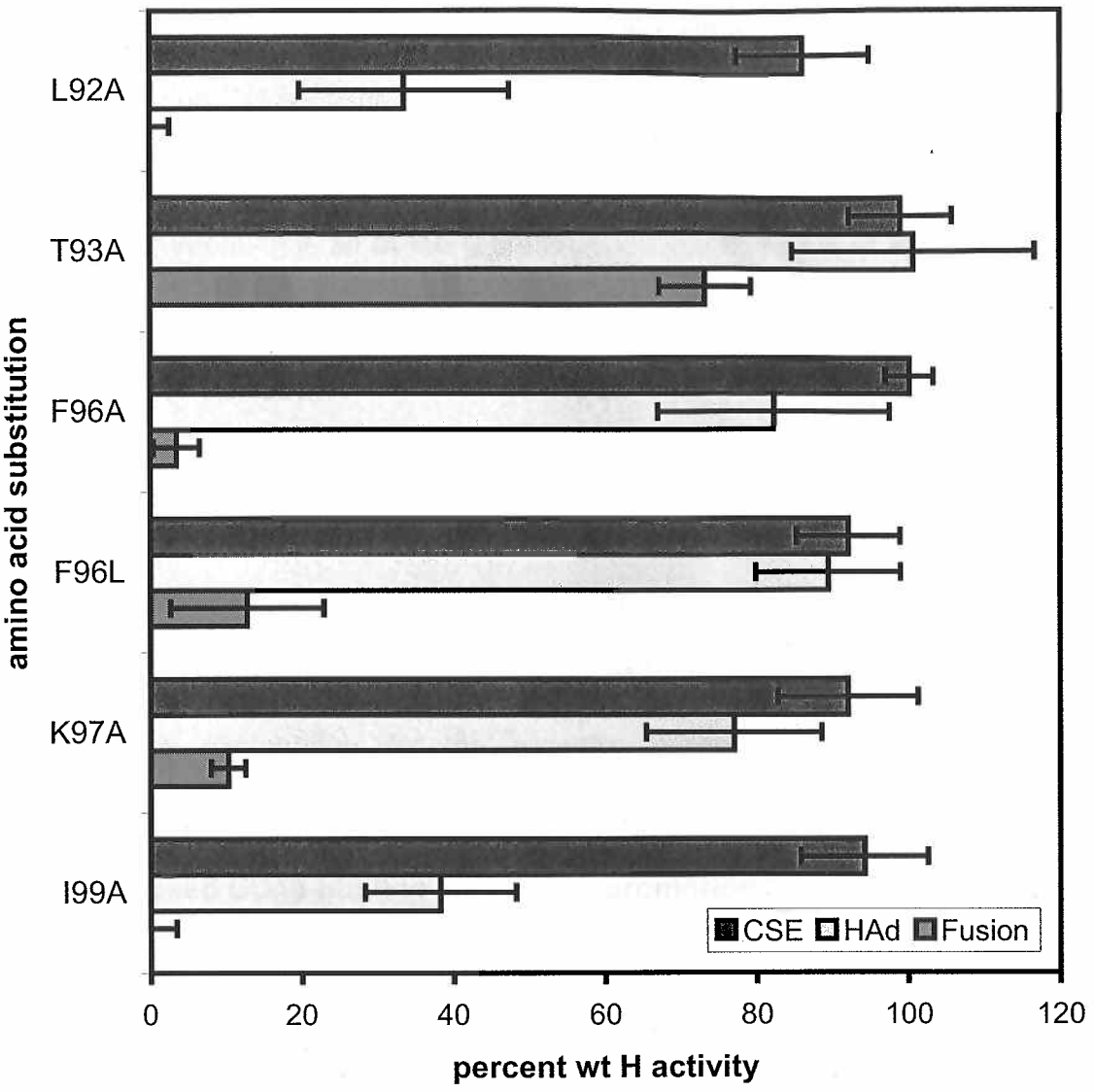



**Figure 47. CSE and functional characteristics of MV H proteins carrying mutations in the intervening regions of the HR domain.**

The experiments were performed as described in the legend to Figure 40. All data points represent the mean of at least three independent experiments and are expressed relative to the activities of the wt proteins.

activities (Fig. 48). The first group (V91A and T93A) includes amino acid substitutions that do not result significantly alter either receptor recognition or fusion promotion. The second group (examples: I84A, L92A, and P94S) is characterized by strong deficiencies in both receptor recognition and fusion promotion. Interestingly, all of the proteins carrying mutations at P94 are in this group, suggesting that the identity of this amino acid is particularly important for maintaining the correct structure and/orientation of the globular head that is required for CD46 binding. The third group (examples: L95A, F96A, and I98A) exhibit significant deficiencies in fusion promotion activity that are not attributable to loss of receptor recognition. All of the MV H proteins carrying mutations in the HR domain have CSE levels similar to that of wt H, suggesting that there must be an alternative explanation for the receptor binding and fusion promotion deficiencies associated with the last two groups.

### 5.2.6 Decreased CD46 binding and fusion promotion do not correlate with altered sedimentation in sucrose gradients.

The stalk region of paramyxovirus attachment proteins is thought to be important for stabilizing the tetrameric structure in the absence of ligand binding (Yuan et al., 2005). Thus, it is possible that mutation of residues in this region could alter the receptor recognition and fusion promotion activities of MV H by interfering with tetramerization. In order to investigate this possible explanation for the phenotypes of the proteins carrying point mutations in the HR domain of the stalk of MV H, sucrose gradient profiles were generated for some of the

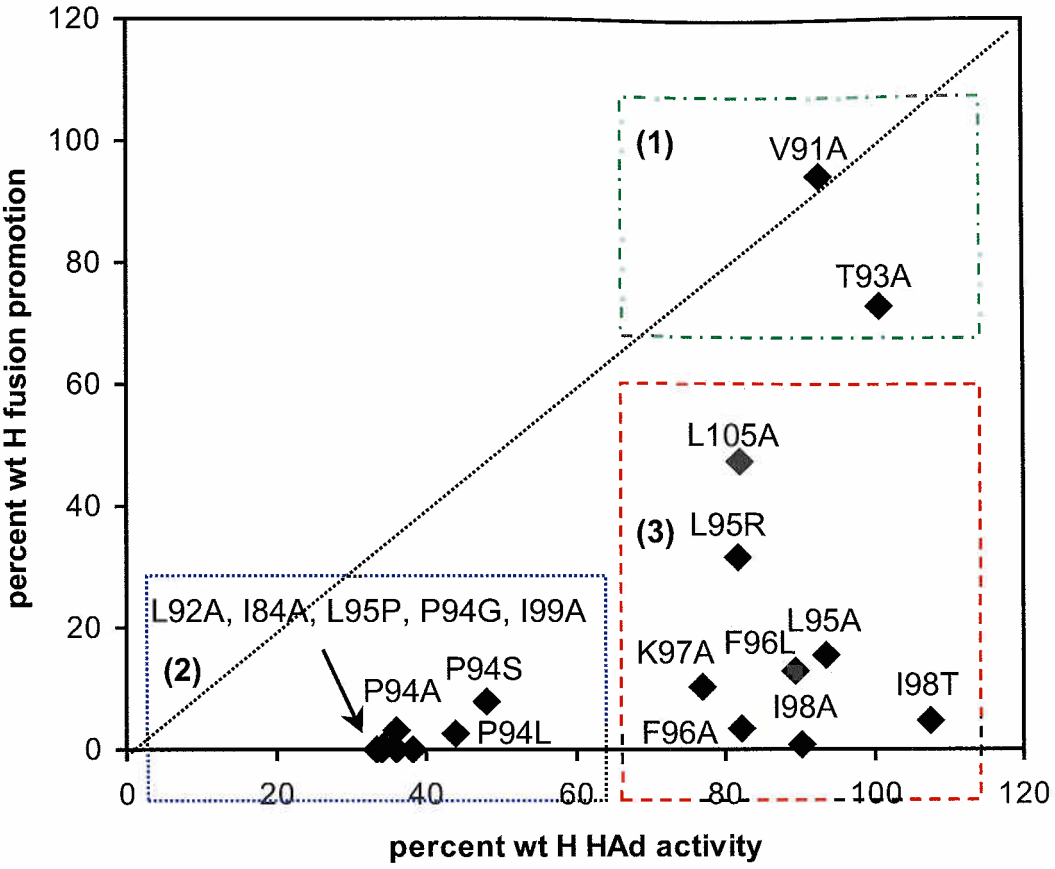



**Figure 48. Plot of hemadsorption versus fusion activity of MV H proteins carrying mutations in the HR region of the stalk.**

Based on this plot, the mutated proteins can be divided into three groups: (1) no significant deficiencies in receptor recognition and fusion promotion, (2) significant deficiencies in both receptor recognition and fusion promotion, and (3) significant deficiencies in fusion promotion, but not receptor recognition.

mutated proteins exhibiting the most extreme functional deficiencies.

Initially, the ability of the sucrose gradient sedimentation technique to distinguish between the tetrameric and dimeric forms of MV H was tested. Figure 49 shows the sucrose gradient sedimentation profiles for native MV H and MV H that has been pre-treated with 0.5% SDS to disrupt non-disulfide linked structures prior to sedimentation. While the native forms of MV H are spread throughout the heavier fractions, peaking in fractions 9 to 13, SDS-treated MV H is predominantly found in fractions 13 to 17 with a peak at 15, indicating that a disruption of non-disulfide linked MV H structures can be detected by this method.

Sucrose gradient sedimentation profiles were generated for five of the mutated proteins with receptor binding and/ or fusion promotion deficiencies, including I84A, L92A, P94A, L95A, and I98T (Fig. 50). Although each of these mutated proteins has a significant functional defect, each exhibits a sucrose gradient sedimentation profile similar to that of wt H with peaks in fractions 9-11. These results suggest that the phenotypes of these mutated proteins do not result from a disruption of oligomerization.

### 5.2.7 Amino acid substitutions in the HR domain of MV H that abolish fusion also abolish hemifusion.

During the membrane fusion process, it is thought that activation of F and insertion of the fusion peptide into the target membrane initially leads to hemifusion, or merger of the lipid bilayers, before fusion pore formation and

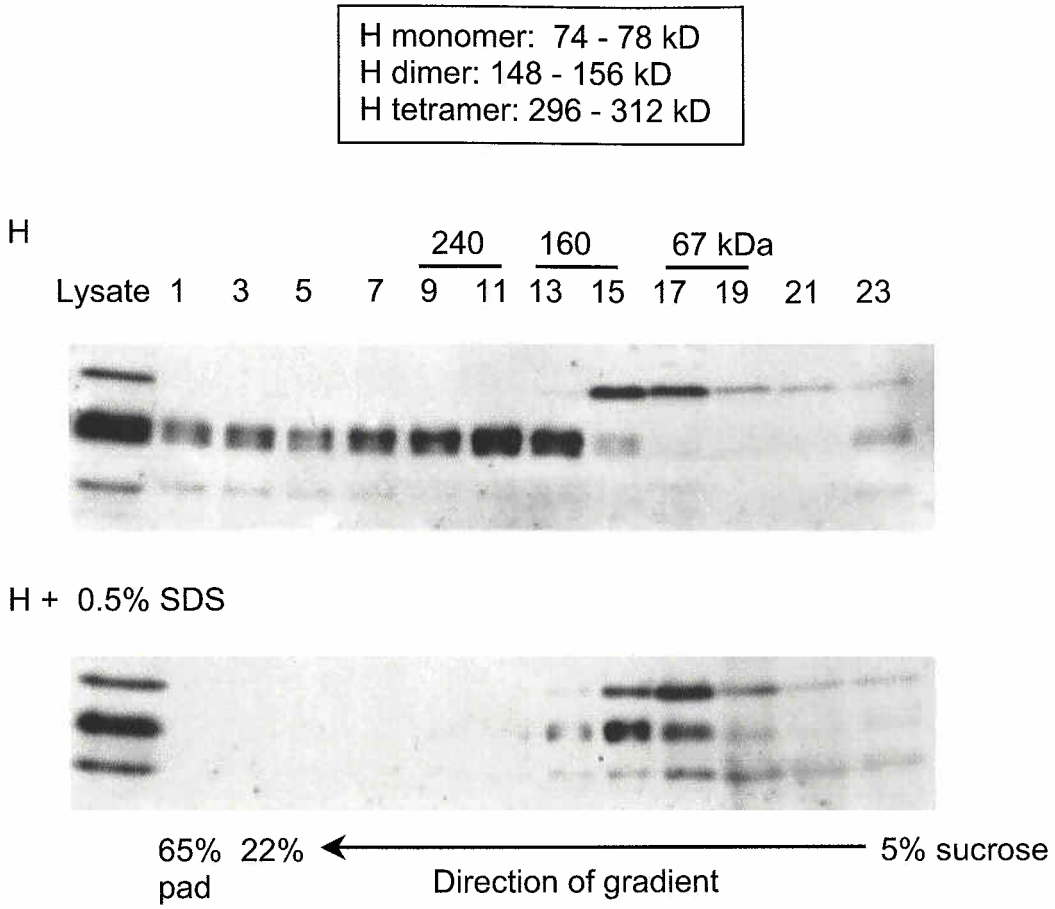



**Figure 49. Sucrose gradient sedimentation profiles of MV H and MV H treated with 0.5% SDS.**

Cells expressing MV H were lysed and layered onto continuous 5-22% sucrose gradients in 0.1% octylglucoside. The proteins in odd numbered fractions were TCA precipitated and analyzed by SDS-PAGE on 10% acrylamide gels under reducing conditions. The proteins were transferred to Immobilon membranes by Western blotting, and MV H was detected with an anti-MV antibody. A companion gradient was run with molecular mass protein markers including bovine albumin (67 kDa), aldolase (160 kDa), and catalase (240 kDa).

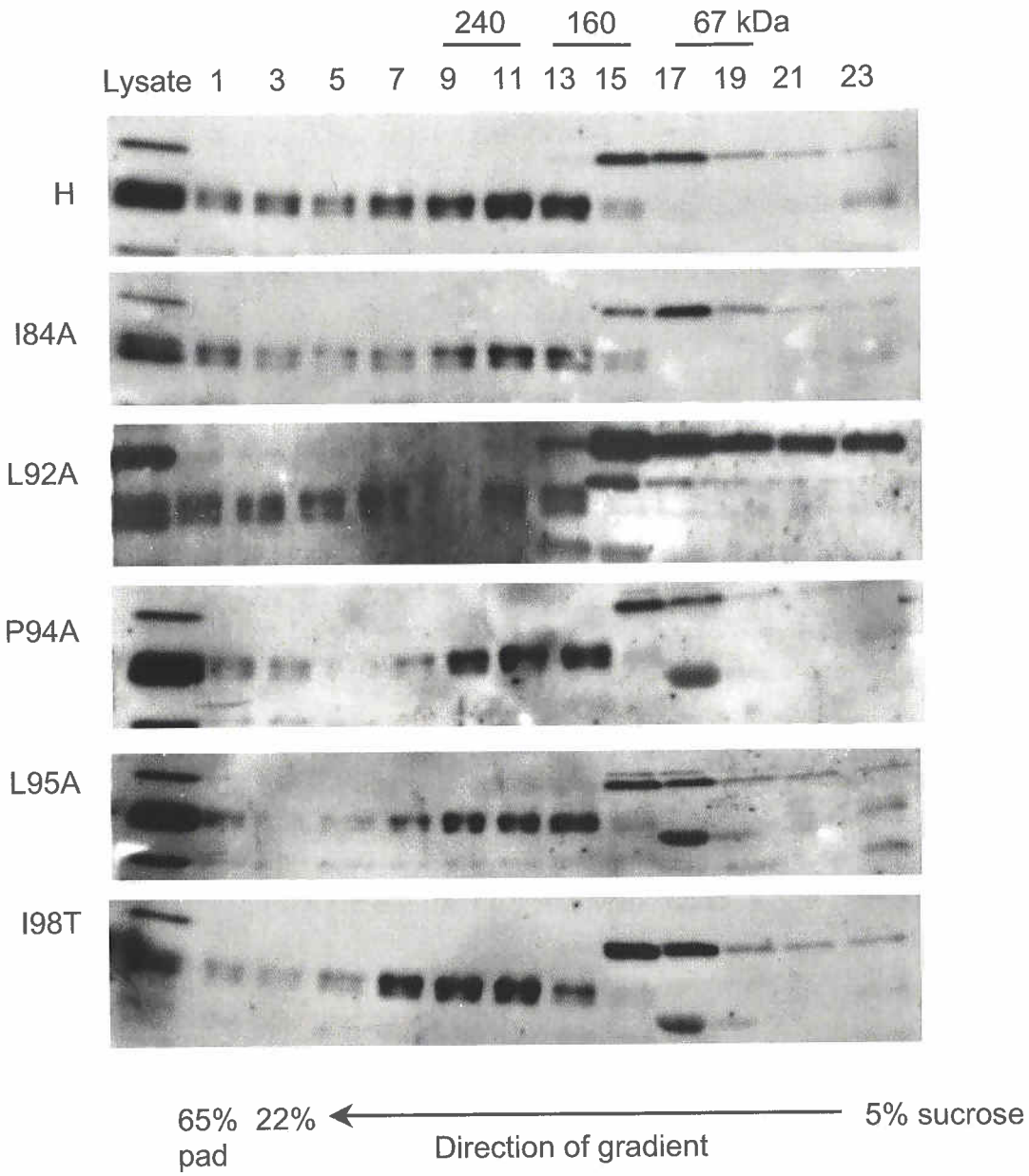



**Figure 50. Sucrose gradient sedimentation profiles of MV H proteins carrying mutations in the HR domain.**

The experiments were performed as described in the legend to Figure 49.

content mixing. Mutation of the conserved proline, P111, between the HRs in the stalk of the hPIV3 attachment protein does not alter receptor binding, but has been found to decrease the rate of triggering of the F protein into its activated form (Porotto et al., 2003). Insertion of the fusion peptide into the target membrane and hemifusion was shown to occur, but more slowly than when F was triggered by wt HN-expressing cells. A hemifusion assay was used to determine if this is also the case for the MV H proteins carrying mutations in the HR that abolished fusion without altering CD46 binding. For this assay, AGM erythrocytes were labeled with a membrane soluble dye known as R18. If hemifusion is triggered by H and F expressed in transfected cells, the dye is distributed between the two cell types. If H does not trigger F, no dye transfer is detectable.

Figure 51 shows the results of a hemifusion assay performed with wt MV F co-expressed with the H proteins carrying substitutions P94A, L95A, I98A, and I98T. The controls for this experiment show that there is dye transfer from the labeled erythrocytes to cells expressing wt H and F, but not when H is expressed with Fcsm. Each of the mutated proteins tested exhibits a significant decrease in the ability to promote fusion. As expected, based on the previous results for hemadsorption activity, the P94A mutated protein does not show significant receptor binding, and thus, does not trigger hemifusion. Despite detectable hemadsorption of the labeled red blood cells, dye transfer is not detectable for the remaining mutants tested with this assay. These results suggest that

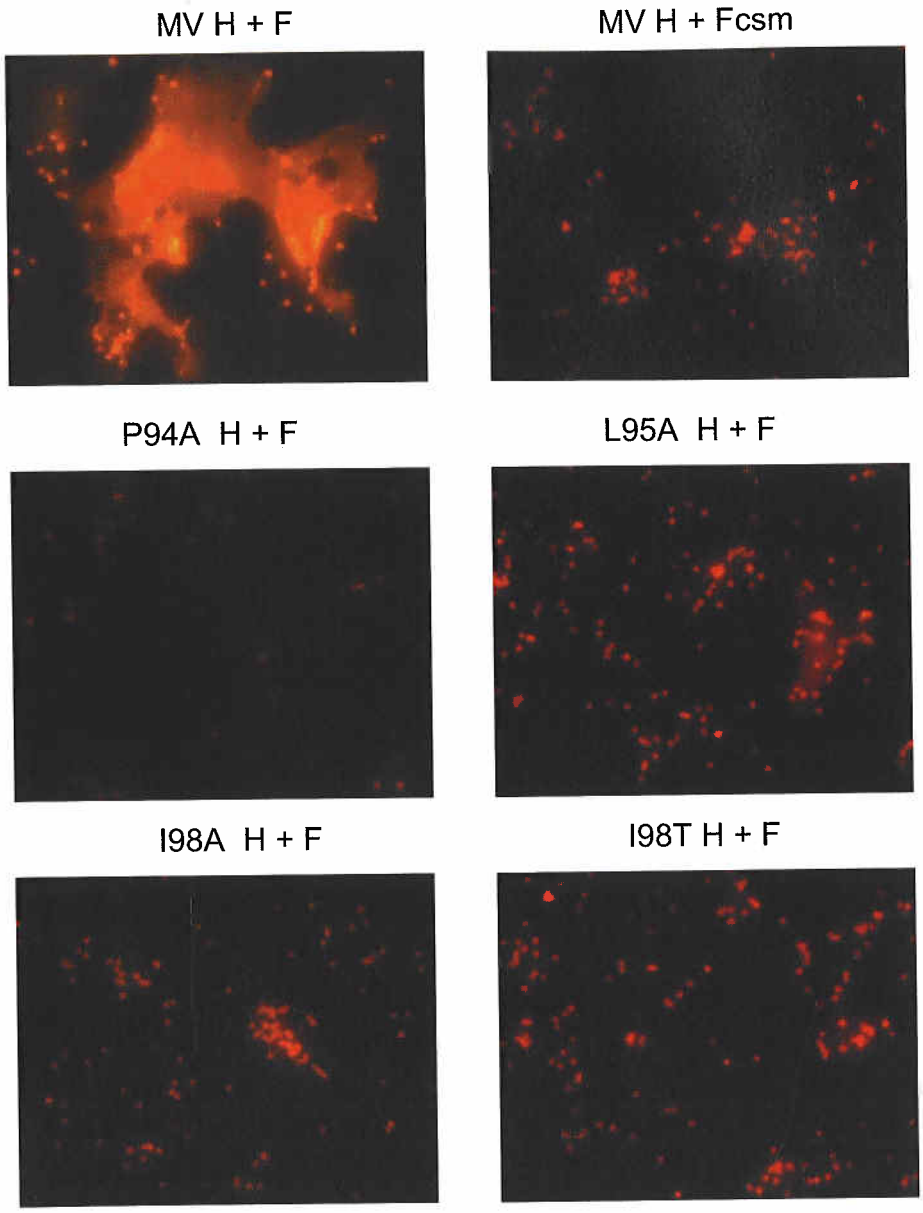



**Figure 51. Promotion of hemifusion by MV H proteins carrying mutations in the HR domain.**

Hemifusion assays were performed using HeLa cells at 18 hours post-transfection with the MV H and F genes. R18-labeled erythrocytes were added to each monolayer, and then the cells were incubated for 30 min on ice. Fusion between the cell monolayers and erythrocytes was initiated by transferring cells to 37°C. After 30 minutes, the cells were washed three times with warm PBS+ to remove unbound erythrocytes. Images were immediately acquired with a 20x objective using fluorescent microscopy.

membrane fusion promotion by these mutated proteins is blocked before MV H triggers F activation to initiate insertion of the fusion peptide into the target membrane.

### 5.2.8 Amino acid substitutions in the HR domain of MV H do not disrupt the physical association of H and F at the cell surface.

As shown in Chapter III, a cell surface interaction between MV H and F in HeLa cells can be detected using a co-immunoprecipitation assay. Mutations in the IR between the heptad repeats in the stalk of the NDV HN protein have been shown to de-stabilize the HN-F interaction at the cell surface (Melanson & Iorio, 2004). In order to investigate this possible explanation for the phenotypes of the MV H proteins carrying point mutations in the HR domain, several of the mutated proteins were tested for the ability to interact with F at the cell surface. For these assays, a cleavage site mutant of F, which is known as Fcsm and is unable to promote fusion in the absence of exogenously added trypsin, was used in order to prevent comparison of IP from fusing and non-fusing monolayers.

Figure 52 shows the results of a co-immunoprecipitation assay for each of the proteins carrying alanine substitutions at the heptadic hydrophobic residues of the HR domain, including I84A, V91A, I98A, and L105A. Despite the significant deficiencies in CD46-binding and/or fusion promotion associated with three out of the four amino acid substitutions, each of the mutated proteins efficiently co-immunoprecipitates with MV F. Additionally, a MV H protein with an amino acid substitution at I98T co-immunoprecipitates with F at a level similar to

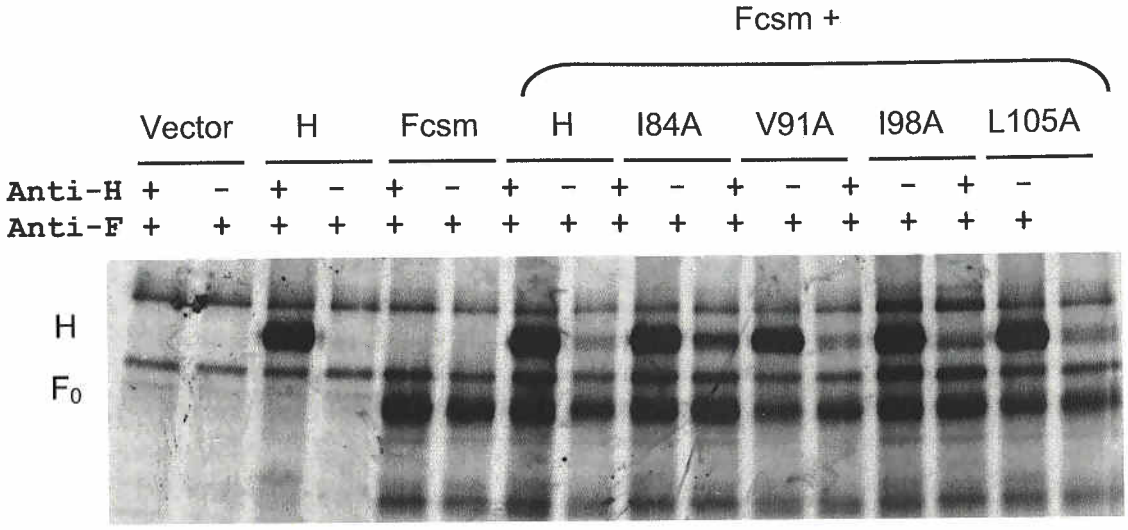



**Figure 52. Co-immunoprecipitation of MV H proteins with mutations in the heptadic residues in the HR region of the stalk.**

Co-immunoprecipitation of MV H, I84A, V91A, I98A, and L105A mutated H proteins with MV Fcsm. At 16 hours post-transfection cells were radiolabeled for 3 hours, and then, they were incubated for five hours with chase medium. The cell surface proteins were biotinylated and lysed. The lysates were split into two equal aliquots and then the proteins were immunoprecipitated with a combination of an antibody against the cytoplasmic tail of MV F and a pair of antibodies against the H ectodomain or the anti-F antibody alone. The immunoprecipitates were collected with Protein G beads and then the captured proteins were re-precipitated with streptavidin beads prior to analysis by SDS-PAGE under reducing conditions.

wt H (not shown).

It is interesting to note that there is variability in the amount of the 78 kDa protein that can be co-immunoprecipitated for each mutated protein. However, the variability does not appear to correlate with functional deficiencies in either receptor binding or membrane fusion promotion. Overall, the results of the co-immunoprecipitation assays for the MV H proteins carrying amino acid substitutions at I84A and I98A, which exhibit a complete abolishment of the ability to promote membrane fusion, suggest that a loss of the ability to interact with the MV F protein does not account for their functional deficiencies.

To determine if the deficiencies in receptor recognition and fusion promotion by mutated proteins, carrying substitutions at the conserved P94 and L95 residues in the second IR, correlate with an inability to interact with MV F at the cell surface, the amount of protein that can be co-immunoprecipitated with MV F was determined for P94S and L95A. As shown in Figure 53, each of the mutated proteins can still be efficiently co-immunoprecipitated with MV F. The results of this experiment suggest that the loss of fusion helper function exhibited by the L95A mutation is not due to with a disruption of the interaction between MV H and F.

Mutated proteins carrying amino acid substitutions at the remaining residues in the second IR, as well as a protein with a substitution at the first residue of the third IR, were also tested for the ability to interact with MV F at the cell surface. Consistent with the previous results, each of the mutated proteins,

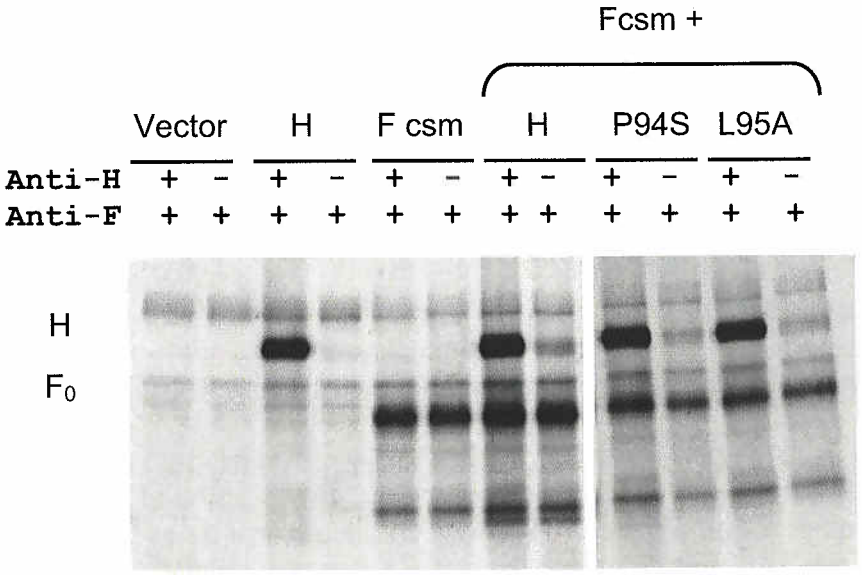



**Figure 53. Co-immunoprecipitation of MV H proteins with mutations P94A and L95A.**

This experiment was performed as described in the legend to Figure 52.

including L92A, T93A, F96A and –L, K97A, as well as I99A, can be co-immunoprecipitated with MV F at the cell surface (Figure 54).

All together, the results of these co-immunoprecipitation assays suggest that amino acid substitutions in the HR domain do not de-stabilize the MV H-F interaction at the cell surface. An interaction at the cell surface between H and F can be detected for mutated H proteins from all three groups of mutated proteins that were identified in Figure 48.

### 5.2.9 Amino acid substitutions in MV H that abolish fusion promotion interfere with the co-immunoprecipitation of a third unidentified protein.

As previously demonstrated in Chapter III, a third band similar in size to $F_1$ is present in the co-immunoprecipitation of MV H with MV Fcsm (Fig. 55). Further examination of the results of some of the co-immunoprecipitation assays testing for an interaction of MV F with H proteins carrying amino acid substitutions in the HR that abolish fusion revealed that this 45 kDa protein is present in significant levels only in samples with an MV H protein that is able to promote fusion at a level of more than 25% of wt H activity. Although, as shown in the previous co-immunoprecipitation assays, the protein is obscured by a non-specific protein band of similar size that is present under the optimal conditions for detection of the H-F interaction, it can be clearly seen in the two gels presented in Figure 56. These two assays involved additional washes between the Protein G and streptavidin isolation steps of the co-immunoprecipitation assay. The identity of this co-immunoprecipitated protein and its connection to

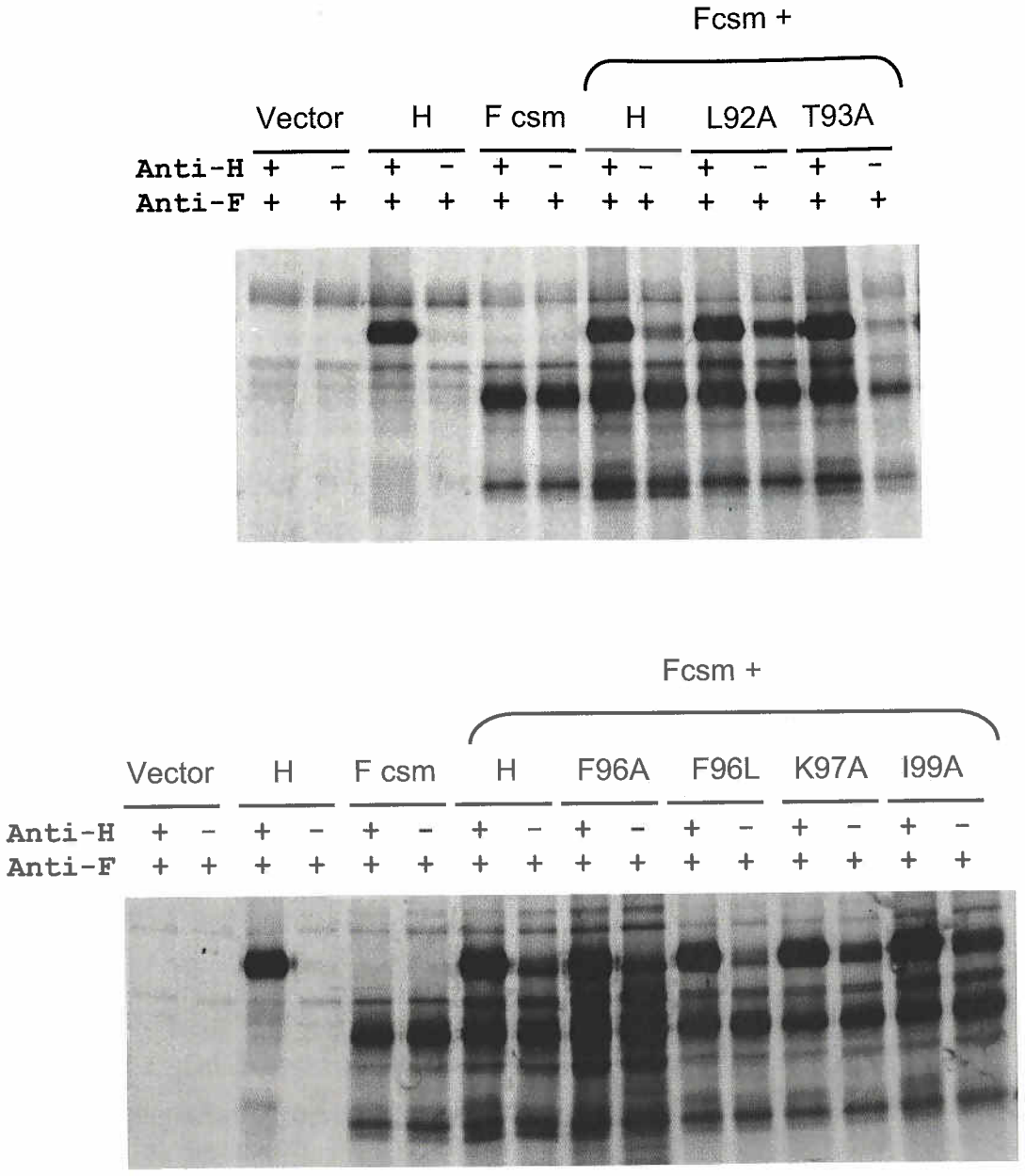



**Figure 54. Co-immunoprecipitation of MV H proteins with mutations at residues in the intervening region of the HR in the stalk including L92A, T93A, F96A, F96L, K97A, and I99A.**

These experiments were performed as described in the legend to Figure 52.

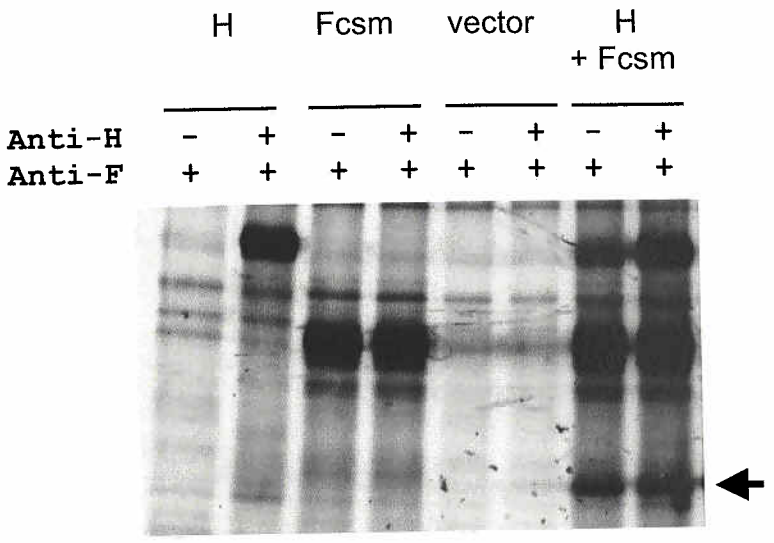



**Figure 55. Co-immunoprecipitation of MV H with MV Fcsm.**

The unidentified protein, Protein X, that is brought down with the H and F proteins is indicated by the arrow. These experiments were performed as described in the legend to Figure 52 with a slightly modified protocol that included additional washes in between the Protein G and streptavidin isolation steps of the surface proteins.

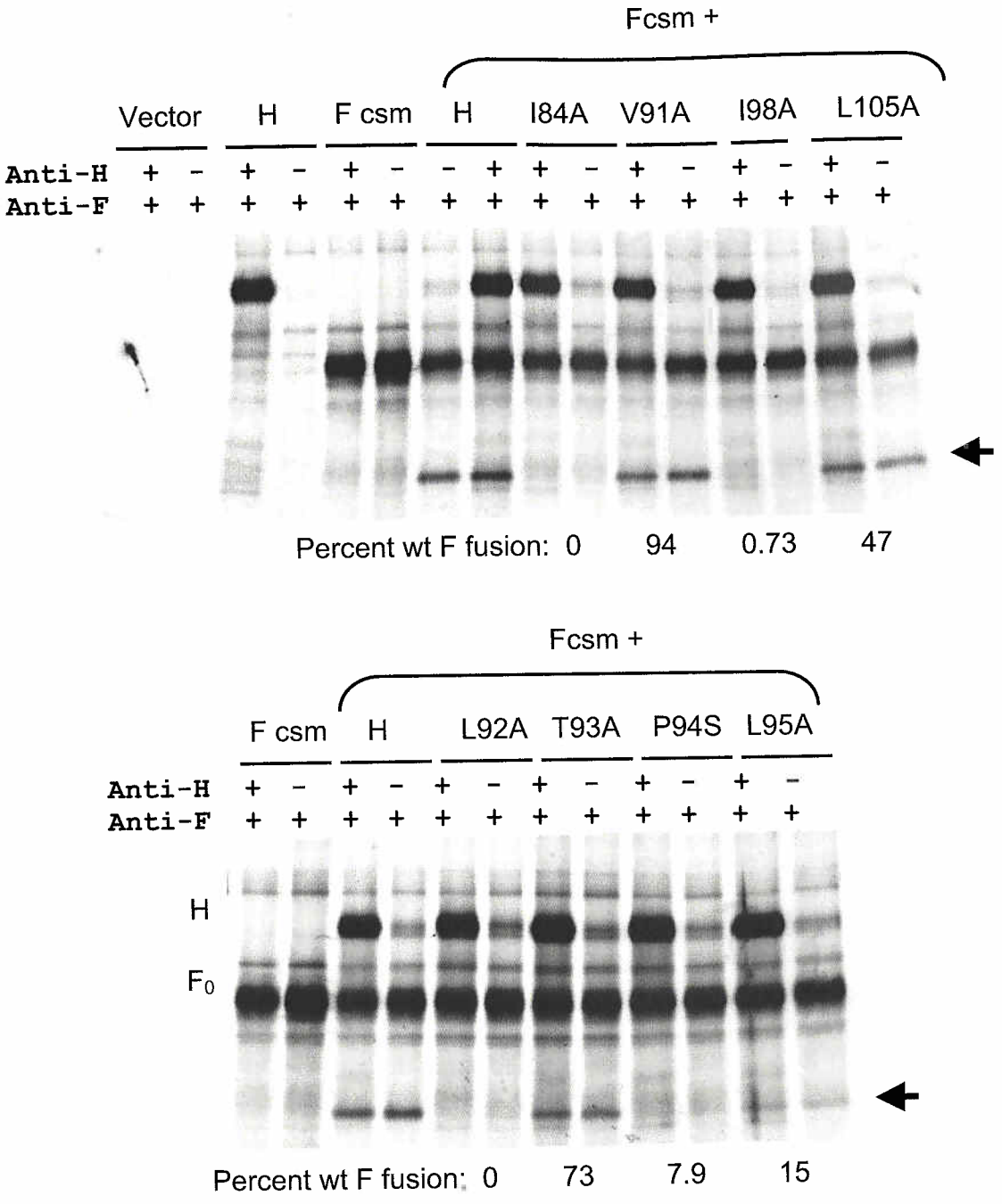



**Figure 56. Co-immunoprecipitation of MV H mutants with MV Fcsm.**

Protein X is indicated by the arrow. These experiments were performed as described in the legend to Figure 52 with a slightly modified protocol that included additional washes in between the Protein G and streptavidin isolation steps of the surface proteins.

the fusion promotion remain unknown. Thus, it will be referred to as Protein X.

### 5.2.10 The HR domain is not the sole determinant of the specificity associated with the MV H-F interaction.

A possible explanation for the deficiencies in fusion activity associated with these mutations in the HR is that the domain determines the requirement for MV F, and thus, the amino acid substitutions interfere with the ability of MV H to specifically trigger MV F activation. If the amino acid sequence of the HR domain in the stalk of morbillivirus H protein is the sole determinant of the specific requirement for the homologous F, one might expect that conversion of this region of MV H to match the sequence in CDV H would inhibit fusion promotion with MV F. Additionally, one might expect that MV H would acquire the ability to promote fusion with CDV F.

Alignment of the MV and CDV H sequences reveals that there are four residues that differ between the proteins in the HR domain (Fig. 45). In order to determine if the HR domain mediates the specificity of the interaction between H and F, these sites were mutated in MV H to the corresponding residues of CDV H. A protein carrying the following mutations was prepared and characterized: I84V, E85H, K89I and V103I. The mutated protein promotes membrane fusion at approximately 55% of the level of wt H activity. The reduction in fusion activity does not correspond to a decrease in CSE (107% of wt H) or receptor binding (99% of wt H). The mutated protein is unable to promote fusion when co-expressed with CDV F (0% wt MV H-F fusion activity). These data are consistent

with the results of the co-immunoprecipitation assays that suggest that the MV H HR domain is not the sole mediator of the specific physical association between H and F at the cell surface that is required for fusion promotion.

**Summary**

In this chapter, two regions of MV H were tested for the ability to mediate the fusion helper function of MV H, as well as the H-F interaction at the cell surface. First, alanine-scanning mutagenesis was used to demonstrate that the region 244 to 250 in the globular head domain is not at the functional interface between H and F. Mutation of residues in this region does not significantly alter CD46 binding or fusion promotion by MV H. Second, site-directed mutagenesis of several residues in a HR domain in the stalk of MV H was found to modulate hemadsorption and/ or fusion promotion activity. These results suggest that changes in the amino acid sequence of the HR domain can modulate the structure of the globular head domain. They also indicate that the region may play a role in mediating the fusion helper activity of MV H. However, mutations in the HR domain were not found to affect the H-F interaction at the cell surface or the specific requirement of MV H for the homologous F protein. Importantly, an unidentified protein, Protein X, was shown to co-immunoprecipitate with MV H and F proteins only when the glycoproteins are capable of fusion promotion.

# CHAPTER VI

## Two regions of measles F involved in fusion protein cleavage and promotion of membrane fusion

### Introduction

Fusion promotion by MV is believed to require a specific physical interaction between the H and F glycoproteins at the cell surface. The H glycoproteins of MV and CDV are to a certain extent interchangeable with respect to fusion, but fusion promotion is most efficient when the H and F proteins are derived from the same virus (Bossart et al., 2002, Cattaneo & Rose, 1993, Stern et al., 1995, von Messling et al., 2001, Wild et al., 1994, Wild et al., 1991). As shown in Chapter III, an interaction between MV H and F, but not MV H and CDV F, can be detected at the cell surface. Based on the results of chimera studies, the amino terminus of the C-rich region in the MV F protein has been proposed to be a region that mediates its specific interaction with the homologous H protein (Wild et al., 1994). However, it has not been shown that the C-rich region directly mediates the H-F interaction. In addition, a single mutation immediately upstream of HR-D in the F protein has been shown to be responsible for the reduced fusion phenotype of the AIK-C vaccine strain of MV. At this time, the roles of the C-rich region and HR-D in MV F in fusion promotion have not been fully investigated.

The **aim** of the research discussed in this chapter is to characterize the roles of the HR-D domain and C-rich region in MV F in fusion promotion. The

**hypothesis** to be tested is that the region(s) of MV F determining the specificity of the H-F interaction also mediate a physical interaction between the two proteins. The **rationale** for studying the HR-D domain in MV F is that mutation of a residue immediately upstream of this region has been proposed to modulate fusion promotion. The **rationale** for studying the C-rich region is that the fusion deficiency resulting from mutations in this region of MV F has been proposed to be the result of its role in mediating an interaction with H. The **approach** used to test the hypothesis is to perform site-directed mutational analyses of the roles of HR-D and the C-rich region in the fusion process.

## Results

### 6.1 Amino acid substitutions in HR-D of MV F cause decreased fusion promotion by altering stability of the trimer.

#### 6.1.1 Alanine-scanning mutagenesis of the residues in HR-D of MV F.

HR-D is located between HR-A and HR-B in the $F_1$ subunit of MV F (Fig. 57A). It consists of a heptadic repeat of hydrophobic residues that spans L281 through V302. In a hypothetical structural model of the MV F trimer, it is located on the side of the molecule and wraps around the head and neck regions (Figure 57B). A single mutation at F278, which is just upstream of HR-D, has been shown to be responsible for the reduced fusion phenotype of the AIK-C vaccine strain of MV (Nakayama et al., 2001). The roles of the heptadic residues in the fusion process were examined by the introduction of an alanine at each position

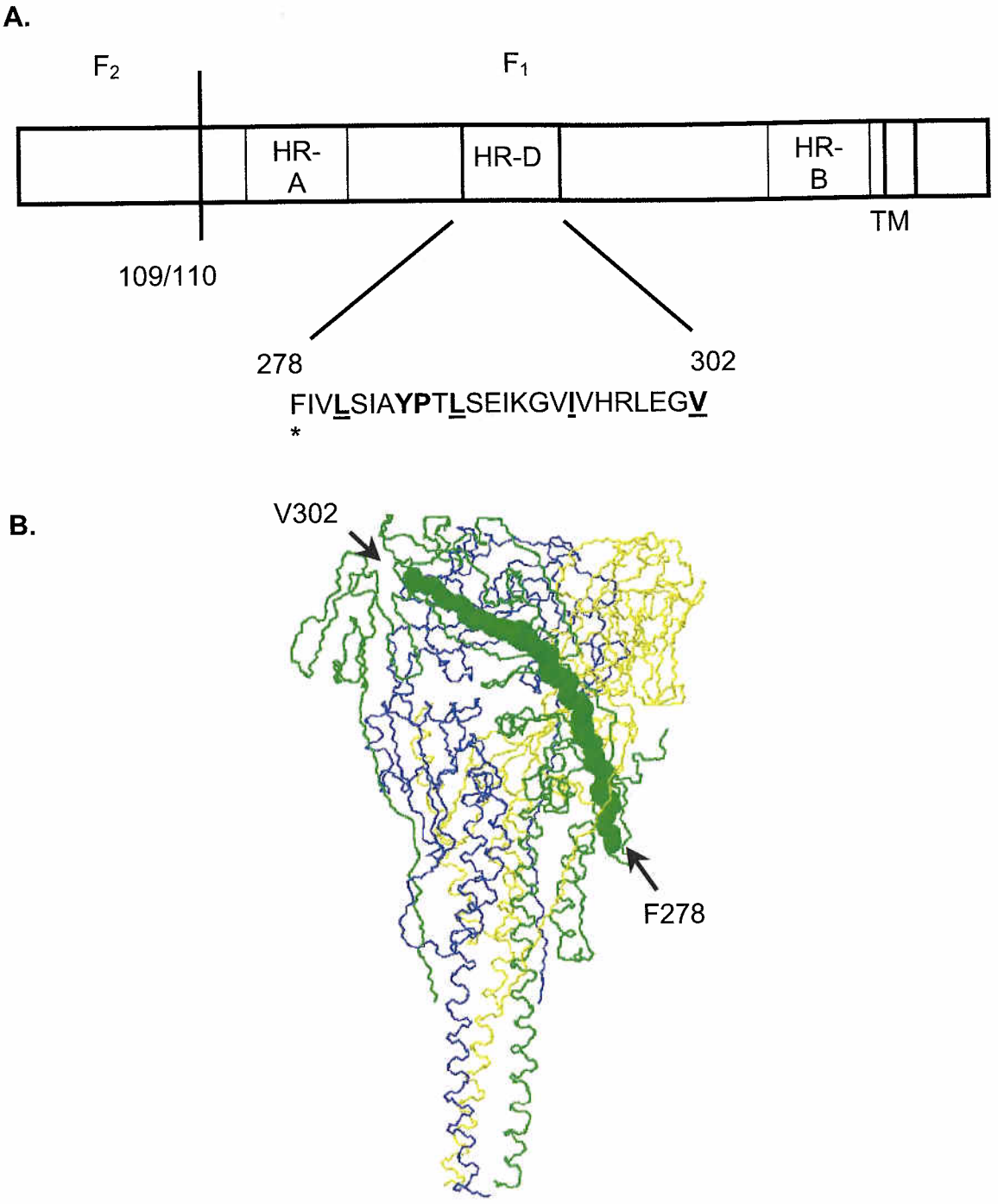



**Figure 57. Location of HR-D in the MV F.**

(A) Diagram of MV F showing the sequence of HR-D. (B) Hypothetical structure of MV F trimer in which the HR-D residues of the green monomer are highlighted (adapted from Plemper et al., 2003).

in the MV F protein and characterization of the mutated proteins. Based on the results of Nakayama et al. (2001), two additional proteins with substitutions F278A and -L were prepared and characterized. Subsequently, two proteins carrying alanine substitutions at residues Y285 and P286 in the first intervening region between the hydrophobic residues were prepared and characterized.

### 6.1.2 Amino acid substitutions in HR-D reduce fusion promotion by MV F at 37°C.

The ability of MV F proteins with amino acid substitutions in HR-D to complement MV H in fusion promotion was quantitated at 37°C using a content mixing assay. Alanine substitutions at each of the heptadic residues L281, L288, I295, and V302 reduced fusion promotion. As shown in Figure 58, while the mutations at L288 and I295 decreased fusion to levels of 45 and 39% of wt, respectively, fusion activity was completely abolished by mutation of either L281A or V302A. Similar to results of previous studies, the F278A and –L mutations also resulted in an essentially complete deficiency in fusion promotion. Additionally, while the Y285A mutation resulted in a moderate decrease in fusion promotion (80% of wt F), the P286A mutation strongly inhibited fusion by MV F (5% of wt F).

### 6.1.3 Decreased fusion promotion resulting from mutations in HR-D correlates with decreased cleavage of $F_0$.

Cleavage of $F_0$ into the $F_1$ and $F_2$ subunits is required for the ability of MV F to promote membrane fusion. Immunoprecipitation (IP) assays were

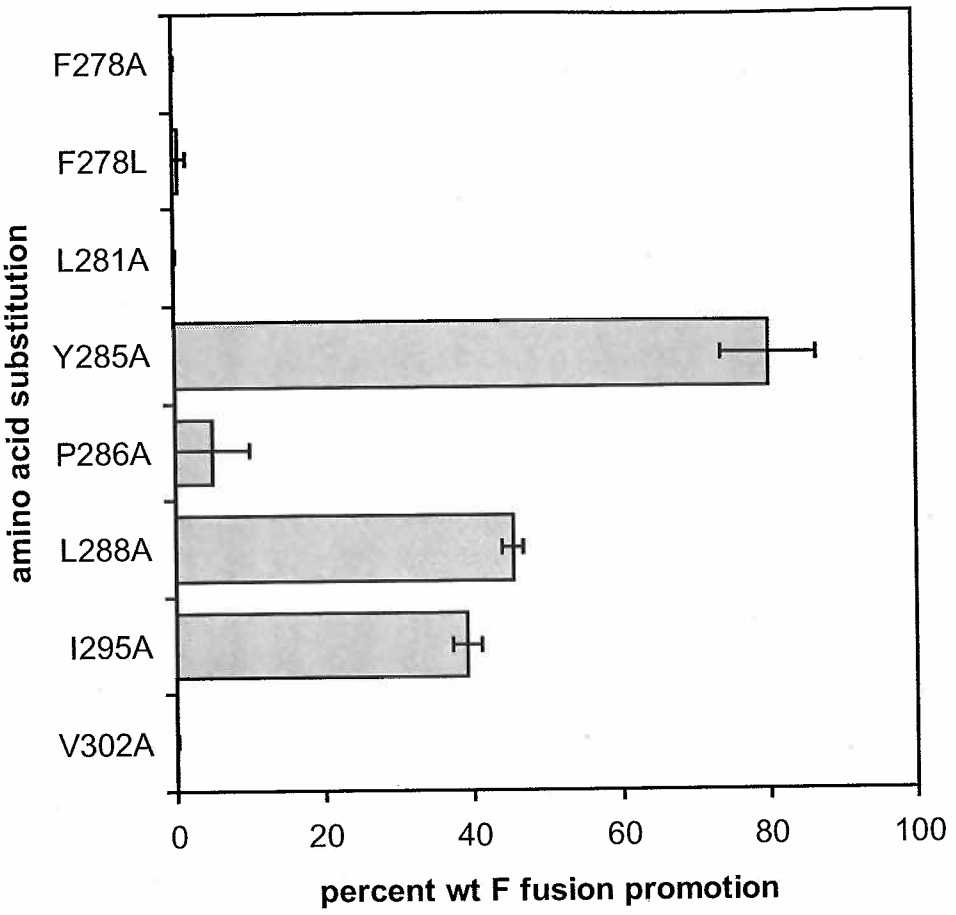



**Figure 58. Fusion promotion activities of MV F proteins carrying mutations in HR-D.**

The ability of the F proteins to complement MV H in the promotion of membrane fusion was determined by a content mixing assay. For each assay the background detected in cells transfected with vector has been subtracted. All data points represent the mean of at least three independent experiments and are expressed relative to the activity of the wt proteins.

used to explore the possibility that the reduced fusion exhibited by some of the HR-D mutated proteins is due to inefficient F cleavage. To test this possibility, transfected cells expressing the mutated proteins were radiolabeled and chased for four hours. The MV F proteins were immunoprecipitated with an antibody against the F tail. As shown in Figure 59, the level of fusion promotion by the mutated proteins correlates with the amount of $F_1$, suggesting that the mutations are interfering with cleavage of $F_0$. For the mutated proteins that promote negligible or no fusion, no cleavage of $F_0$ is detectable. In contrast, the Y285A-mutated F protein, which fuses at 80% of wt F activity, is efficiently cleaved. F proteins carrying mutations that result in intermediate levels of fusion, L288A and I295A, are also cleaved with intermediate efficiencies. Thus, the ability of proteins carrying mutations in HR-D to promote fusion correlates with their susceptibility to cleavage.

### 6.1.4 Expression at a lower temperature rescues the fusion promotion activity of some of the MV F proteins with mutations in HR-D.

A recent study by Doyle et al. (2006) identified mutations in HR-B that were reported to destabilize the pre-fusion structure and transport of the MV F trimer. It was demonstrated that expression of the mutated proteins at a reduced temperature restored both transport competence and fusion promotion. In order to examine the possibility that mutations in HR-D similarly reduce the stability of MV F, the ability of the mutated proteins to promote fusion at 30°C was quantitated. Figure 60 shows that incubation at the lower temperature restores,

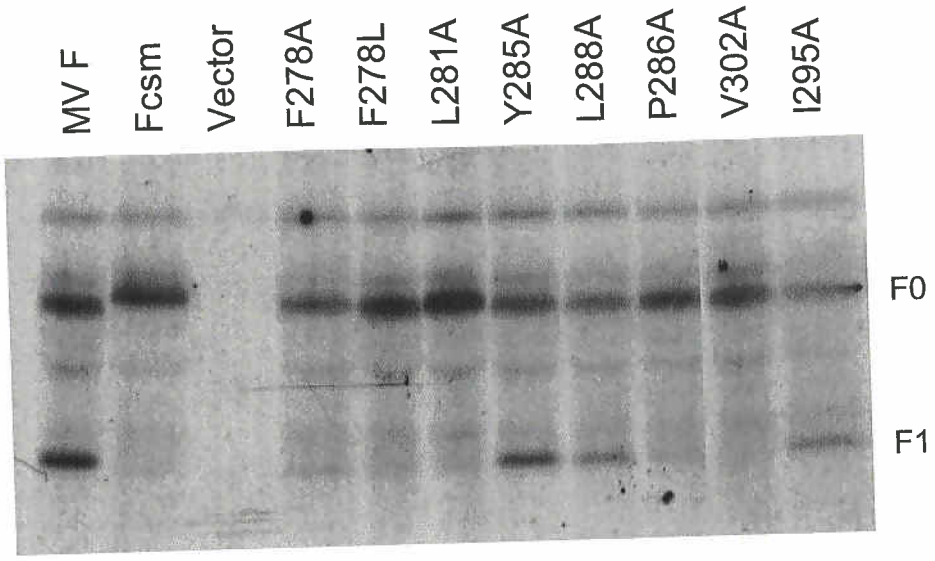



**Figure 59. IP of MV F proteins carrying mutations in HR-D.**

Cells transfected with MV F, Fcsm, vector, or a mutated F gene were labeled for 3 hours and incubated in chase medium for 4 hours prior to lysis. The proteins were immunoprecipitated with an antibody against the cytoplasmic tail of F.

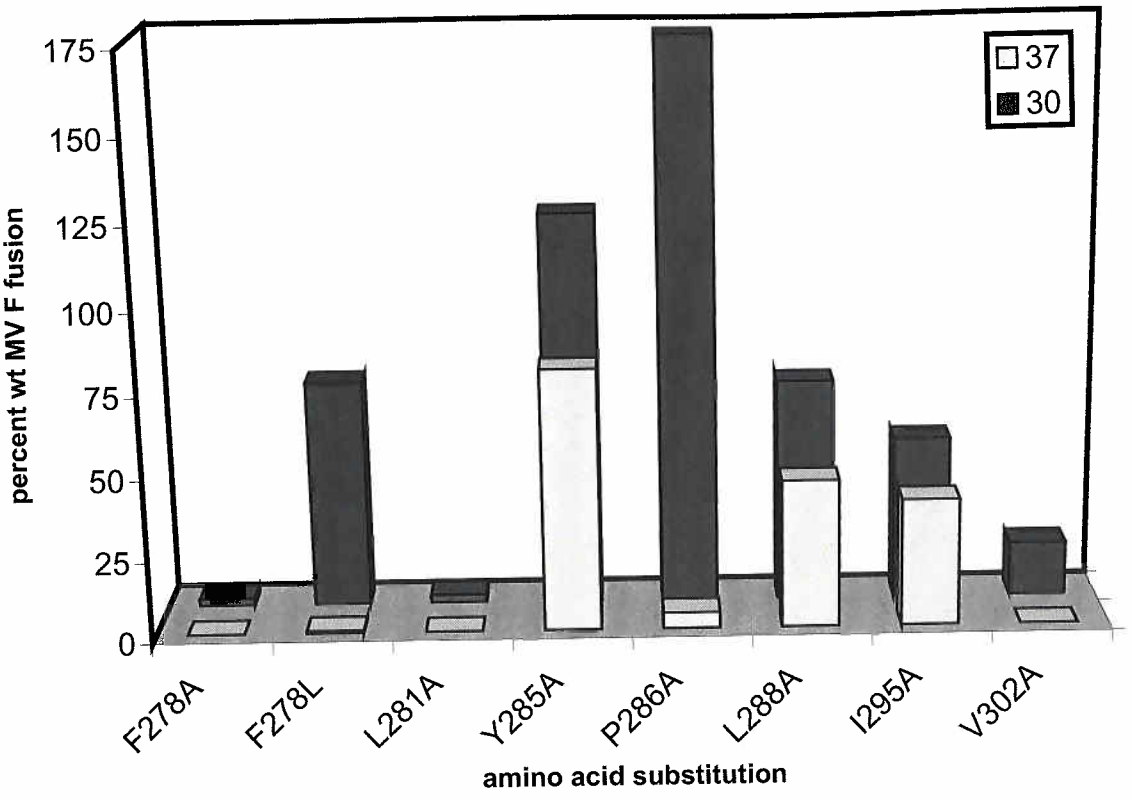



**Figure 60. Comparison of the fusion promotion activities of MV F proteins carrying mutations in HR-D at 30°C and 37°C.**

The ability of the F proteins to complement MV H in the promotion of membrane fusion was determined by a content mixing assay. For each assay the background detected in cells transfected with vector has been subtracted. All data points for 37°C represent the mean of at least three independent experiments and are expressed relative to the activity of the wt proteins. All data points for 30°C represent the mean of at least three replicates in a single experiment and are expressed relative to the activity of the wt proteins at 30°C.

in some cases quite substantially, the fusion promotion activity of several of the mutated proteins. Fusion is enhanced by expression at 30°C for proteins carrying the individual F278L, Y285A, P286A, L288A, I295A, and V302A mutations in HR-D. Most notably, the F278L and P286A mutated F proteins, both of which promote fusion at less that 5% of wt F activity at 37°C, exhibit fusion activities of 75 and 172%, respectively, of wt F at 30°C. Furthermore, the gain of fusogenic activity correlates with increased amounts of cell surface $F_1$ (Fig. 61), suggesting that the mutations are preventing efficient cleavage of $F_0$ at 37°C.

## 6.2 Amino acid substitutions in the C-rich region of MV F cause decreased fusion promotion.

### 6.2.1 Quantitation of membrane fusion by heterologous pairs of MV and CDV glycoproteins.

In order to demonstrate the specific requirement for expression of H and F from the same virus for maximum fusion activity in our system, the ability of MV and CDV F to promote fusion with MV and CDV H was compared using the content mixing assay at 37°C in HeLa cells and BHK cells. As shown in Figure 62, MV F is most efficient at promoting fusion when co-expressed with MV H in HeLa cells, and CDV F is most efficient when co-expressed with CDV H in BHK cells. MV F is able to complement CDV H to a lesser degree, resulting in approximately 25% of the fusion promotion of CDV F. In contrast, CDV F is

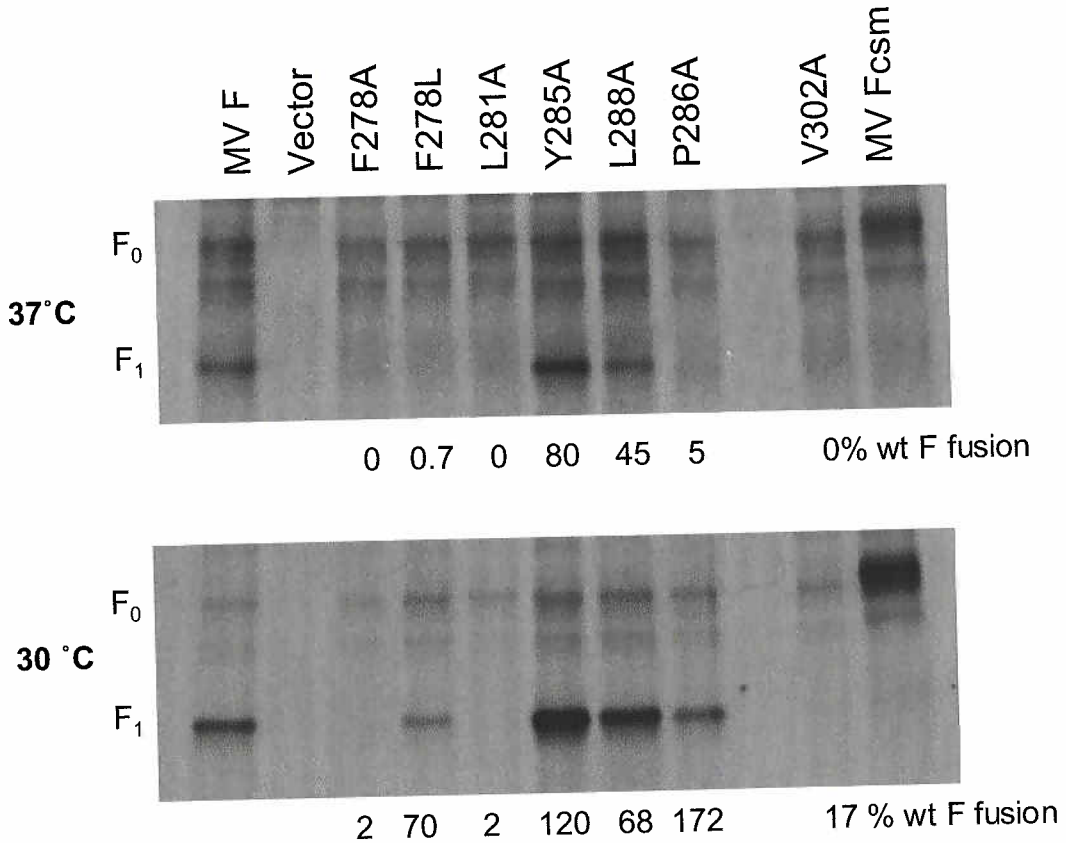
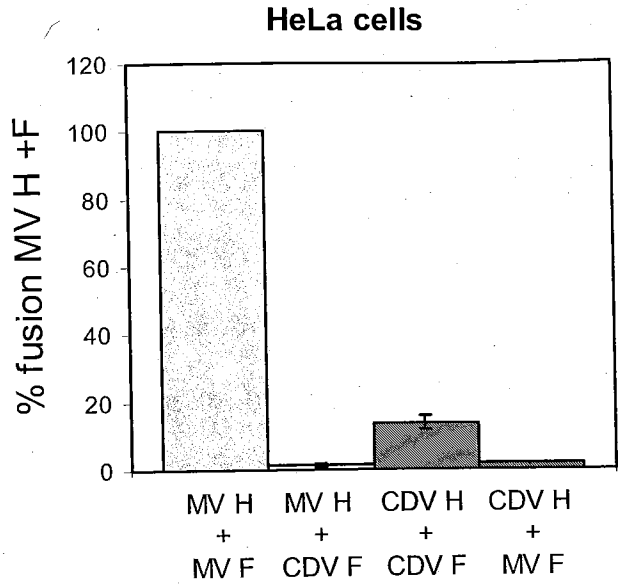



**Figure 61. Comparison of the IP of MV F proteins carrying mutations in the HR-D at 30°C and 37°C.**

At 20 hours post-transfection, cells were labeled for 5 hours. The cell surface proteins were biotinylated and then the cells were lysed. The proteins were immunoprecipitated with an antibody against the cytoplasmic tail of F, the immune complexes were collected with Protein G beads, and then, the captured proteins were re-precipitated with streptavidin beads prior to analysis by SDS-PAGE under reducing conditions. (A) IP after expression at 37°C. (B) IP after expression at 30°C.

A.




B.

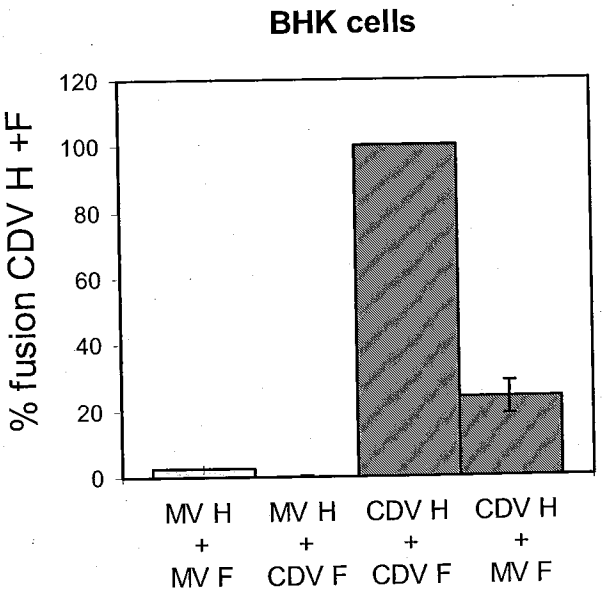



**Figure 62. Comparison of the fusion promotion activities of homologous and heterologous pairs of the MV and CDV glycoproteins.**

The ability of the F proteins to complement H proteins in the promotion of membrane fusion was determined by a content mixing assay. For each assay, the background obtained with vector has been subtracted. All data points represent the mean of at least three replicates in a single experiment and are expressed relative to the activity of the wt proteins. (A) Fusion promotion in HeLa cells. (B) Fusion promotion in BHK cells.

unable to promote fusion with MV H.

### 6.2.2 Site-directed mutagenesis of residues in the C-rich region of MV F.

The C-rich region is located between HR-D and HR-B in the $F_1$ subunit of MV F (Fig. 63A). It consists of a series of eight cysteines, conserved in both number and spacing among paramyxovirus F proteins. As shown in Figure 63B, the C-rich region is located in the head domain of a hypothetical structure of MV F and it appears to form portions of the axial and radial channels, as well as part of the trimer subunit interfaces. Based on the analysis of MV-CDV F chimeras, it has been suggested that the first 44 amino terminal residues in the C-rich region determine the specificity of the interaction between MV H and F (Wild et al., 1994). However, the investigators did not directly test whether the F chimeras are able to physically interact with MV H.

The role of the amino terminus of the C-rich region in the specific requirement of MV F for MV H for fusion promotion was tested by site-directed mutagenesis. Based on the assignment of disulfide bridges in the F protein of Sendai virus, the amino terminus of the C-rich region is predicted to form two loop structures (Loop 1 and 2) with two intervening sequences (IS 1 and 2) (Fig. 64A) (Iwata et al., 1994). As shown in Figure 64B, in each of the loop and IS regions, there are residues that differ between MV and CDV F. Initially, these residues were divided into four groups based on their proximity to each other in the sequence, and then, each group was mutated to the corresponding amino

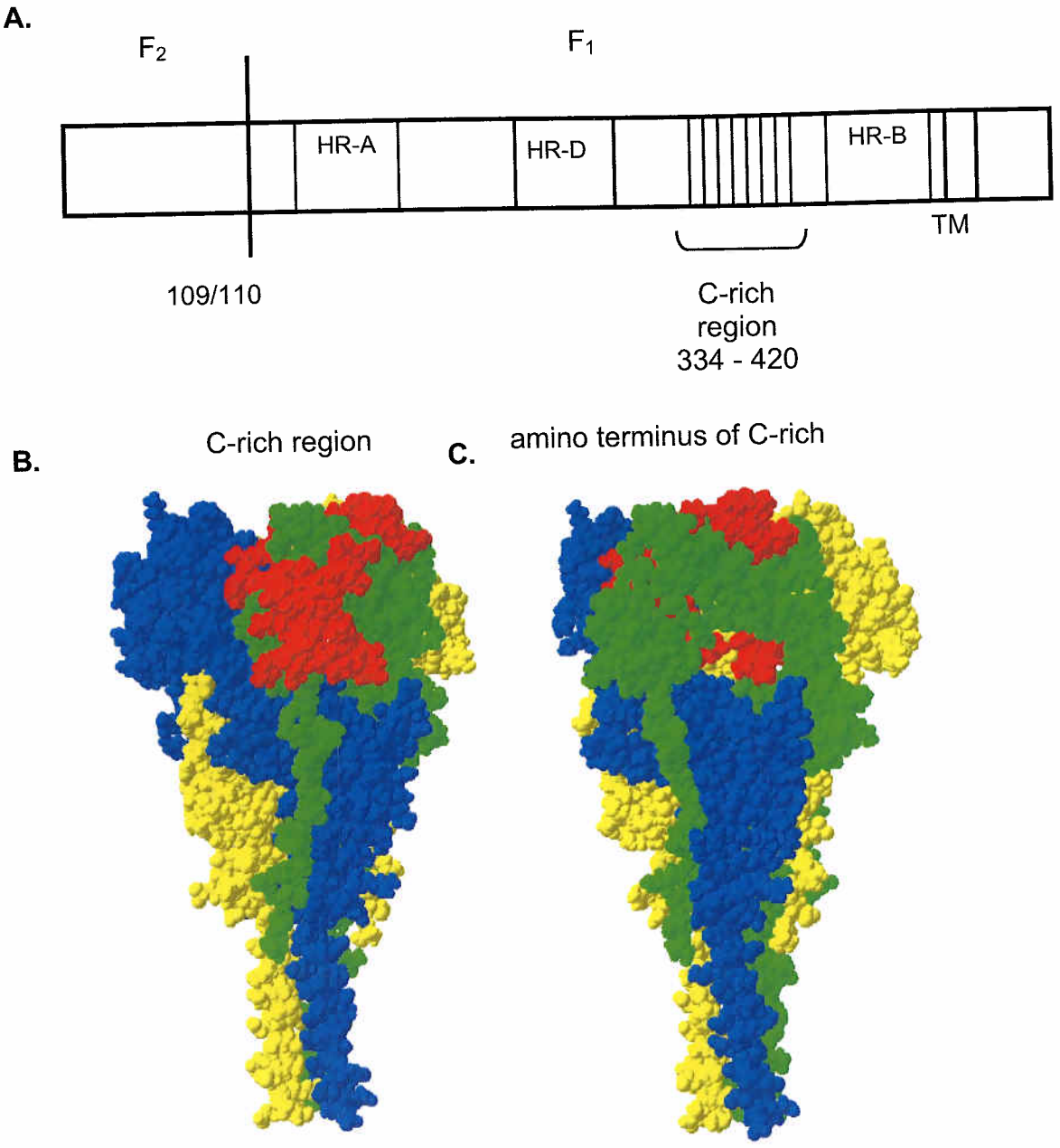
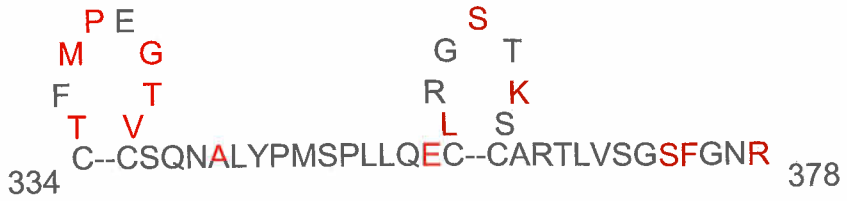



**Figure 63. Location of the C-rich region in the MV F.**

(A) Diagram of MV F showing the location of the C-rich region. (B) Hypothetical structure of MV F trimer in which the C-rich region of the green monomer is highlighted in red (adapted from Plemper et al., 2003). (C) Hypothetical structure of MV F trimer in which the amino terminus of the C-rich region of the green monomer is highlighted in red.

A.




B.

MV F: CTFMPEGTVCSQNALYPMSPLLQECLRGSTKSCARTLVSGSFGNR
CDV F: CVFVSESAICSQNSLYPMSPLLQQCIRGDTSSCARTLVSGTMGNK

Loop 1 * IS 1 Loop 2 IS 2

**Figure 64. Amino terminus of the C-rich region of MV F.**

(A) Diagram of MV F showing the predicted disulfide-linked looped structure of the amino terminus of the C-rich region (adapted from Wild, et al. 1994). The residues that are not conserved between MV F and CDV F are highlighted in red. (B) Alignment of the amino acid sequences of the amino termini of the C-rich regions of MV F and CDV F. Residues that differ between the proteins are presented in bold and the divisions used for mutagenesis are shown underlined or marked with an asterisk, including Loop 1, intervening sequence (IS) 1, Loop 2, and IS 2.

acid in CDV (Fig. 64B). Thus, four proteins carrying the following substitutions were prepared and characterized: 1) T335V, M337V, P338S, G340S, T341A, and V342I; 2) A347A; 3) E357Q, L359I, S362D, and K364S; and 4) S374T, F375M, and R378K.

### 6.2.3 Amino acid substitutions in the C-rich region reduce fusion promotion by MV F at 37°C.

The ability of the MV F proteins with amino acid substitutions in the C-rich region to complement MV H in fusion promotion at 37°C was quantitated. As shown in Figure 65, each of the mutated proteins exhibits a significant decrease in the ability to promote fusion compared to wt F, ranging from 3% for IS 1 to 34% for Loop 1. Due to the proposed role for this region in mediating the specificity of the H-F interaction, each of the mutated proteins was also tested for the ability to complement CDV H in fusion promotion. Unlike wt MV F, which promotes fusion with CDV H at a level of 25% of CDV F activity, the four proteins carrying mutations in the C-rich region are completely unable to promote a detectable level of fusion with CDV H (data not shown).

IP assays were used to determine if the decreased fusion promotion by the mutated proteins is related to altered expression and cleavage of the F protein. As shown in Figure 66, for three of the four mutants, all except Loop 1, the level of fusion promotion appears to be related to the extent of cleavage of $F_0$. The F protein carrying a mutation in IS1, which exhibits the lowest level of fusion promotion at 37°C (3% of wt F activity), is also cleaved the least.

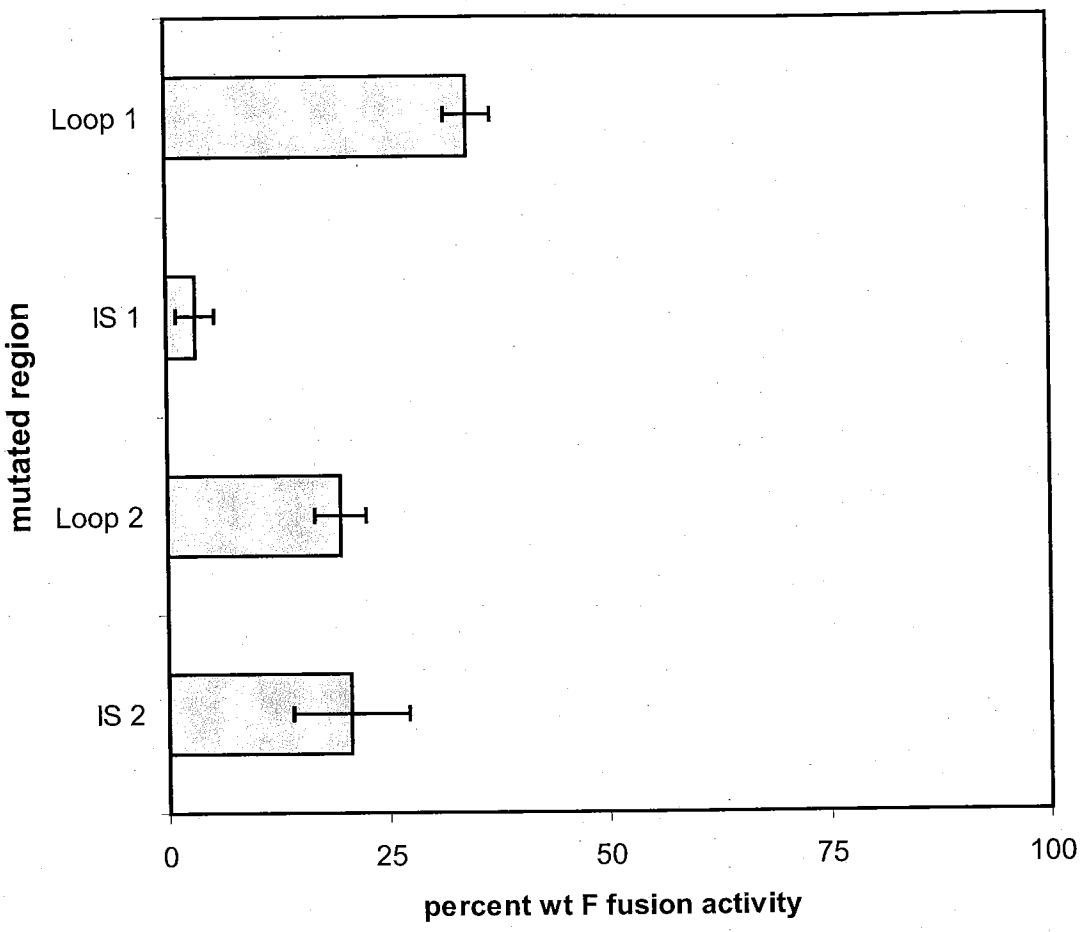



**Figure 65. Fusion promotion activities of MV F proteins carrying mutations in the amino terminus of the C-rich region.**

The ability of the F proteins to complement MV H in the promotion of membrane fusion was determined by a content mixing assay. For each assay the background detected in cells transfected with vector has been subtracted. All data points represent the mean of at least three independent experiments and are expressed relative to the activity of the wt proteins.
level of wt F.

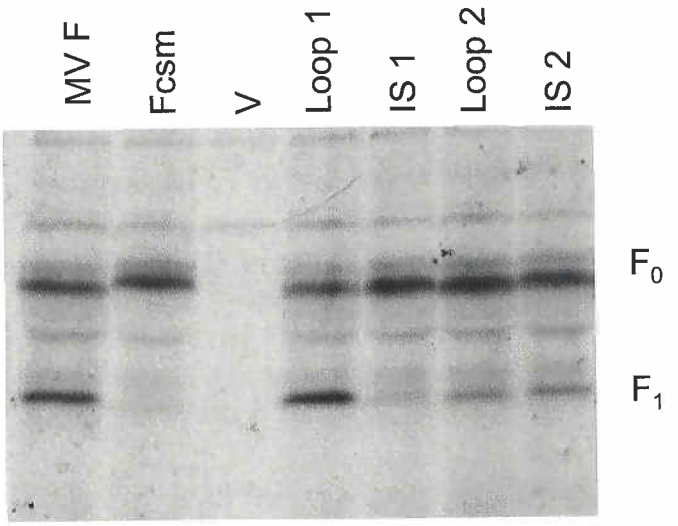



**Figure 66. IP of MV F proteins carrying mutations in the amino terminus of the C-rich region.**

Cells transfected with MV F, Fcsm, vector (V), or a mutated F gene were labeled for 3 hours and incubated in chase medium for 4 hours prior to lysis. This experiment was performed as described in the legend to Figure 65.

The remaining mutated protein, which carries substitutions in Loop 1 and promotes fusion at 34% of wt MV F activity with MV H, is cleaved efficiently. The Loop 2 and IS 2 mutated proteins, which both promote fusion at approximately 20% of wt F activity, are cleaved more efficiently, but still not at an efficiency comparable to that of wt MV F. These results suggest that the deficiencies in fusion promotion exhibited by three of the four mutated proteins are related to defects in the cleavage of $F_0$ at 37°C.

### 6.2.4 Mutation of amino acids in IS1, Loop 2, and IS2 of the C-rich region results in decreased cleavage of $F_0$.

In order to investigate the possibility that mutations in the C-rich region affect the processing and/or cleavage of F, the ability of the mutated proteins to promote fusion with MV H in fusion promotion at 30°C was quantitated. Figure 67 shows that incubation at the lower temperature dramatically enhances the fusion promotion activity of the proteins carrying mutations in IS1, Loop 2, and IS2. Fusion is enhanced by 31-fold, 4.8-fold, and 6.6-fold, respectively, at 30°C versus 37°C. However, the fusion promotion activity of the mutated protein with substitutions in Loop 1 is not enhanced at the lower temperature.

IP was used to investigate the cleavage and surface expression of the mutated proteins at 30°C. As shown in Figure 68A and B, expression of the mutated proteins carrying substitutions in IS1, Loop 2, and IS 2 at 30°C enhances their cell surface expression and cleavage. As expected, the F protein with substitutions in Loop 1 is efficiently cleaved at both temperatures.

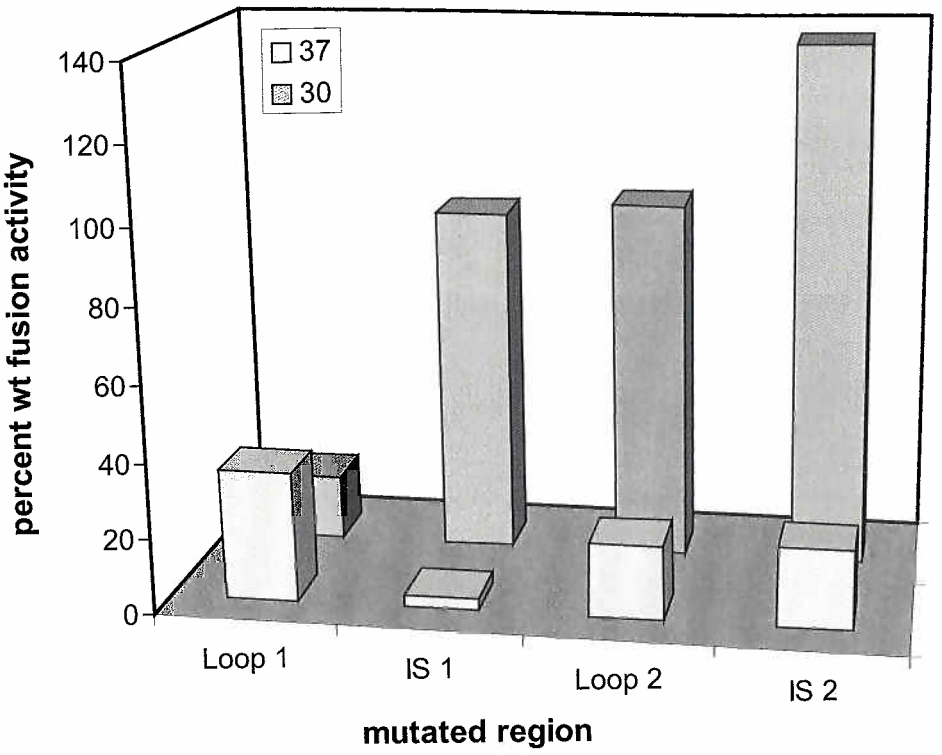



**Figure 67. Comparison of the fusion promotion activities of MV F proteins carrying mutations in the amino terminus of the C-rich region at 30°C and 37°C.**

The ability of the F proteins to complement MV H in the promotion of membrane fusion was determined by a content mixing assay. For each assay the background detected in cells transfected with vector has been subtracted. All data points for 37°C represent the mean of at least three independent experiments and are expressed relative to the activity of the wt proteins. All data points for 30°C represent the mean of two independent experiments and are expressed relative to the activity of the wt proteins at 30°C.

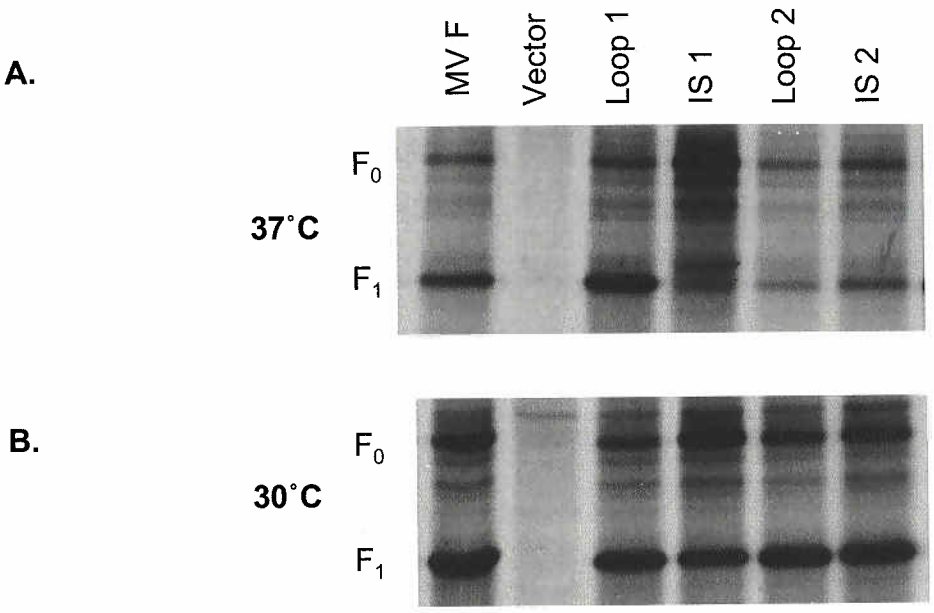



**Figure 68. Comparison of the IP of MV F proteins carrying mutations in the amino terminus of the C-rich region at 30°C and 37°C.**

This experiment was performed as described in the legend to Figure 67.

Taken together, these data indicate that the amino acid sequences of IS1, Loop 2, and IS2 in the C-rich region are important for efficient cleavage of $F_0$. Because fusogenic activity of F proteins carrying mutations in these regions correlates with their susceptibility to cleavage, the role of these segments of the C-rich region in the specific interaction with the homologous H protein was not investigated further.

### 6.2.5 Site-directed mutagenesis of the residues in Loop 1 of the C-rich region of MV F.

The protein carrying mutations in Loop 1 exhibits diminished fusion that is not related to a defect in cleavage. This suggests that, unlike the other segments of the C-rich region, Loop 1 may be involved in the H-F interaction. As shown in Figure 64, there are six amino acids in Loop 1 of the C-rich region that differ between MV and CDV. In order to determine which of the amino acid substitutions is responsible for the fusion deficiency exhibited by the Loop 1 mutated F protein, each of residues was mutated individually to the corresponding residue in CDV F. Thus, proteins carrying the following substitutions were prepared and characterized: T335V, M337V, P338S, G340S, T341A, and V342I.

These mutated MV F proteins were tested for the ability promote fusion with MV H. The mutated proteins carrying substitutions T335V, M337V, P338S, T341A, and V342I each exhibit moderately reduced fusogenic activity, ranging from 65% to 83% of wt F activity. The activity of the G340S-mutated F is similar

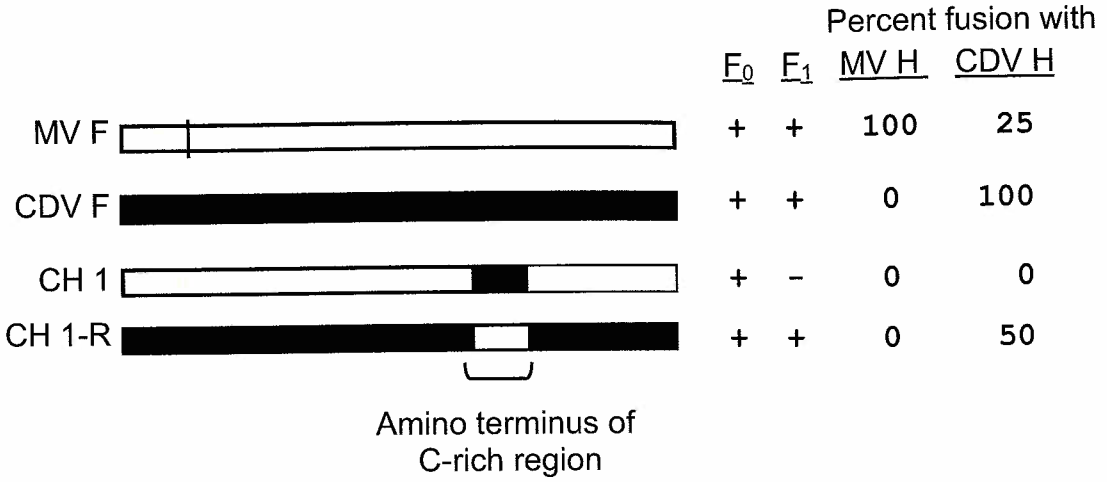
to wt F. The $F_1$ form of the proteins can also be detected at a level similar to wt F by IP after labeling and a four-hour chase (data not shown). These results suggest that multiple substitutions in Loop 1 are responsible for the fusion deficiency of the mutated F protein.

### 6.2.6 Replacement of the amino terminus of the C-rich region of CDV F with the corresponding region of MV F does not significantly alter H-F specificity.

The results of the mutagenesis of individual regions within the amino terminus of the C-rich region of MV F are not consistent with a role for this region in determining the specificity of the MV H-F interaction. Thus, in order to try to confirm the results of Wild et al. (1994), a chimeric protein (CH 1), in which the amino terminus of the C-rich region of CDV F was replaced with the corresponding region of MV F, was prepared by site-directed mutagenesis and characterized for the ability to promote membrane fusion with MV H and CDV H (Figure 69). The reciprocal chimeric protein (CH 1-R) was also prepared and characterized.

The ability of the chimeric proteins to complement CDV H and MV H in fusion promotion was determined. As seen in Figure 69A and C, contrary to published findings (Wild et al., 1994), neither of the chimeras is able to complement MV H for fusion promotion at 37°C. Interestingly, the CDV F protein carrying the amino terminus of the MV F C-rich region is able to promote membrane fusion with CDV H, albeit at a reduced level compared to the wt CDV

**A.**




**B.**

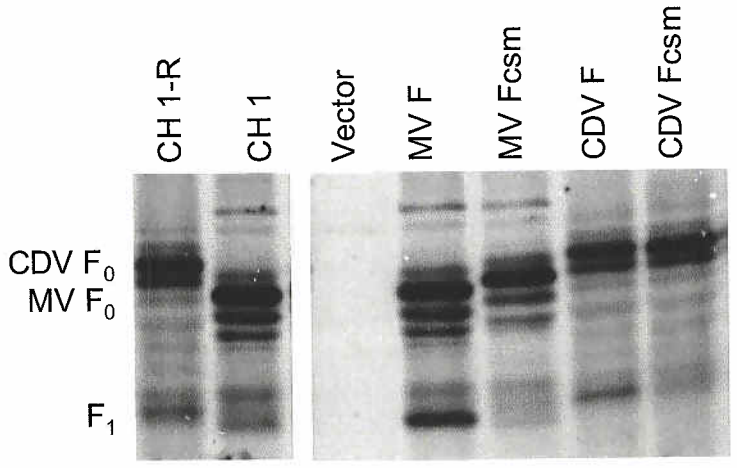
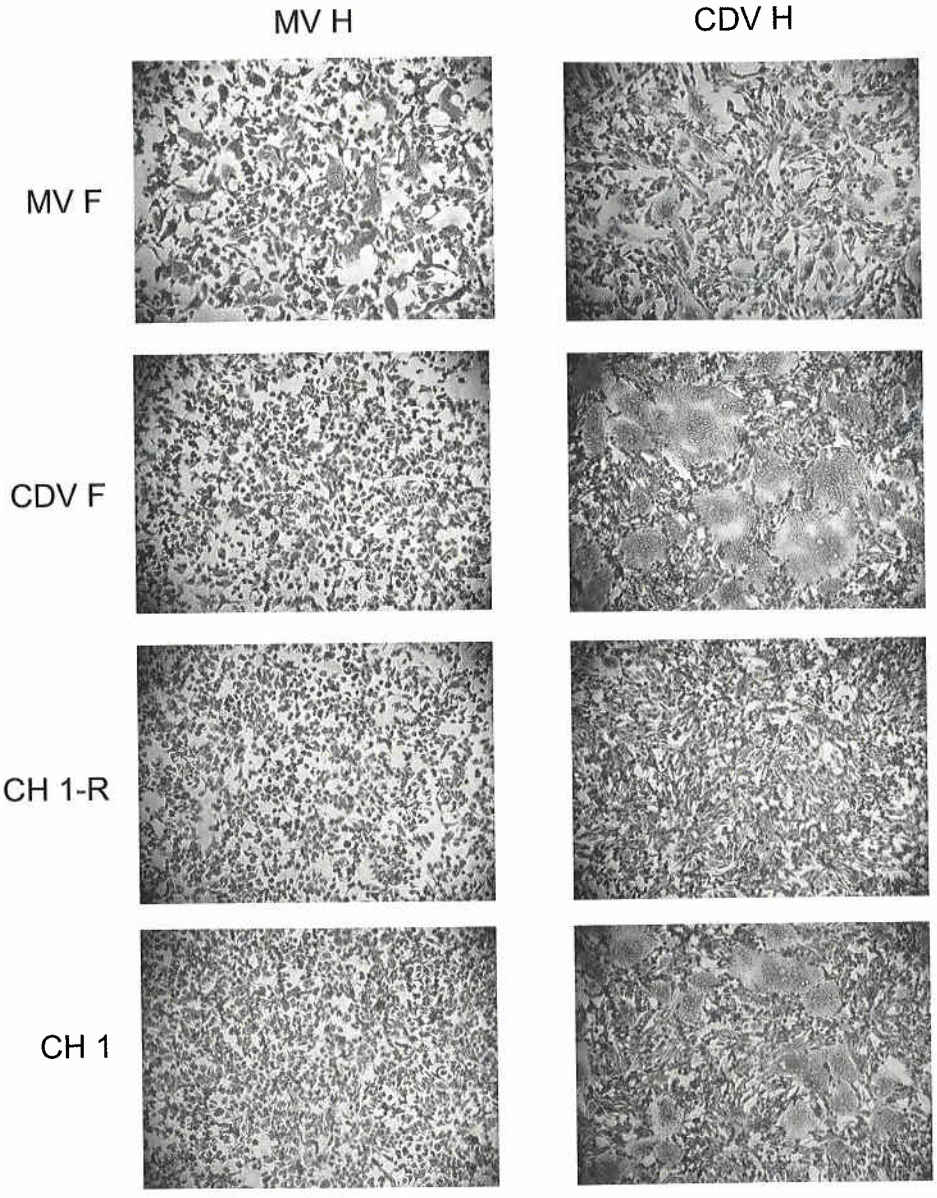



**Figure 69. Expression and fusion promotion by MV/ CDV F protein chimeras carrying the C-rich domain of the heterologous virus.**

(A) Diagram of CH 1-R and CH 1 protein chimeras and summary of their fusion properties. Fusion promotion with MV H was determined using a content mixing assay. Fusion promotion with CDV F was determined by visual analysis of Giemsa stained monolayers. (B) IP of chimera proteins. This experiment was performed as described in the legend to Figure 65.

C.




**(Fig. 69 continued).** (C) Fusion promotion MV H and CDV H co-expressed with MV/ CDV F protein chimeras carrying the C-rich domain of the heterologous virus. At 20 hours post-transfection, the monolayers were fixed and stained with Giemsa stain. HeLa cells were used for expression of the F proteins with MV H. BHK cells were used for expression of the F proteins with CDV H.

F protein. This is also contrary to the expected results based on the earlier findings. The complementary chimera is unable to promote fusion with CDV H.

IP of the chimeras, after radioactive labeling and a four-hour chase, shows that the reduction in the ability of the chimeras to promote membrane fusion correlates with reduced cleavage of $F_0$ (Fig. 69B). These results are inconsistent with the reported role of the C-rich region of MV F in determining the specificity of the H-F interaction. In the original study of the MV-CDV F chimeras (Wild et al., 1994), the cleavage of the proteins was not confirmed. These data suggest that cleavability of F is a determinant of the ability of the chimeras to promote fusion.

In order to assess the possibility that alteration of the amino acid sequence in the C-rich region reduces the pre-fusion stability or processing of the chimeras, the ability of the mutated proteins to complement MV H in fusion promotion was assessed at 30°C. As shown in Figure 70, incubation at a reduced temperature enhances the fusogenicity of the F protein chimeras. The fusogenic activity of the chimera in which the C-rich region of MV F is replaced with that of CDV F is significantly increased at 30°C relative to 37°C, further consistent with the idea that the amino acid sequence of the region is important for cleavage of $F_0$. Most notably, this chimera (CH 1), fuses at 50% of wt F at 30°C, while it exhibits no detectable fusion activity at 37°C. The ability of the complementary chimera (CH 1-R) to promote fusion with MV H is not significantly enhanced by incubation at a reduced temperature. These results indicate

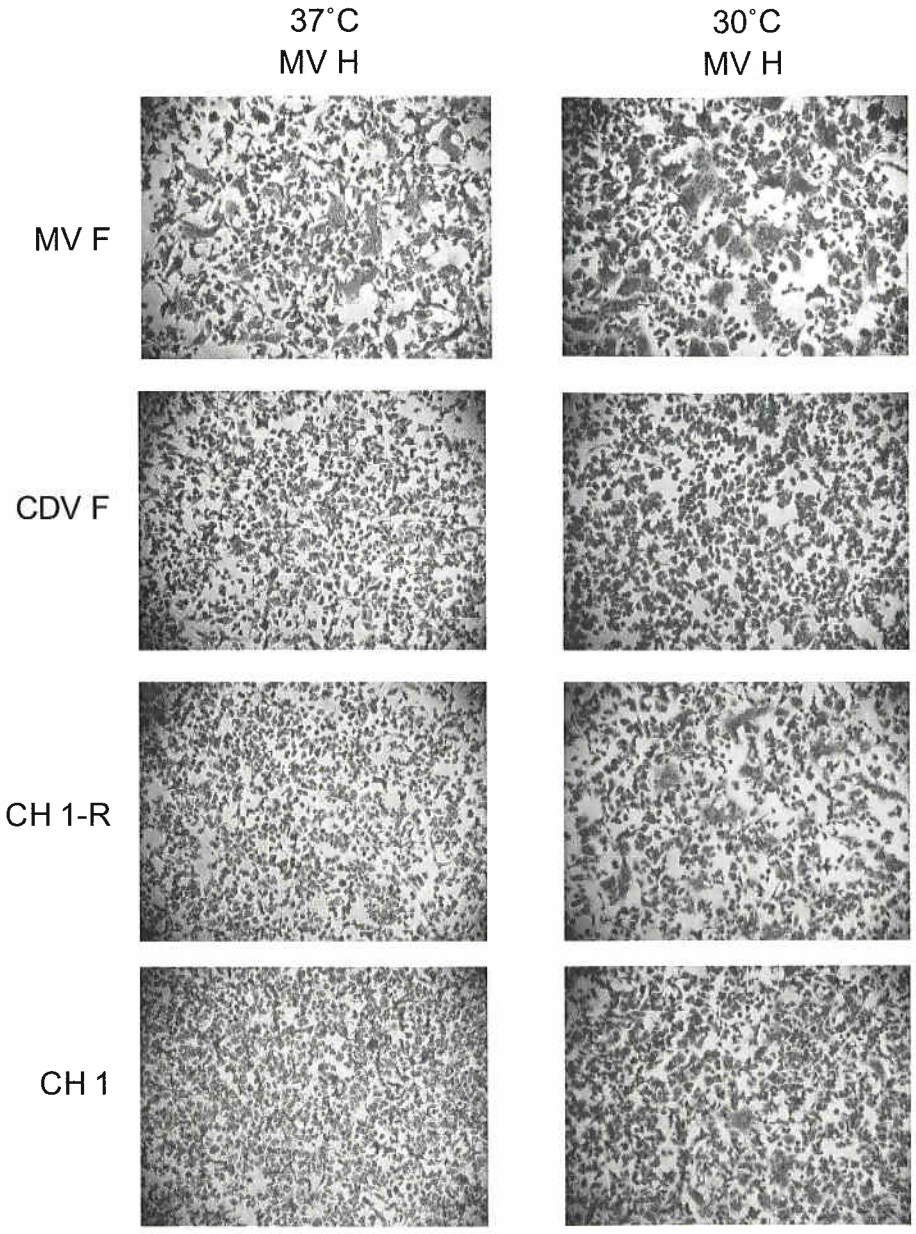
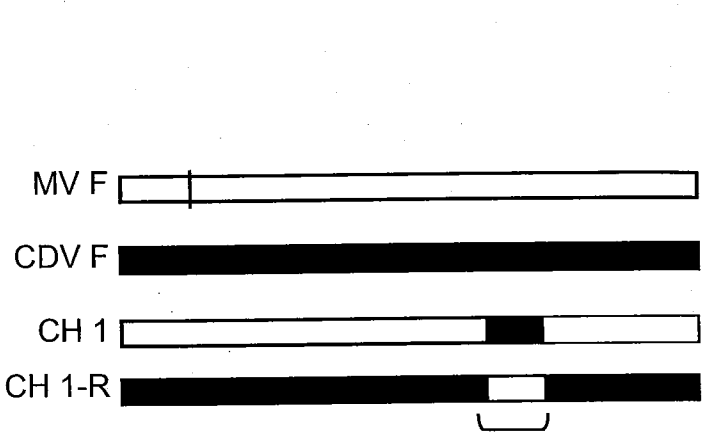



**Figure 70. Fusion promotion by MV H and MV/ CDV F protein chimeras carrying the C-rich domain of the heterologous virus at 37°C versus 30°C.**

At 20 hours post-transfection, the monolayers were fixed and stained with Giemsa stain.




| | $F_0$ | $F_1$ | Percent fusion with MV H 30°C | 37°C |
|---|---|---|---|---|
| MV F | + | + | 100 | 100 |
| CDV F | + | + | <5 | 0 |
| CH 1 | + | – | 50 | 0 |
| CH 1-R | + | + | <10 | 0 |

**(Fig. 70 continued).** Fusion promotion by MV H and MV-CDV F protein chimeras at 30°C versus 37°C. The amount of fusion promotion was assessed by visual inspection of Giemsa stained monolayers.

that, though the C-rich region of MV F may play a role in fusion, it cannot be the sole determinant of the H-F specificity.

**Summary**

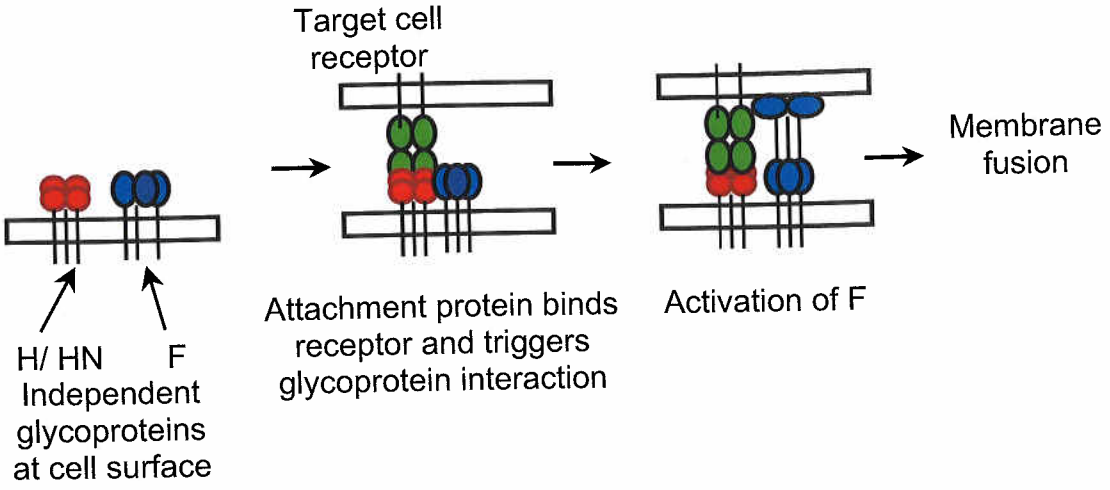
In this chapter, the roles of two regions of MV F in fusion promotion with MV H were characterized. Earlier work had suggested that the HR-D and C-rich region of MV F are involved in mediated fusion promotion. In particular, the C-rich region was proposed to mediate the specific H-F interaction. However, evidence presented in this chapter clearly shows that the fusogenicity of most F proteins carrying mutations in these two regions is a function of their susceptibility to cleavage. It was also found that the first loop of the amino terminus of the C-rich region has a role in fusion promotion that is not related to cleavage of $F_0$. However, evidence is presented that indicates that the C-rich region is not the sole mediator of the specific MV H-F interaction.

# CHAPTER VII

## Discussion

Two different models have been proposed for the initial steps of the mechanism of membrane fusion promoted by the paramyxovirus glycoproteins (Fig. 71). In the first model, the attachment and fusion proteins arrive at the cell surface independently and the specific interaction between the two proteins that is required for fusion promotion is triggered by receptor binding (Lamb, 1993). This model is supported by evidence that a complex between NDV HN and F can be detected at the cell surface only when HN is able to bind to its receptor, sialic acid-containing glycoproteins and/or -lipids (Deng et al., 1999; Li et al., 2004; Melanson & Iorio, 2004). In the second model, the attachment and fusion proteins form an intracellular complex and arrive at the cell surface together. This model proposes that the attachment protein maintains F in a pre-fusion metastable state prior to receptor recognition. In this model, receptor binding by the attachment protein causes dissociation of the complex, resulting in fusion activation of the F protein. The basis for this model is the reported detection of complexes of the attachment and fusion proteins in the ER (Plemper et al., 2001, Stone-Hulslander & Morrison, 1997). Additionally, it has recently been reported that an interaction can be detected between NDV HN and F in cells depleted of cell surface sialic acid by neuraminidase treatment (McGinnes & Morrison, 2006). The issue is further complicated by the fact that the mechanism of membrane

**Model 1: Paramyxovirus glycoproteins arrive at the cell surface independently.**




**Model 2: Paramyxovirus glycoproteins form an intracellular complex and arrive at the cell surface together.**

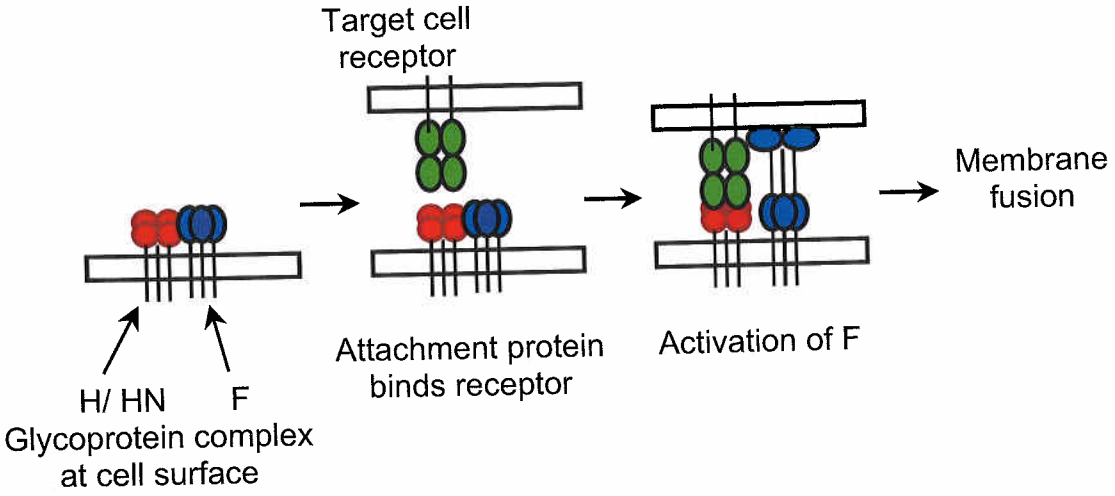



**Figure 71. Two models of mechanism of paramyxovirus membrane fusion promotion.**

(A) Model 1: Paramyxovirus glycoproteins arrive at the cell surface independently. Receptor recognition by the attachment protein triggers an interaction with the fusion protein. (B) Model 2: Paramyxovirus glycoproteins form an intracellular complex and arrive at the cell surface together. Receptor recognition by the attachment protein triggers fusion protein activation through conformational changes.

fusion promotion may differ from one paramyxovirus to another based, in part, on functional/ structural differences among the viral glycoproteins. Most significantly, the morbilliviruses differ from most other paramyxoviruses in receptor usage, certainly an important aspect of the first step of fusion activation.

As for most paramyxoviruses, membrane fusion promotion by measles requires co-expression of the attachment and fusion proteins (Wild et al., 1991). In the first step of the process, MV H mediates receptor binding to specific protein receptors, CD46 or CD150, in order to bring the two membranes into close proximity prior to F activation. There is evidence that MV H has an additional role in the fusion process that involves triggering F activation through a specific interaction between the two proteins. It has been shown that the attachment glycoproteins of MV and CDV are, to a certain extent, interchangeable, but fusion promotion is most efficient with homologous H and F proteins (Bossart et al., 2002, Cattaneo & Rose, 1993, Stern et al., 1995, von Messling et al., 2001, Wild et al., 1994). Additionally, monoclonal antibodies to MV H have been characterized that block infectivity and inhibit fusion without interfering with receptor recognition, indicating that MV H contributes something to fusion promotion in addition to receptor binding (Fournier et al., 1997, Hu et al., 1993).

Despite the considerable amount of effort put forth to study the process, many aspects of the first steps of membrane fusion remain unclear. First, while there is evidence that fusion involves a specific interaction between H and F, the

cellular site(s) of the interaction has not been fully investigated. Second, the relationship between receptor recognition by the attachment protein and the interaction between it and the fusion protein has not been clearly elucidated. Third, the regions of the glycoproteins that determine the specific interaction have not been completely defined. Further examination of the early steps of entry into and spread among host cells holds the potential to direct the development of new anti-viral strategies for the control of infection by this important human pathogen.

### 7.1 Re-examination of the detection of an intracellular MV H-F complex.

Expression of ER retention signal-tagged F proteins from hPIV2 and hPIV3 resulted in down-regulation of the level of cell surface expression of not only the homologous HN protein, but also those of the attachment proteins of heterologous viruses, including measles (Tanaka et al., 1996, Tong & Compans, 1999). The lack of specificity of this phenomenon is inconsistent with the demonstrated virus specific nature of the glycoprotein interaction that is required for promotion of membrane fusion. Although MV H and F are efficiently transported to the cell surface when expressed independently, co-transfection of either ER-retained protein with its wild-type partner was reported to result in a decrease in the kinetics of the processing of the non-tagged protein, consistent with the formation of an intracellular complex between the two proteins (Plemper et al., 2001). Based on these results, it was concluded that MV H and F hetero-

oligomerize in the ER. However, it was not established whether the putative co-retention was specific to the two measles glycoproteins.

Consistent with the formation of an intracellular complex between H and F, the cell surface expression and receptor binding of wt MV H is slightly reduced by co-expression of an excess of ER-tagged MV F relative to co-expression with wt F. However, the reduction in surface expression is non-specific in that it can also be induced by co-expression of NDV F. Also, cell surface expression of NDV HN was reduced by co-expression of an excess of MV F. Additionally, no significant difference could be detected in the extent of intracellular processing of either wt glycoprotein when co-expressed with a five-fold excess of its ER-tagged partner. The significant down-regulation in fusion promotion by the wt glycoproteins when they are both co-expressed with an ER-tagged form of one of them (ex. H + H-ER + F) likely results from homo-oligomerization rather than hetero-oligomerization. Indeed, a significant decrease in MV H receptor binding results from co-expression with ER-tagged H. These findings suggest that the ER-retention approach may not be capable of demonstrating the existence of a specific intracellular interaction between MV H and F.

However, it cannot be ruled out that there is an intracellular association of the proteins of insufficient strength to be detectable with the co-retention approach in our system. A potential explanation for the disparity between the results of this study and those of Plemper et al. (2001) is that a minor decrease in the kinetics of intracellular processing of H and F caused by ER co-retention

could be masked by the overall high levels of expression of MV H and F obtained with the vaccinia virus expression system. Based on a comparison of the amounts of the H and F proteins that can be immunoprecipitated in the two systems, the level of expression in the CMV promoter-based expression system used by Plemper et al. (2001) appears to be much lower than that of the vTF7-3 system. Indeed, the H and F proteins are expressed at such low levels in the CMV promoter-based system that it is difficult to visually detect the delay in processing of the untagged proteins that is said to result from co-retention by the ER-tagged heterologous proteins (Plemper et al., 2001).

Another possible reason for the difference in the ability to detect ER co-retention is that the original experiments involved the use of FLAG epitope-tagged measles glycoproteins. Although the FLAG epitope does not directly alter the processing of the proteins, it is possible that it could strengthen an intracellular interaction between the H and F proteins to a level detectable by the co-retention method. However, a study by the same group demonstrated that the addition of a FLAG epitope to the cytoplasmic tail of MV H actually weakened an interaction between H and F (Plemper et al., 2002). Importantly, these experimental differences cannot account for the lack of specificity that is associated with the co-retention method, necessitating the use of a different approach to address the issue of the potential intracellular interaction between the MV glycoproteins.

## 7.2 Detection of a MV H-F complex at the cell surface.

Co-immunoprecipitation assays have been used to demonstrate glycoprotein interactions at the cell surface (Deng et al., 1999, Li et al., 2004, Malvoisin & Wild, 1993, McGinnes & Morrison, 2006, Melanson & Iorio, 2004, Yao et al., 1997). However, chemical cross-linking was required for the detection of a complex between MV H and F, leaving open the possibility that they are in close proximity at the cell surface without physically associating (Malvoisin & Wild, 1993). More recently, a Western blot-based co-immunoprecipitation assay was used to study variations in the strength of the interaction between MV H and F (Plemper & Compans, 2003, Plemper et al., 2002, Plemper et al., 2003). However, this type of assay has two drawbacks. First, it does not distinguish between intracellular and cell surface interactions between the two proteins. Second, this protocol involves display of the protein that is co-precipitated, but not the primary protein used for capture. Clearly, it is desirable to be able to focus on the relationship between the H and F proteins at the cell surface, using a method that quantitates the amount of complex formation between the two proteins at the site where fusion promotion takes place.

Treating cells with a membrane-impermeable form of biotin has been used to selectively identify proteins expressed on the cell surface. The biotinylated proteins can be immunoprecipitated with specific antibodies followed by precipitation with streptavidin beads. This assay detects cell surface expressed radiolabeled forms of both the primary protein used for co-immunoprecipitation

and proteins that interact with it. This technique has been used to demonstrate the formation of virus-specific glycoprotein complexes at the cell surface for both hPIV3 and NDV (Deng et al., 1999, Li et al., 2004, Malvoisin & Wild, 1993, McGinnes & Morrison, 2006, Melanson & Iorio, 2004, Yao et al., 1997). Consistent with a cell surface interaction between MV H and F, the combination of surface biotinylation and co-immunoprecipitation detects a H-F complex in HeLa cells. Approximately 21% (± 9%) of the total amount of MV H at the cell surface can be captured with MV F using an antibody against the latter protein.

Three important controls establish the specificity and physiological relevance of the H-F complex detected at the cell surface by co-immunoprecipitation. First, consistent with the inability of co-expressed MV H and CDV F to promote membrane fusion, MV H cannot be co-precipitated with CDV F. Second, the H-F complex cannot be detected when the proteins are expressed independently in separate cell monolayers and the lysates are mixed together. Third, the complex can be detected when the proteins are expressed in a different cell line, CHO-CD46, using a virus-free expression system, eliminating the possibility that the co-immunoprecipitation is an artifact of the use of vaccinia virus.

Co-immunoprecipitation of fusion-competent MV H and F proteins results in precipitation of a third cell surface protein, Protein X, which migrates slightly faster than $F_1$. At this time, the identity of this protein remains unknown. Its molecular weight (approximately 43 kDa) rules out the possibility that it is either

the 55 or 65 kDa isoform of CD46 known to be expressed by HeLa cells (Maisner et al., 1994). It is possible that Protein X is a cellular protein that interacts with MV H and/or F during the fusion process. Cellular proteins have been shown to contribute to paramyxovirus membrane fusion. For example, RhoA, a small GTP binding protein, has been shown to interact with the F protein of RSV and Rho GTPase signaling increases fusion promotion by the Hendra and SV5 glycoproteins (Pastey et al., 1999, Pastey et al., 2000, Schowalter et al., 2006). While the molecular weight and cell surface localization of Protein X eliminates Rho A (22 kDa) as a possible candidate, it could conceivably be another protein in the Rho GTPase signaling pathway.

At this time, it also cannot be ruled out that Protein X is a form of either MV H or F. Two alternative forms of paramyxovirus F proteins have previously been reported. The first is a form of the ectodomain of measles F that is cleaved just upstream of the transmembrane region and is thought to facilitate pore formation (von Messling et al., 2004). However, in Fcsm, the molecular weight of this form would be approximately 48 kDa, and thus, it would migrate slower than $F_1$ in the MV cell surface co-immunoprecipitation assays. The second form is an alternative topological form of NDV F in which both the amino terminal end, including the signal sequence, and carboxy terminal end of the protein have been translocated to the extracellular surface (McGinnes et al., 2003, Pantua et al., 2005). However, our lab is unable to detect a polytopic form of MV F in transfected cells using an antibody against the F tail and flow cytometry (data not

shown). Additionally, given the glycosylation pattern of MV F, in which all of the glycosylation sites are located in $F_2$, and the length of the signal sequence, this alternative form of MV Fcsm would migrate slightly slower than $F_0$. It is also possible that Protein X is a previously undetected degradation product of either H or F resulting from the conformational changes in the proteins triggered by MV H receptor recognition.

### 7.2.1 Detection of a MV H-F complex at the cell surface in the absence of cellular receptors.

For paramyxoviruses, the relationship between receptor recognition and the attachment-fusion protein interaction required for fusion promotion has not been clearly elucidated. A recent study by McGinnes & Morrison (2006) suggests that NDV HN and F are associated at the cell surface prior to receptor recognition. In this study, it was reported that a HN-F interaction was detected by co-immunoprecipitation of the glycoproteins from cells that had been treated with neuraminidase to remove surface sialic acid. Additionally, incubation of the neuraminidase-treated transfected cells with untreated cells resulted in a decrease in the amount of HN-F complex that could be detected, leading to the conclusion that ligand binding leads to complex dissociation.

However, there are problems associated with this approach. Most notably, the only evidence presented of sialic acid depletion is the inability of NDV HN and F to promote membrane fusion, certainly not a sensitive read-out for the presence or absence of the receptor. The study does not demonstrate

that incubation with neuraminidase removes all of the sialic acid from the treated cells. Also, the sialic acid on the cell surface of the treated cells is likely replenished on a continual basis through the process of normal protein expression prior to cell lysis. Failure to completely eliminate sialic acid from the cell surface would leave open the possibility that there could be enough HN ligand binding activity to trigger a HN-F interaction, but not enough to generate a measurable level of membrane fusion. Additionally, while it was shown that some of the co-immunoprecipitated material was derived from the cell surface, the assays used to test receptor-deficient cells do not distinguish between intracellular interactions of the two proteins and those that take place at the cell surface. Thus, this method does not conclusively demonstrate that NDV HN and F interact at the cell surface in the absence of receptor binding.

Due to the lack of a naturally receptor-free expression system for NDV glycoprotein expression, an alternative approach is to test NDV HN proteins lacking the capacity to bind sialic acid for the ability to interact with F at the cell surface. Multiple NDV HN proteins with amino acid substitutions in the neuraminidase active site have been shown to lack detectable receptor binding and fusion activity (Iorio et al., 2001). These mutated proteins are indistinguishable from wt NDV HN by a large panel of conformation-specific MAbs. Importantly, a majority of the attachment-deficient HN proteins did not form detectable HN-F complexes at the cell surface, suggesting that the NDV HN and F interaction is triggered by receptor binding (Li et al., 2004).

However, a NDV HN protein carrying a mutation of I175E that also abolishes receptor binding and fusion activity has been shown to interact with F at the cell surface (Li et al., 2004). It is thought that this mutation results in structural changes in the protein that mimic the post-attachment conformation, resulting in constitutive interaction with the F protein. This conclusion is supported by data obtained with an I175E mutated HN protein from another strain of NDV, which, despite 50% more fusion activity than wt HN, has less than 50% of its receptor binding activity (Connaris et al., 2002). Nevertheless, it cannot be ruled out that the attachment-deficient HN proteins, lacking a detectable interaction with F, are somehow structurally different from the wt HN protein. Thus, the interaction of NDV HN and F remains a controversial subject.

Unlike a majority of the other paramyxoviruses, which utilize sialic acid, the morbilliviruses bind to specific cellular proteins to initiate membrane fusion with host cells, making it possible to more easily investigate the relationship between the attachment and fusion proteins in the presence and absence of receptors. At this time, two receptors, CD46 and CD150, have been identified for measles. Chinese hamster ovary (CHO) cells do not express CD46 or CD150, and thus, are not permissive to MV binding or infection (Dorig et al., 1993). Although these cells properly express and process MV H and F, they are not susceptible to membrane fusion promoted by the glycoproteins. These characteristics make the CHO cell line a suitable tool for examining the MV H-F interaction at the cell surface in the absence of receptors.

Similar levels of MV H can be co-immunoprecipitated with MV F from the surfaces of parental CHO cells and stably transfected cells that constitutively expresses human CD46 (CHO-CD46), indicating that binding to CD46 is not the trigger for the H-F interaction. Co-immunoprecipitation assays reveal that approximately 17% of the total amount of MV H is co-precipitated with MV F from the surface of CHO-CD46 cells. In direct comparison, 19% of the total amount of cell surface MV H is co-precipitated with MV F from CHO cells lacking measles receptors. Significantly, these results indicate that MV H and F interact in the absence of, and thus prior to, receptor binding.

### 7.2.2 Detection of a MV H-F complex at the cell surface in the absence of receptor recognition activity.

Following the identification of CD46 as a cellular receptor for MV, several groups have mapped multiple regions of MV H that may be involved in receptor recognition. Consistent with the detection of a complex between MV H and F at the cell surface in the absence of the expression of cellular receptors, a cell surface interaction can also be detected between F and attachment-deficient H proteins. Despite significant decreases in receptor binding activity, MV H proteins carrying mutations in putative CD46-binding sites co-immunoprecipitate efficiently with F from the surface of HeLa cells. This includes a mutated H protein carrying alanine substitutions for residues 473-477, which has no detectable receptor binding activity, but still forms a complex with F at the cell surface.

Replacement of the amino acids 473-477 with alanine residues, in combination with the Y481N mutation, eliminates the 78 kDa glycosylation isoform of MV H, as well receptor recognition, but does not disrupt the cell surface H-F complex. The 74 kDa isomer of MV H can be isolated and is unable to agglutinate erythrocytes, suggesting that addition of sialic acid to the protein is necessary for the interaction between MV H and CD46 (Ogura et al., 1991). The ability of this isoform to interact with MV F at the cell surface provides additional support for a model in which the H-F interaction occurs prior to receptor recognition.

Taken together, the results of the cell surface co-immunoprecipitation experiments with the measles glycoproteins strongly suggest that the H and F proteins interact at the cell surface in the absence of, and prior to, receptor binding by H. This suggests that, unlike complex formation by NDV HN and F, the MV H-F interaction is not triggered by receptor recognition.

It has been suggested that differences between the morbilliviruses and the other paramyxovirus genera in terms of receptor usage and glycoprotein functions may result in virus-specific requirements for regulation of the initiation of fusion promotion (Plemper, et al. 2001). There are two significant differences in the receptor recognition properties of MV H and NDV HN. First, while MV H has two main activities in membrane fusion, including receptor recognition and triggering the F protein, NDV HN also possesses neuraminidase activity.

Second, while MV H binds to specific cellular proteins, NDV interacts with the ubiquitous sugar sialic acid.

It is possible that independent expression of the NDV glycoproteins at the cell surface provides an additional level of control over initiation of fusion to prevent triggering of the F protein if NDV HN binds inappropriately to sialic acid that is not associated with a target cell, such as that in saliva or respiratory tract mucus. MV H may be less likely to encounter the soluble form of its receptor, and thus, the measles glycoproteins may not need to remain independent at the cell surface in order to prevent premature activation of the F protein.

Virus-specific requirements for regulation of the initiation of fusion promotion may also be based in differences in the stabilities of the pre-fusion meta-stable state of the MV and NDV F proteins. The second model for the mechanism of paramyxovirus membrane fusion proposes that the attachment protein maintains F in a pre-fusion state prior to receptor recognition. Receptor binding by the attachment protein is then thought to cause dissociation of the complex, resulting in fusion activation of the F protein. If MV F has a lower threshold of activation compared to that of NDV F, it may require an interaction with MV H prior to receptor recognition to prevent inappropriate spontaneous activation. Consistent with this hypothesis, it has been demonstrated that the strength of the proposed intracellular interaction between MV H and F is inversely related to fusogenicity (Plemper et al., 2002). The interaction between the glycoproteins is stronger in measles virus strains with reduced cytopathicities

and fusion promotion activities, consistent with H maintaining F in an inactive, pre-fusion state.

Overall, the results of the cell surface co-immunoprecipitation experiments with the measles glycoproteins suggest that the mechanism of measles virus induced membrane fusion is similar to that proposed in the second model of paramyxovirus fusion (Figure 71). Two lines of evidence that demonstrate that the proteins form a complex in the absence of receptor recognition by MV H support the hypothesis that MV H and F interact prior to receptor recognition. First, MV H can be co-immunoprecipitated with F in the absence of cellular receptors. Second, attachment-deficient MV H proteins can be co-immunpreciptitated with F from the cell surface.

## 7.3 The specific MV H-F interaction may be mediated by multiple regions of the glycoproteins.

Identification of the domains of the paramyxovirus attachment and fusion proteins that mediate their membrane fusion activities is an important part of understanding the mechanism of fusion. Specific domains in both MV H and F have been identified that are thought to be involved in fusion promotion. For example, a single mutation in MV F, located just upstream of HR-D, has been shown to be responsible for the reduced fusion phenotype of the AIK-C vaccine strain of MV (Nakayama et al., 2001). Additionally, it has been proposed that the C-rich region in the MV F protein determines the specificity of the H-F interaction.

Two regions of MV H have been proposed to mediate its fusion helper function, including I98 in the stalk and 244 to 250 in the head. Despite the importance of the proposed roles of these regions of the H and F proteins in fusion promotion, they have not been fully characterized.

### 7.3.1 The integrity of a heptad repeat in the stalk of MV H is important for different aspects of fusion promotion.

In measles H, region 244 to 250 was identified as a linear epitope in the globular region of MV H that binds to a monoclonal antibody, which inhibits membrane fusion without altering receptor recognition (El Kasmi et al., 1998, Fournier et al., 1997). Based on this evidence, it was suggested that this region mediates an interaction between the MV H and F proteins. However, the involvement of these residues in fusion promotion was not directly tested. Although it cannot be definitively ruled out that the region 244-250 has a role in membrane fusion promotion, the results of alanine-scanning mutagenesis of the residues in this region and characterization of the mutated proteins suggest that this region does not have a significant role in the receptor binding or fusion promotion activities of MV H. The region 244-250 is positioned on the model of MV H in a loop that protrudes into the putative center of symmetry of the tetramer (Deroo et al., 1998). It is possible that antibodies directed against this region may inhibit fusion by interfering with the stability of the H tetramer. Antibody binding in this region could also inhibit membrane fusion by sterically hindering either conformational changes in H or the H-F interaction.

Chimera and mutagenesis studies of the attachment proteins of other paramyxoviruses suggest that the stalk region both determines the specificity of, and directly mediates, the HN-F interaction (Deng et al., 1997, Deng et al., 1999, Deng et al., 1995, Melanson & Iorio, 2004, Melanson & Iorio, 2006, Tanabayashi & Compans, 1996, Tsurudome et al., 1995, Wang et al., 2004). Additionally, site-directed mutagenesis of individual residues within the stalk has led to the identification of specific amino acids involved in triggering of F by HN, as well as the site of the HN-F interaction (Melanson & Iorio, 2004, Porotto et al., 2003). These residues are located in domains known as heptad repeats (HRs), which are motifs with hydrophobic amino acids at every seventh residue core position. In most paramyxoviruses, the HR domain in the attachment protein stalk consists of two HR motifs separated by an intervening region containing a highly conserved proline-leucine doublet. Unlike those of most other paramyxoviruses, the morbillivirus attachment proteins have six residues in this intervening region, such that the entire domain constitutes a single HR.

Through construction and testing of H chimeras for fusion promotion, an amino acid difference at position 98, which is located at a core position in the HR domain, was found to be responsible for the lack of fusion in a persistently infected cell line (Hummel & Bellini, 1995). While it was demonstrated that this residue is involved in fusion promotion, it was not determined if it is important for receptor recognition. Although it is located in the stalk, the mutation could alter the conformation of the regions of the globular domain that are responsible for

receptor binding. Indeed, mutation of some of the individual heptadic hydrophobic residues in the NDV HN HRs, as well as residues in the intervening region, has been shown to diminish fusion promotion, but was also found to alter receptor recognition and neuraminidase activity (Melanson & Iorio, 2004, Stone-Hulslander & Morrison, 1999, Wang & Iorio, 1999). Alanine and threonine substitutions for I98 strongly decrease fusion with no significant effect on either surface expression or receptor binding, consistent with the residue modulating the fusion helper function of MV H. However, while mutation of the residue disrupts fusion promotion prior to triggering of MV F, it does not affect the H-F interaction at the cell surface. Therefore, the effect of substitutions at position 98 on the fusion helper function appears to be indirect, possibly preventing conformational changes in H that are required for it to activate F. Alternatively, it is conceivable that more than one region of MV H mediates the H-F interaction at the cell surface.

Two highly conserved residues have been identified in the intervening region (IR) between the HRs of NDV, including P93 and L94 (Wang & Iorio, 1999). Mutation of these residues, as well as other amino acids in the IR, resulted in a strong decrease in fusion promotion that correlated with disruption of the HN-F interaction (Melanson & Iorio, 2004). An hPIV3 HN protein carrying a mutation of the conserved proline, P111S, has been reported to have a defect in triggering of F with fusion occurring at a reduced rate compared to the wt proteins (Porotto et al., 2003). Although the IR of measles is one amino acid

shorter than those of NDV and hPIV3 HN, the proline and leucine residues are conserved. Similar to the effects in NDV, mutation of P94 and L95 in MV H strongly decreases fusion promotion. In contrast to the results with hPIV3 P111S, mutation of P94 in MV H inhibits fusion prior to triggering of F. In fact, each of the MV H proteins carrying amino acid substitutions at P94 exhibits a significant decrease in receptor binding, as well as fusion, suggesting that the mutations alter the structure and/or orientation of the globular head. Unlike NDV HN proteins carrying mutations for the conserved proline and leucine, MV H proteins with amino acid substitutions for these residues can still be detected in complexes with F at the cell surface.

Also in direct contrast to NDV, mutation of individual residues in the HR domain of MV H does not disrupt the specific H-F interaction at the cell surface, indicating that multiple regions of MV H may be involved in the formation of the H-F complex. However, it cannot be ruled out that the HR in MV H mediates a physical interaction with F. It is possible that multiple residues in the domain are involved in the interaction such that mutation of a single amino acid is not sufficient to dissociate the H and F proteins. Additionally, an interaction involving another region of the H protein could be strong enough to mask a disruption of a physical interaction mediated by residues in the HR.

It is possible that a second region of H-F interaction resides in the globular head of MV H. The mutation V451E in the globular head has a significant effect on membrane fusion promotion that is not entirely attributable to altered receptor

recognition activity. This suggests that an unidentified region in the globular head domain, which either includes V451 or is structurally influenced by it, may be involved in mediating the H-F interaction. However, the region is not the sole determinant of the interaction, since the V451E mutation also fails to disrupt the formation of the H-F complex at the cell surface.

Overall, it can be concluded that changes in the amino acid sequence of the HR domain can modulate the structure and/or orientation of the globular head domain, but, more importantly, that the region plays a role in mediating the fusion helper activity of MV H. However, mutations in the HR domain do not affect the H-F interaction at the cell surface or solely determine the specific requirement of MV H for the homologous F protein. The effect of mutations in the HR on the fusion helper function appears to be indirect, possibly preventing conformational changes in H that are required for it to activate F. Alternatively, it is conceivable that the HR domain mediates an interaction between the glycoproteins, but that an additional region(s) of F-interaction in MV H prevents disruption of H-F complex formation. The ability of mutations in the HR repeat to disrupt the receptor recognition and/or F triggering functions of MV H without altering the H-F interaction at the cell surface is consistent with an interaction between MV H and F prior to receptor recognition and triggering of fusion promotion.

### 7.3.2 Characterization of the functions of two regions of MV F in fusion promotion.

The measles AIK-C vaccine was developed by attenuation of the Edmonston strain. It is a temperature-sensitive strain, which grows optimally at 33°C and exhibits reduced fusion promotion/ poor growth at 40°C. A single mutation in MV F, F278L, which is located just upstream of HR-D, has been shown to be responsible for the reduced fusion phenotype of the AIK-C vaccine strain of MV (Nakayama et al., 2001). However, the mutation did not appear to contribute to the temperature-sensitivity of the strain and its role in fusion promotion was not further characterized.

In the Edmonston strain F protein, mutation of F278 to leucine or alanine abolishes fusion promotion at 37°C. The fusion deficiency of the mutated protein can be directly attributed to a lack of $F_0$ cleavage. In contrast to the AIK-C strain, fusion promotion, as well as $F_0$ processing, of the MV F carrying the F278L mutation can be significantly rescued by incubation at 30°C. Similar results were obtained for amino acid substitutions in the HR-D domain of MV F. The correlation between restoration of fusogenic activity and acquisition of cleavage susceptibility suggests that the amino acid sequence of HR-D is important for efficient cleavage of $F_0$ at 37°C.

It has been suggested that the amino terminus of the C-rich region determines the specificity of the interaction between MV H and F based on the analysis of MV-CDV F chimeras (Wild et al., 1994). This study concluded that

the first 44 residues of the C-rich region are responsible for the specificity of MV F for MV H. However, there are problems with this study that weaken this conclusion. First, not all chimeras containing the amino terminus of the C-rich region were able to promote fusion with MV H, suggesting that other regions could be involved in determining the specificity of the H-F interaction. Second, no actual data was presented; fusion promotion was reported as (+) or (–) with no information provided with respect to the levels of fusion promotion associated with each construct. Third, fusion with CDV H was not tested. This is an important point because, although fusion promotion is most efficient when the H and F proteins are derived from the same virus and strain, the MV and CDV H proteins are, to a certain extent, interchangeable for fusion with MV F. Fourth, and probably most importantly, efficient cleavage and processing of the chimeric proteins were not verified. Although some of the chimeras were detected by cell surface immunofluorescence, it remains possible that they were not cleaved. Finally, the investigators concluded that the C-rich region is the site of the MV H-F interaction without directly testing whether the F chimeras are able to physically interact with MV H.

Based on the assignment of disulfide bridges in the F protein of Sendai virus, the amino terminus of the C-rich region is predicted to form two loop structures (Loop 1 and 2) with two intervening sequences (IS 1 and 2). In each of these regions, there are residues that differ between MV and CDV F. Mutation of the divergent residues in each region to the corresponding amino acid in CDV

F significantly inhibits promotion of membrane fusion with both MV and CDV H. This, but itself, argues that this region is not the sole determinant of H specificity. Most importantly, the deficiencies in fusion promotion exhibited by three of the four mutated proteins, including IS 2, Loop 2, and IS 2, are related to defects in the cleavage of $F_0$. Expression of the proteins at a lower temperature dramatically enhances both the cleavage of $F_0$ and the fusion promotion activity of the proteins carrying mutations in IS1, Loop 2, and IS2. However, fusion promotion is not enhanced for the mutated protein with substitutions in Loop 1, which is efficiently cleaved at both temperatures. These results suggest that the amino acid sequences of IS1, Loop 2, and IS2 in the C-rich region are important for proper cleavage of $F_0$ and/or surface expression of $F_1$. In contrast, the residues in Loop 1 appear to have a different role in fusion promotion. Still, the MV F protein carrying the Loop 1 sequence of CDV F is unable to promote fusion with CDV H at 37°C, again suggesting that this region is not the sole mediator of the H-F interaction.

Consistent with the results presented by Wild et al. (1994), a chimeric protein in which the amino terminus of the C-rich region of MV F was replaced with the corresponding region of CDV F was unable to promote fusion with MV H. However, the level of fusion promotion with CDV H was also reduced. Similar results were obtained with the reciprocal chimera in which the amino terminus of the C-rich region of CDV F was replaced with the corresponding region of MV F. It was unable to promote fusion with MV H and exhibited reduced fusion

promotion with CDV H. Expression of the proteins at a lower temperature does not enhance fusion promotion with the heterologous H protein. These results support a role for the C-rich region of MV F in fusion promotion, but indicate that it cannot be the sole determinant of the H-F specificity.

Overall, it can be concluded that changes in the amino acid sequence of the HR-D and C-rich domains can modulate fusion promotion by MV F. However, this appears to be unrelated to the virus-specific interaction with the homologous H protein. Rather, it seems to be more directly related to the structure and function of the F protein. A majority of the mutations in these regions inhibited fusion by preventing cleavage activation of $F_0$ and cell surface expression of $F_1$. It is possible that the mutations disrupt proper folding of the F protein at 37°C such that the cleavage site can no longer be recognized by proteases. Additionally, Loop 1 plays a role in fusion promotion by MV F. However, it is not the sole determinant of the specific H-F interaction, suggesting that, consistent with the results for MV H, multiple regions of MV F are involved in the formation of the H-F complex.

## 7.4 Mechanism of membrane fusion promotion by measles glycoproteins.

The data presented in this dissertation support a model of measles membrane fusion in which the H and F proteins form a complex prior to receptor recognition (Figure 72). Although the existence of an intracellular interaction between the two proteins could not be confirmed using the ER-retention

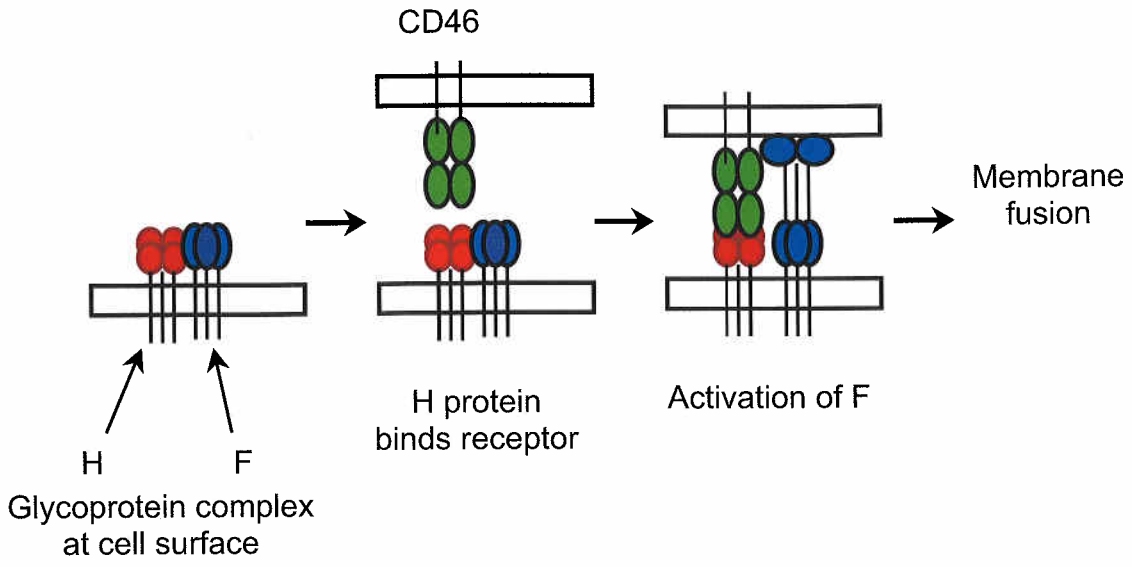



**Figure 72. Models of mechanism of measles virus membrane fusion promotion.**

Measles glycoproteins interact at the cell surface prior to receptor recognition by H. Receptor binding by H triggers F activation. The H-F interaction is mediated by multiple residues in both proteins.

approach, a MV H-F complex can be detected at the cell surface in the absence of receptor recognition. Thus, this alternative approach strongly suggests that MV H and F arrive at the surface in a complex. These results are in direct contrast to those obtained by our lab for NDV, which indicate that the HN-F cell surface interaction is triggered at the cell surface by receptor binding (Li et al., 2004, Melanson & Iorio, 2004).

As a result of the interaction prior to receptor binding, the attachment of MV H to its cellular receptor must induce conformational changes that trigger activation of the F protein. Similar to NDV, this communication between the H and F proteins appears to be mediated in part by a HR domain in the stalk of the attachment protein. Additionally, the functional interaction between MV H and F may be mediated in part by Loop 1 of amino terminus of the C-rich region of the fusion protein. However, the exact role of this region of the F protein in fusion promotion remains to be determined.

In NDV HN, it has also been demonstrated that single amino acid changes in the HR of the stalk can disrupt both the triggering of F and the HN-F cell surface interaction without altering receptor recognition (Melanson & Iorio, 2004). In contrast, in no case has a definitive link between any single amino acid difference in MV H or F and an inability to form the cell surface H-F complex been demonstrated, suggesting that cell surface interaction between MV H and F proteins may be mediated by more that one region of each protein.

Importantly, the data presented in this dissertation indicate that although paramyxoviruses may all use the same general process for promotion of membrane fusion, the mechanism may vary in multiple aspects. A more complete understanding of the means by which measles promotes membrane fusion may allow for development of specific strategies aimed at interfering with the early stages of infection. Although, the results of the studies presented in this dissertation shed light on the early steps of the mechanism of membrane fusion by MV H and F, additional work is necessary to identify the regions of H and F involved in the specific interaction between the two proteins. Further, investigation of cellular factors involved in the membrane fusion process is also important for a complete understanding of measles entry into and spread among host cells.

# APPENDIX

## Primers used for site-directed mutagenesis

**Table 2. Site-directed mutagenesis primers for generation of ER-tagged MV H and F.**

| *Mutation | †Enzyme | Primer sequence (5' to 3') |
|---|---|---|
| MV H RRRRR | Aat II | GTCGTTAGGGTGCAAGATCATCCACAATG**CGCCGTAG**<br>**ACGTCGA**TCACCACAACGAGACCGGATAAATGCCTTC |
| MV F KSKTH | Dra I | CTTACGGGAACATCAAAATCCTATGTAAGGTCTTTA**AA**<br>**ATCAAAAACACAC**TGAACCTCTACAACTCTTGAAACAC |

*Mutation indicates the amino acid residues introduced into MV H or F protein. Mutated or added codons are highlighted in bold. †Enzyme indicates the restriction enzyme site introduced into the gene. Restriction enzyme site in the primer sequence is underlined.

**Table 3. Site-directed mutagenesis primers for generation of MV and CDV Fcsm proteins.**

| *Mutation | †Enzyme | Primer sequence (5' to 3') |
|---|---|---|
| MV F RNHNR | Nhe I | GAGTGTAGCT**AGC**AGTAGG**AACCACAAC**AGATTTGCG GGAG |
| CDV F GRQGR | Kas I | CATTAGGGTCAGGT**GGGCGCC**AAGGACGTTTTGCAG GAG |

*Mutation indicates the amino acid substitution(s) in the F proteins. Mutated codons are highlighted in bold. †Enzyme indicates the restriction enzyme site introduced into the gene. Restriction enzyme site in the primer sequence is underlined.

**Table 4. Primers used for site-directed mutagenesis of putative MV H receptor recognition regions.**

| *Mutation | †Enzyme | Primer sequence (5' to 3') |
|---|---|---|
| V451E | Ssp I | CCACAACAAT**GAA**TATTGGCTGAC |
| Y481N | Ear I | CAAGGTTAGTCCC**AAT**CTGTTCAATGTC |
| S544G | Acc I | GTGGTTTATTACGTATAC**GGA**CCAAGTCGC TCATTTTC |
| S546G | Eag I | CGTTTACAGCCCC**GGC**CGCTCATTTT CTTAC |
| I473A | Sty I | CAACACATTGGAATGG**GCT**CCTAGGTTCAAGGTT AGTC |
| P474A | Nru I | CATTGGAGTGGATC**GCG**AGATTCAAG GTTAG |
| R475A | Dra I | GAGTGGATACCG**GCA**TTTAAAGTTAGTCCC |
| F476A | Sty I | GAGTGGATACCTAGG**GCC**AAAGTTAGTC |
| K477A | Sty I | GGATACCTAGGTTC**GCG**GTTAGTCCC**TAC** |
| 473-477A Y481N | Pst I | GTAATCAACACATTGGCGTGG**GCGGCCGCAGCT GCA**GTTAGTCCT**AAC**CTCTTC |
| 473-477A | PstI | GTAATCAACACATTGGCGTGG**GCGGCCGCAGCT GCA**GTTAGTCCTTACCTCTTC |
| F431A | BspE I | CAAAATTGCTTCAGGT**TCC**GGACCATTTGATCAC |

*Mutation indicates the amino acid substitution(s) in the H protein. Mutated are highlighted in bold. †Enzyme indicates the restriction enzyme site introduced into the gene. Restriction enzyme site in the primer sequence is underlined.

**Table 5. Primers used for site-directed mutagenesis of MV H region 244-250.**

| *Mutation | †Enzyme | Primer sequence (5' to 3') |
|---|---|---|
| S244A | BssH II | CTGAGCAGCAAGCGC**GCA**GAGTTGTCAC |
| L246A | Stu I | GCAAAAGGTCTGAG**GCC**TCACACCTGAGC |
| S247A | FspI | CAAAAGGTCAGAGCTT**GCG**CAACTGAG |
| L249A | None | CAGAGTTGTCACAA**GCG**TCCATGTACCGAG |
| S250A | Msc I | GTTGTCACAACTG**GCC**ATGTACCGAG |
| E245A | Afe I | CAGCAAAAGGTCA**GCG**CTATCACAACTGAG |
| Q248A | Afe I | GTCAGAGTTGTCA**GCG**CTGCGCATGTACCGAGTG |
| S244A/ L246A | Hind III | CTGAGCAGCAAAAGG**GCA**GAA**GCT**TCACAACTGA G |
| S244A/ E245A | Hae II | CTGAGCAGCAAAAGG**GCGGCG**CTATCACAACTGA G |

*Mutation indicates the amino acid substitution(s) in the H protein. Mutated codons are highlighted in bold. †Enzyme indicates the restriction enzyme site introduced into the gene. Restriction enzyme site in the primer sequence is underlined.

**Table 6. Primers used for site-directed mutagenesis of MV H heptad repeat.**

| *Mutation | †Enzyme | Primer sequence (5' to 3') |
|---|---|---|
| I98A | Dra I | CTGACACCACTATTTAAA**GCC**ATCGGTGATGAAG |
| I84A | Hae II | GATGTAACTAACAGC**GCT**GAGCACCAGGTC |
| L105A | Hae II | GATGAAGTGGGC**GCT**AGGACACCTCAG |
| P94A | Dra I | GACGTGCTGACA**GCT**CTCTTTAAAATCATCGGTG |
| L95A | Dra I | CGTGCTGACACCT**GCC**TTTAAAATCATC |
| V91A | Dra I | GTCAAGGAC**GCG**CTAACACCACTCTTTAAAATCATC |
| L92A | Dra I | GTCAAGGACGTA**GCG**ACGCCACTCTTTAAAATCATC |
| I99A | Dra I | CACCACTCTTTAAAATC**GCC**GGTGATGAGGTGGGCCTG |
| P94L | Dra I | GACGTGCTGACA**TTA**CTCTTTAAAATCATCGGTG |
| P94S | Dra I | GACGTGCTGACA**AGC**CTCTTTAAAATCATCGGTG |
| L95P | Dra I | CGTGCTGACACCA**CCC**TTTAAAATCATC |
| L95R | Dra I | CGTGCTGACACCA**AGG**TTTAAAATCATC |
| F96L | BsaH I | GGACGTGCTGACGCCTCTCT**TG**AAAATCATCGGTG |
| P94G | Age I | GACGTGCTGACC**GGT**CTCTTCAAAATCATC |
| F96A | Nru I | CTGACACCACTC**GCG**AAAATTATAGGTGATGAAG |
| T93A | Kas I | GTCAAGGACGTGCTG**GCG**CCTCTCTTCAAAATC |
| I98T | Pvu I | CCACTCTTCAAA**ACG**ATCGGTGATG |

*Mutation indicates the amino acid substitution(s) in the H protein. Mutated codons are highlighted in bold. †Enzyme indicates the restriction enzyme site introduced into the gene. Restriction enzyme site in the primer sequence is underlined.

**Table 7. Primers used for site-directed mutagenesis of MV F heptad repeat.**

| *Mutation | †Enzyme | Primer sequence (5' to 3') |
|---|---|---|
| V302A | Xho I | GTGATTGTCCACCGGCTCGAGGGC**GCA**TCATACAACATAG |
| L281A | Nru I | CCTACTTCATTGTC**GCG**AGTATAGCCTATC |
| I295A | ApaL I | GAGATTAAGGGTGTG**GCT**GTGCACCGGCTAGAG |
| Y285A | AcI I | GTCCTCAGTATAGCC**GCT**CCAACGTTGTCCGAGATTAAG |
| L288A | AlwN I | GTATAGCCTATCCGACA**GCG**TCTGAGATTAAG |
| F278A | Sca I | GTCGACACAGAGTCATAT**GCC**ATAGTACTCAGTATAGCC |
| F278L | Sca I | GACACAGAGTCCTAC**TTA**ATAGTACTCAGTATAGCCTATC |
| P289A | AcI I | CAGTATAGCCTAC**GCA**ACGTTGTCCGAGATTAAG |

*Mutation indicates the amino acid substitution(s) in the F protein. Mutated codons are highlighted in bold. †Enzyme indicates the restriction enzyme site introduced into the gene. Restriction enzyme site in the primer sequence is underlined.

**Table 8. Primers used for site-directed mutagenesis of F protein C-rich region.**

| *Mutation | †Enzyme | Primer sequence (5' to 3') |
|---|---|---|
| **MV F** Loop 1 | Hae II | GTCATCGTGT**GTT**TTC**GTGTCA**GAG**AGCGCTATA**T GCAGCCAAAAT |
| IS 1 | EcoR I | CTGTGTGCAGCCAGAATTCCTTGTACCCGATG |
| Loop 2 | Nsi I | CTCTGCTCCAA**CAA**TGC**ATC**CGCGGT**GAC**ACT**AG C**TCCTGTGCTCGTA |
| IS 2 | BspE I | CTCGTATCCGGA**ACCATG**GGGAAC**AAG**TTCATTTTATC |
| T335V | Pvu II | CGAATTTTGATGAGTCCAGCTGT**GTT**TTCATGCC |
| P338S | BspH I | CGTGTACTTTCATG**AGC**GAAGGAACTGTGTG |
| T341A | Pst I | GCCAGAGGGA**GCA**GTCTGCAGCCAAAATG |
| M337V | BspE I | CGTGTACTTTC**GTT**CCGGAAGGGACTCTGTG |
| V342I | Nde I | CATGCCAGAGGGAACC**ATA**TGTAGCCAAAATGCCT TGTACCCGATGAG |
| G340S | Sca I | CTTTCATGCCAGAG**AGT**ACTGTGTGCAGCC |
| **CDV F** Loop 1 | BsrG I | GAGTCATCTTGT**ACA**TTC**ATGCCA**GAG**GGAACCG TA**TGTAGCCAGAAC |
| IS 1 | None | GTAGCCAGAAC**GCC**TTGTATCCCATGAGCCCAC |
| Loop 2 | BamH I | CACTCTTACAG**GAA**TGC**CTC**AGAGGA**TCC**ACT**AAA** TCTTGTGCTCGGAC |
| IS 2 | Cla I | CCTTGGTATCTGGA**TCCTTC**GGCAAT**CGA**TTTATTC TGTC |

*Mutation indicates the amino acid substitution(s) in the F protein. Mutated codons are highlighted in bold. †Enzyme indicates the restriction enzyme site introduced into the gene. Restriction enzyme site in the primer sequence is underlined.

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