Studies Concerning the Interaction of Measles Virus with Its Cellular Receptors

By

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In spite of advances in vaccine development, measles virus is still the most common infectious killer of children in developing countries. The Edmonston isolate of measles virus has been used to generate attenuated vaccines. However, recent failures of these vaccines have led to a 20% increase in the rate of mortality and have prompted the World Health Organization to re-evaluate methods with which to control this important pathogen. CD46 was previously shown to be a primate-specific receptor for Edmonston strain of measles virus. In this thesis, we first studied the inability of the New World monkey erythrocytes to hemagglutinate in the presence of measles virus. We found that the CD46 molecules from the lymphocytes and erythrocytes of the New World monkeys all lacked the SCR1 coding region adjacent to the amino terminal signal peptide. We also generated artificial mutations by replacing either charged or hydrophobic amino acids with alanine or serine residues into the SCR1 and SCR2 domains of CD46. In addition, we mapped the antigenic epitopes of 5 monoclonal antibodies which are known to inhibit the binding of measles virus H protein to CD46. These results were used to model the SCR1 and SCR2 domains of CD46 from an analogous region in another complement regulatory protein, Factor H, whose three dimensional structure has been previously reported. Finally, we have evidence indicating that wild type isolates of measles virus use another receptor besides CD46 for viral entry. The common marmoset (New World monkey) was shown to be susceptible to wild type measles virus infections in spite of the SCR1 deletion. In addition, a direct binding assay using insect cells expressing the wild type measles virus H protein did not bind to rodent cells expressing CD46 but adhered to marmoset and human B cells. We were also able to demonstrate that residue 481 of the H protein determines whether the virus uses CD46 or the unknown receptor on B cells for viral entry, raising the possibility that populations of measles virus can use one or the other receptor during the normal process of infection.

Acknowledgments

I would like to dedicate this thesis to my parents who have sacrificed many fine things in life for their children. Their love, understanding and support are forever appreciated.

I am especially grateful to my supervisor and friend, Chris Richardson, for showing me the way in science and, from time to time, life as well. I will remember to carry on life in a "safe and prudent" manner. These memories will be forever cherished. I also extend my gratitute to Dr. David Rose and Dr. Gil Privé for their helpful suggestions.

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Table of Contents

List of Figures

CHATER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

List of Tables

List of Publications

1. **Hsu, E. C., R. E. Dorig, F. Sarangi, A. Marcil, C. Iorio, and C. D. Richardson.** 1997. Artificial mutations and natural variations in the CD46 molecules from human and monkey cells define regions important for measles virus binding. J Virol. **71:**6144-54.

2. **Hsu, E. C., S. Sabatinos, F. J. Hoedemaeker, D. R. Rose, and C. D. Richardson.** 1999. Use of site-specific mutagenesis and monoclonal antibodies to map regions of CD46 that interact with measles virus H protein [In Process Citation]. Virology. **258:**314-26.

3. **Hsu, E. C., F. Sarangi, C. Iorio, M. S. Sidhu, S. A. Udem, D. L. Dillehay, W. Xu, P. A. Rota, W. J. Bellini, and C. D. Richardson.** 1998. A single amino acid change in the hemagglutinin protein of measles virus determines its ability to bind CD46 and reveals another receptor on marmoset B cells. J Virol. **72:**2905-16.

List of Abbreviations

AcNPV	*Autographa californica* nuclear polyhedrosis virus
AIDS	Acquired immunodeficiency syndrome
CAR	Coxsackie/adenovirus receptor
cDNA	complementary DNA
CDV	Canine distemper virus
CNS	Central nervous system
DAF/CD55	Decay accelerating factor
DDB1/XPE	DNA damaged binding protein 1
DTH	Delayed-type hypersensitivity
EBV	Epstein-Barr virus
ED	Edmonston
ELISA	Enzyme-linked immunosorbent assay
ERM	Ezrin/radixin/moesin
ES	Embryonic stem cells
F	Fusion protein
FACS	Flow cytometry
FITC	fluorescein isothiocyanate
H	Hemagglutinin
HN	Hemagglutinin/Neuraminidase
HIV	Human immunodeficiency viruses
IL-12	Interleukin-12
kDa	KiloDaltons
L	Large protein
M	Matrix protein
MV	Measles virus

MCP/CD46	Membrane cofactor protein
MIBE	Measles inclusion body encephalitis
Min.(s)	Minutes
mRNA	Messenger RNA
NDV	Newcastle disease virus
N/NP	Nucleocapsid protein
ONPG	*o*-nitrophenylgalactoside
P	Phosphoprotein
PFU	Plaque forming units
PCR	Polymerase chain reaction
RBC	Red blood cells
RPV	Rinderpest virus
RT-PCR	Reverse transcriptase polymerase chain reaction
Sec.(s)	Seconds
SCR	Short consensus region
SSPE	Subacute sclerosing panencephalitis
STP	Serine/threonine/proline
SV5	Simian virus 5
VSV	Vesicular stomatitis virus
WT	Wild type

Chapter 1: General Introduction

1.1 Historical Perspective

Measles virus is still known as the greatest infectious killer of children, affecting more than 50 millions and killing nearly 1.4 millions each year (54). The first description of the measles virus is credited to Rhazes in 840. However, it is uncertain whether Rhazes was able to distinguish measles from smallpox (82). Thus, the discovery of measles is credited to Sydenham who clearly differentiated measles virus from smallpox virus with a detailed description of measles symptoms (54, 82). Nonetheless as early as 1757, Francis Home, a Scottish doctor, was able to demonstrate the transmission of a rash causing disease by inoculating healthy individuals with a blood sample from patients with the rash (128). In addition, Boldberger and Anderson transmitted measles using filtered respiratory secretions from human patients to macaque monkeys. The modern epidemiology of measles virus was attributed to Peter Panum who described the epidemic of a contagious disease, which was transmitted through the respiratory tract. More importantly, Panum was able to recognize its fourteen-day incubation period and the life long immunity acquired by those who contracted measles virus (54). This revelation led to the hypothesis that measles virus is a "disease of civilization" since a large and dense reservoir is required to sustain the presence of measles in the human population. Consequently, measles probably evolved from the closely related rinderpest virus, a cattle virus, found in populated areas of the Middle East and India where cows and humans live in very close proximity (54).

The spread of measles resulted in high morbidity and mortality in India, China and Europe. Measles virus was subsequently transmitted to the New World through European explorers. Together with smallpox, the introduction of measles virus to the American Indian population led to devastating epidemics, which caused the demise of nearly 56 million people in America. To further study the pathology

of measles, Enders and Peebles (1954) successfully propagated the virus in tissue culture by inoculating blood samples from David Edmonston, a young patient, into human primary kidney cells (54). The Edmonston strain of the measles inoculate was subsequently adapted to different cell lines, and eventually led to the development of a live attenuated measles vaccine (52, 82).

1.2 The *Paramyxoviridae* Family

Measles virus is a member of the *Paramyxoviridae* family, which contain genomes consisting of non-segmented, single stranded RNA. This genomic RNA serves as a template for messenger RNA transcription, and is by convention refered to as negative sense. Transcription is performed by the viral polymerase, which is packaged along with the viral genome. Replication of the viral genome occurs only after viral proteins have been synthesized using the anti-genome as the template (92). The classification of the *Paramyxoviridae* is primarily based on viral morphology, genome organization, viral protein functions, and sequence similarity of the viral genome. This family is divided into two subfamilies, Paramyxovirinae and Pneumovirinae, which are distinguished by the number of proteins they encode and by the morphology of their nucleocapsids. The two subfamilies encompass 4 different genuses: paramyxovirus, rubulavirus, morbillivirus and pneumovirus. All the viruses in the family are characterized by pleomorphic, enveloped virions ranging from 150 to 350 nm in diameter (24, 84, 85). The lipid bilayer membrane of the virus is derived from the host's cell membrane. The viral envelope contains viral surface glycoproteins, 8 to 12 nm in length, which are visible by electron microscopy. These glycoproteins are primarily responsible for the attachment and entry of the virion into the cell. Consequently, the viruses of the Paramyxovaridae family are restricted in their host range among vertebrates (131).

The viral genome of the paramyxoviruses is organized in the manner depicted in Figure 1.1 (12). The genome is around 15.5 kilobase in length. It contains both 3' leader and 5' trailer extracistronic regions, each approximately 50 nucleotides in length. These regions are used for the regulation of transcription and replication. The genome contains 6 genes with the exception of the rubulaviruses and pneumoviruses, which contains 7 genes and 10 genes, respectively. One gene produces a single mRNA that utilizes different open reading frames to encode several proteins. In addition, the viral genome also contains intergenic regions, which separate the individual genes. In the case of morbilliviruses and parainfluenzaviruses, the intergenic regions are exactly three nucleotides. Conversely, rubulaviruses and pneumoviruses have intergenic sequences that may vary between 1 to 56 nucleotides.

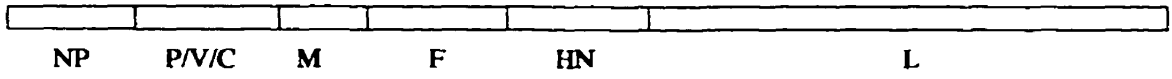
The first gene encoded by the viruses in the *Paramyxoviridae* family, with the exception of pneumoviruses, is the nucleocapsid protein (NP). It is the most abundant viral protein present in infected cells and has three different functions: First, it encapsidates the genome, protecting the RNA from RNase degradation. It also associates with the viral polymerase during viral transcription and replication. Finally, it interacts with the matrix protein to facilitate viral assembly (12, 13). In terms of pneumoviruses, the NP protein is preceded by two small non-structural proteins, 1C and 1B. The function of these two proteins is still unknown.

In most *Paramyxoviridae*, the gene located downstream of NP is the P gene. The P gene encodes three different viral proteins through the use of overlapping open reading frames: the phosphoprotein (P), the C protein, and the V protein. On the other hand, the pneumovirus P gene encodes only the phosphoprotein. In the viral polymerase complex, the P protein along with the large protein (L) and NP protein play a major role in the transcription and

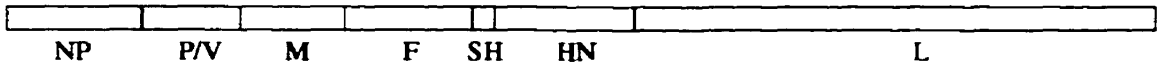
Figure 1.1 *Paramyxoviridae* family map

The *Paramyxoviridae* family contains viruses with non-segmented, single stranded RNA genomes. This family is divided into two subfamilies, Paramyxovirinae and Pneumovirinae, which are distinguished by the number of proteins they encode and by the morphology of their nucleocapsids. The two subfamilies are subdivided into 4 different genuses: paramyxovirus, rubulavirus, morbillivirus and pneumovirus. The genes are drawn to scale and separated by vertical lines.

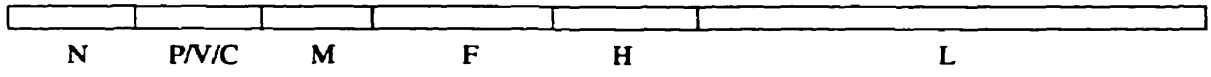
Paramyxoviruses-Sendai virus



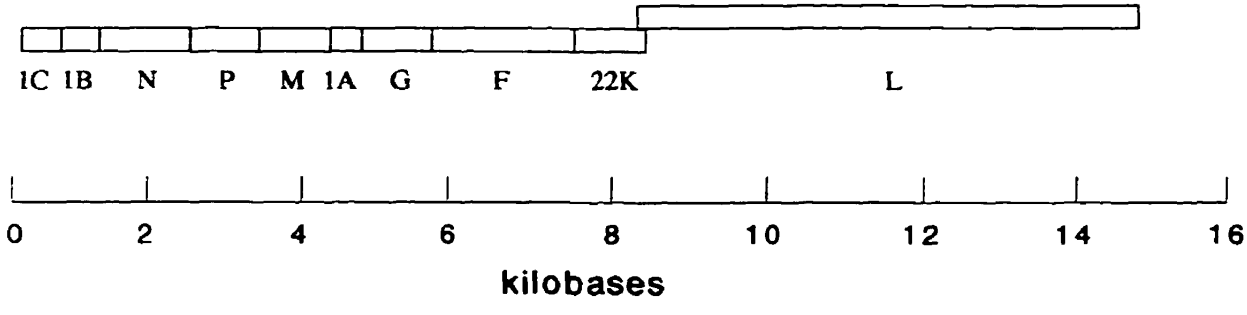
Rubulaviruses-SV5



Morbilliviruses-Measles virus



Pneumoviruses-Respiratory Syncytial virus



replication of viral RNA. An essential role for these three proteins in the replication complex has been shown by a genome replication reconstitution experiment (65). The C protein is a non-structural protein, which is translated from the P mRNA using an open reading frame different from that of P. However, recent experimental evidence has suggested that a small amount of C proteins are also associated with the virions (90, 130). The function associated with C protein is still not clear. *In vitro* evidence has indicated that C protein appears to inhibit mRNA synthesis while having no effect on viral genome replication. Therefore, the C protein may be important in regulating the balance between protein synthesis and genome replication (27). Another non-structural protein, V protein, is also translated from the P mRNA through use of an alternative start codon. Similar to the situation with C protein, the function of the V protein remains an enigma. Nonetheless, a role for the V protein in regulating the transition between viral transcription and replication has been suggested (28). In addition, recent evidence has shown an interaction between the V protein and a DNA repair protein, DDB1/XPE. This interaction may suggest that other functions may be associated with the V protein (94).

In the paramyxovirus virion, the matrix protein (M) is the most abundant protein. Through electron microscopy, the M protein can be observed to underlay the lipid bilayer of the virion (40, 91). In addition, the M protein has been demonstrated to interact with the nucleocapsid protein and viral glycoproteins (14, 143). The M protein is probably the most important protein for organizing and packaging the infectious virion during viral maturation. This observation is based upon evidence derived from the persistent measles infection associated with subacute sclerosing panencephalitis (SSPE). These studies indicated that in the absence of the M protein, virus is not able to assemble and package (66).

Viruses in the *Paramyxoviridae* family contain fusion protein (F) on the surface of the virion. The F protein is usually synthesized as an inactive precursor, which is then processed by a host cell protease to create an active fusion protein (64, 147). The amino-terminus of the fusion protein is very hydrophobic and highly conserved among the different viruses in the family (119). The fusion protein is ultimately responsible for the mixing of the viral and target cell membranes to permit viral entry after initial attachment. In addition, the fusion proteins, together with the hemagglutinin/attachment glycoprotein, are also responsible for producing multinucleated giant cells or syncytia between infected cell and adjacent cells.

The attachment proteins (H, HN or G) of the viruses in the *Paramyxoviridae* family are responsible for the first step of viral infection. These proteins are responsible for virus attachment to specific cellular receptor proteins. The HN and H proteins from the paramyxovirus, Rubulavirus and Morbillivirus genera have hemagglutinin activity, which causes red blood cells to clump, or to agglutinate. The HN of human parainfluenzavirus and simian virus 5 appear to utilize sialic acid containing molecules as the receptor, while the H protein of measles virus binds to CD46, a complement regulatory protein (54). The HN proteins also have neuraminidase activity, which prevents self-aggregation of viral particles, cuts through mucous in respiratory tract, and elutes virions in the non-productive infection. In addition, the H and HN proteins can also increase the fusion activity of the F protein when both proteins are co-expressed (92). In contrast, the G protein of the pneumovirus possesses neither the hemagglutinin activity or neuraminidase activity. The G protein simply acts as the attachment protein for the virus (144, 171).

The last gene of the Paramyxovaridae genome encodes the large protein (L). The L protein is the least abundant protein in the virion and functions as part of the

viral polymerase complex (11, 178). *In vitro* studies have demonstrated that the presence of L, P and NP are required for viral polymerase activity (27, 57). How this polymerase complex functions is still unknown.

1.3 Diseases Associated With Measles Virus

1.3.1 Acute Measles Virus Diseases

The "classical" disease associated with measles virus begins about 10 days after initial infection with the pathogen. During this 10 day incubation period, no symptoms of the viral disease are observed (54). An infected child then develops respiratory disease, which can be mistaken for the common cold. A few days after, the symptoms will intensify into high fever, cough, coryza, and eyelid membrane. This phase of measles is accompanied by the development of Koplik spots, white-gray maculae with a pale red circumference, which are visible on the oral mucosa and the conjunctivae. Approximately 14 days after the initial infection to measles virus, a maculopapular rash appears on the forehead of the patient while the Koplik spots begin to subside. In a few days, the rash spreads over the entire body. The onset of the rash also coincides with the mounting of an immune response, which eventually leads to the clearance of the virus. In most cases, the rash will disappear completely about 10 days after the initial appearance, but the cough may persist for weeks and asthenia may last for a month.

Measles virus is usually spread by aerosol and enters the human body through the respiratory tract. The initial replication of measles virus takes place in the tracheal and bronchial epithelial cells (142, 155). A few days after the initial infection, the virus then spreads to the local lymphatic system probably via the infected pulmonary macrophages (79, 152). The amplification of the virus in these regional lymph nodes results in the appearance of lymphoid or reticuloendothelial

giant multinucleated cells called syncytia, first described by Warthin and Finkeldey (170). Viral replication then leads to primary viremia, resulting in the dissemination of virus through the blood of the patient. The virus primarily infects monocytes in the blood and then spreads to the various organs. Lymphoid organs and tissues such as thymus, spleen, lymph nodes, appendix and tonsils are the predominate site of secondary viral replication which results in secondary viremia. At this point, the virus scatters throughout the body infecting skin, conjunctivae, kidney, lung, gastrointestinal tract, respiratory mucosa, genital mucosa and liver. The measles rash occurs when the dermal endothelial cells are infected. The infection then spreads to the overlaying epidermis causing syncytia formation, perivascular mononuclear infiltration and edema. Epidemilogical evidence indicates that infected patients are most contagious a few days before the onset of the rash (82). The transmission usually takes place through the release of virus from the infected mucous membranes of the respiratory tract.

1.3.2 Additional Complications Associated with Measles Virus

Beyond the acute measles virus infection, there are other complications associated with this pathogen. One of the complications is the creation of a modified measles, which implies that the acute disease has been attenuated by the presence of anti measles antibodies (37). This disease usually develops when infected children either still have the protection of maternal antibodies or have received an immunoglobulin injection treatment. However, the presence of the antibody is not sufficient to stop further infection. Children developing a modified measles disease usually have longer incubation periods, about 20 days. Although the patient, also develop rashes, the symptoms in general, are milder.

Measles virus may also cause rare complications among different organs. For example, young patients may develop laryngotracheobronchitis, and infants

may experience bronchiolitis obliterans, which are infections of the respiratory system (124). In terms of the cardiovascular system, measles is associated with myocarditis and pericartitis (82). In addition, measles can sometimes cause gastroenteritis in the developing world, especially in children under 2 years of age (107).

Other complications, which can arise from measles virus, affect the central nervous system. There are different forms of neural disease caused by the virus, including measles inclusion body encephalitis (MIBE) and subacute sclerosing panencephalitis (SSPE). MIBE has been found to affect predominantly immunocompromised patients. The incubation period varies from 1 month to 6 months, and the clinical manifestation of the disease is non-specific encephalitis accompanied by convulsive seizures. Patients suffering from inclusion body encephalitis usually die within 2 weeks following the onset of symptoms (111). Polymerase chain reaction (PCR) analysis of necropsy sections from brain tissues has revealed the presence of the measles viral genome (111).

Subacute sclerosing panencephalitis (SSPE) is a rare complication of the measles infection that manifests into persistent neuronal disease many years after the initial acute infection. SSPE usually strikes children who were infected with acute measles at a very young age, usually less than 2 years old. During a 7 to 10 year dormant stage, the virus enters the central nervous system (CNS) and, following this latent period, progressive cerebral dysfunction occurs over a period of several weeks or months. The initial symptoms include personality changes and myoclonic jerks of the head and limbs. Subsequently, a progressive loss of vision occurs due to cortical blindness or optic atrophy. Patients finally fall into a coma ultimately leading to death. SSPE is characterized by a slow deterioration over a period of 2 years following the onset of symptoms. The presence of measles virus in neurons has been confirmed by the abundance of anti-measles antibodies in the

cerebrospinal fluid. Neuropathology from SSPE patients indicated demyelination and lesions of the cerebral cortex, hippocampus, cerebral cortex, basal ganglia, brain stem and spinal cord. The presence of measles virus in neuronal tissue was verified by fluorescent staining with anti-measles antibodies (68, 69).

Measles virus replication in the CNS of SSPE patients is usually defective since rescue of the infectious virus can only be accomplished by fusing brain tissue with immortalized cell lines such as Vero cells (70). Brain tissue from SSPE patients has revealed that the virus cannot bud from the surface of the infected cell. Rather, the virus spreads within the CNS by transmission of the ribonucleoprotein from cell to cell (126). Studies have shown that measles virus isolated from SSPE patients is likely to cause neurological disease after intracerebral injection in animals, such as mice (122) (54). These results indicate that the measles virus found in SSPE has adapted to grow efficiently in neuronal tissue (122). Sequence analysis of viral RNA from SSPE patients shows that the viral genome of the measles virus that causes SSPE is different from the virus that causes acute measles infection. This is especially evident in the coding regions of the matrix (M), the hemagglutinin (H) and the fusion (F) proteins (174). For example, the genetic sequence encoding for the M protein contains numerous U to C substitutions (174). Furthermore, the expression level of certain viral proteins has also been altered in the SSPE patients. In general, the expression level of the M protein is nearly undetectable either due to lack of synthesis or protein instability (154, 157). There are some SSPE strains of measles virus that do express the M protein. However, M protein in these strains fails to interact with the nucleocapsid protein (NP), and localizes predominantly to the cytosol (60). Conversely, the M protein of the wild-type measles virus accumulates near the plasma membrane due to interactions with the nucleocapsid protein and viral glycoproteins. The abnormality of M protein

function in SSPE may be important for the establishment of persistent measles virus infections in the CNS of SSPE patients.

Furthermore, changes in the envelope proteins, H and F, may also contribute to persistent measles virus infections seen in SSPE patients. Although not as abundant as the M protein, mutations in the coding regions of the H and F proteins have also been identified. Some SSPE strains of measles virus appear to have a deletion of the cytoplasmic region of the H protein, a region required for cell to cell fusion in measles infections. Some other strains appear to have a deletion or mutation in the cytoplasmic region of F protein. While this alteration of the F protein may not affect fusion, the budding of infectious viral particles appears to be impaired (22, 148). A defective immune response against measles virus in SSPE patients may also contribute to a persistent measles virus infection in the CNS (45, 78). In addition, passive transfer of the anti-measles antibodies appears to induce persistent measles virus infections and SSPE (133).

High titers of measles virus antibodies have also been linked to patients with multiple sclerosis, implying a relationship between the two. There is an epidemiological correlation between the number of multiple sclerosis cases and measles infection. However, measles virions have not been isolated from patients with multiple sclerosis patients (50). Similarly, an increase in measles virus antibodies has also been associated with chronic active hepatitis (82). In this case, measles virus has been isolated from patients with chronic hepatitis (43, 83). Measles has also been associated with Paget's Disease and osteosclerosis (52, 105). However, no strong evidence has been established to support a direct link between measles and these diseases. In addition, measles virus vaccine has previously been implicated in autism but recent evidence from epidemiological study showed that there was no direct causal correlation (158).

1.4 Measles Immunization

The first attempt to immunize people against measles virus was performed by Home in 1749 when he directly introduced the virus into skin. He reasoned that the virus, which normally causes respiratory disease, would be less severe if it were inoculated through the skin. However, immunization of this kind was generally not effective (63). In the early twentieth century, attempts to generate a measles vaccine were also unsuccessful. The ability to cultivate measles virus in tissue culture provided a breakthrough towards the development of a measles vaccine. In North America, the inactivated vaccine and the widely used live-attenuated virus were produced from the Edmonston strain of the measles virus, which was grown in cultured cells.

The inactivated measles vaccine used in the United States was generated by treating the Edmonston strain of the measles virus with formalin (169). The vaccine produces moderate levels of neutralizing antibodies against measles, but offers limited protection since antibody titers decline very rapidly after immunization (41, 80). In addition, some individuals who have received the formalin inactivated measles vaccine are susceptible to the development of atypical measles when exposed to the wild-type measles virus (112). The first live attenuated measles vaccine, Edmonston B, was generated by sequentially passing the wild type Edmonston strain of measles virus in human renal cell, primary amnion cells and, finally chick embryo fibroblasts. When the Edmonston B vaccine was tested in monkeys, the animals did not exhibit viremia, nor did they develop symptoms associated with measles infection. Most importantly, the monkey produced measles virus antibodies, which offered protection against subsequent challenge by the wild type virus. However, the children who received this vaccine developed a fever and rash. Further passage of the Edmonston B measles through chick embryo fibroblasts in 1965 (Schwarz vaccine) produced an

even more attenuated virus which did not produce any symptoms in children (151). In recent years, the Schwarz vaccine has become the standard measles vaccine used throughout the world. In addition, other attenuated vaccine strains such as CAM, AIK-C and Shanghai-191 have been produced using similar approaches (127).

The effectiveness of the new live attenuated measles vaccine was enormous in the developed countries, such as the United States and Canada (10). The children in industrialized nations usually receive their first dose of measles vaccine between the age of 12 to 15 months. This vaccine decreased the incidence of measles infections by 95% in these regions (37). In the developing countries, measles vaccines are being administered to children of 9 months of age due to earlier exposure to the virus. However, the presence of maternal antibodies against measles virus in children of this age neutralized the attenuated vaccine before seroconversion was established in the children. In an attempt to yield measles virus immunity at an early age, higher vaccine titers (nearly 100 fold of the original dosage) were used. Although administration of the high titer measles vaccine did improve the seroconversion in young infants, the mortality rate of the children who received the vaccine also increased over the 2 to 3 years following the initial vaccination (39). The increased mortality was caused by the immunosuppressive nature of the high titer measles vaccine, which resulted in secondary infections such as pneumonia, diarrhea or parasitic infection (168). The immunity acquired after vaccination is not absolute. Therefore, in some instances, secondary vaccination is required to produce life long immunity (103, 125). In recent years, failures of the measles vaccine have also been reported in developed countries, such as the United States and Canada (10). Nearly 55,000 cases and 120 measles related deaths were reported in the United States between 1989 and 1991 (10). The failure of the vaccine is primarily due to the administration of a single dose vaccine instead of the recommended two dose regimen.

1.5 Measles Related Therapy

Although various medications have been used to reduce the severity of symptoms caused by an acute measles infection, there is still no effective antiviral treatment for measles. Historically, there were various studies suggesting that a high dose of vitamin A during an acute measles infection decreases the rate of morbidity and mortality (8, 25, 26, 46). Other therapeutic agents such as bromodeoxyuridine, azaguanin, interferon and ribavirin have been used for the treatment of SSPE (56). However, the effectiveness of these medications has been difficult to evaluate due to the reduced incidence of this disease.

Small hydrophobic peptides resembling the N-terminal amino acid sequences of the F_1 polypeptides of the fusion protein have been shown to inhibit the fusion activity of paramyxoviruses such as MV, Sendai virus, CDV, NDV, and SV5 as well as retroviruses such as HIV. One of these peptides, carbobenzoxy-D-phenylalanyl-L-phenylalanyl-L-nitroarginine was able to inhibit MV induced plaques and syncytia formations (106). However, the peptides did not seem to inhibit the binding of virus to cells since the virus still retained the ability to hemagglutinate monkey red blood cells in the presence of the inhibitory peptides (136). Experiments which tested the most effective amino acid combinations and conformational requirements for specific inhibition by these peptides have revealed that Z-D-Phe-LPhe-L-Gly is the most potent combination for inhibiting measles infection (137). In addition, our group has also demonstrated that the peptide's inhibitory effect does not involve disruption the specific interaction between measles virus with its cellular receptor but is related to changes in the heptad repeat region of the measles virus F protein. Thus, the peptide may impair the folding or activation of the fusion protein (Richardson, unpublished data).

Other approaches have also been investigated as potential measles virus therapies (88, 146, 153). Koschel *et al.* attempted to use measles anti-sense RNA

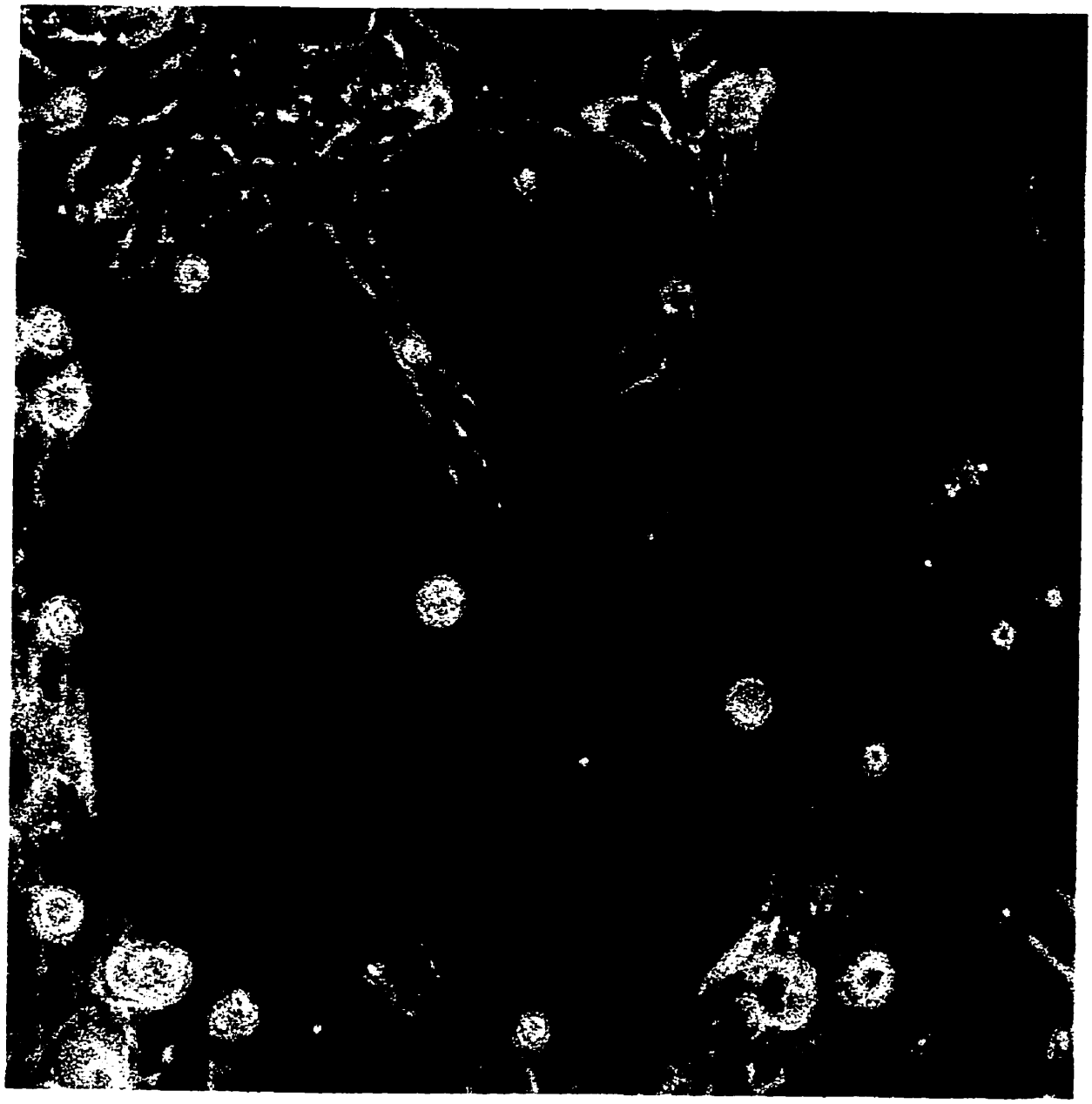
to cure cells persistently infected with measles virus. Using vectors expressing anti-sense mRNAs complementary to the measles nucleoprotein (N) or hemagglutinin (H) genes, they were able to show significant inhibition of both measles virus syncytia formation and virus release *in vitro* (88). In addition, Seya *et al.* and Sato *et al.* have generated both soluble CD46 and measles H proteins, which resulted in an inhibition of measles virus infection (146, 153).

1.6 Differences Between Wild-Type and Vaccine Strains of Measles

Confusion has arisen in distinguishing between wild-type and vaccine strains of measles virus. In recent years, the distinction between the two has been based upon the method of virus propagation in tissue culture following initial isolation of the pathogen from blood or throat swabs of measles infected patients. Historically, measles virus isolated from patients was grown by continuous passage through monkey kidney cell lines such as Vero and CV-1. However, "adaptation" after several passages of virus was required before cytopathic effects (syncytia) were observed in culture (Figure 1.2). Starting in the early 1990s, all new measles virus isolates were propagated in a marmoset lymphoblastic cell line, B95-8 (86). In this cell line, it is possible to grow the measles virus without a period of adaptation. Therefore, the wild-type strains of measles refer to those measles isolates that have been grown in B95-8 cells or human B lymphoid cell lines, whereas the vaccine strains of measles refers to virus that has been adapted to grow in either Vero or HeLa cell lines.

Figure 1.2 Syncytia formation of measles virus infected cells

Measels virus infected cells can be identified by the presence of cytopathic effect, called syncytia formation. In the measles virus infected cells, viral glycoproteins are expressed on their cell surface. The presence of H, F proteins and the viral receptor (CD46) can effectively promote fusion between infected cells and their adjacent cells resulting in syncytia formation.

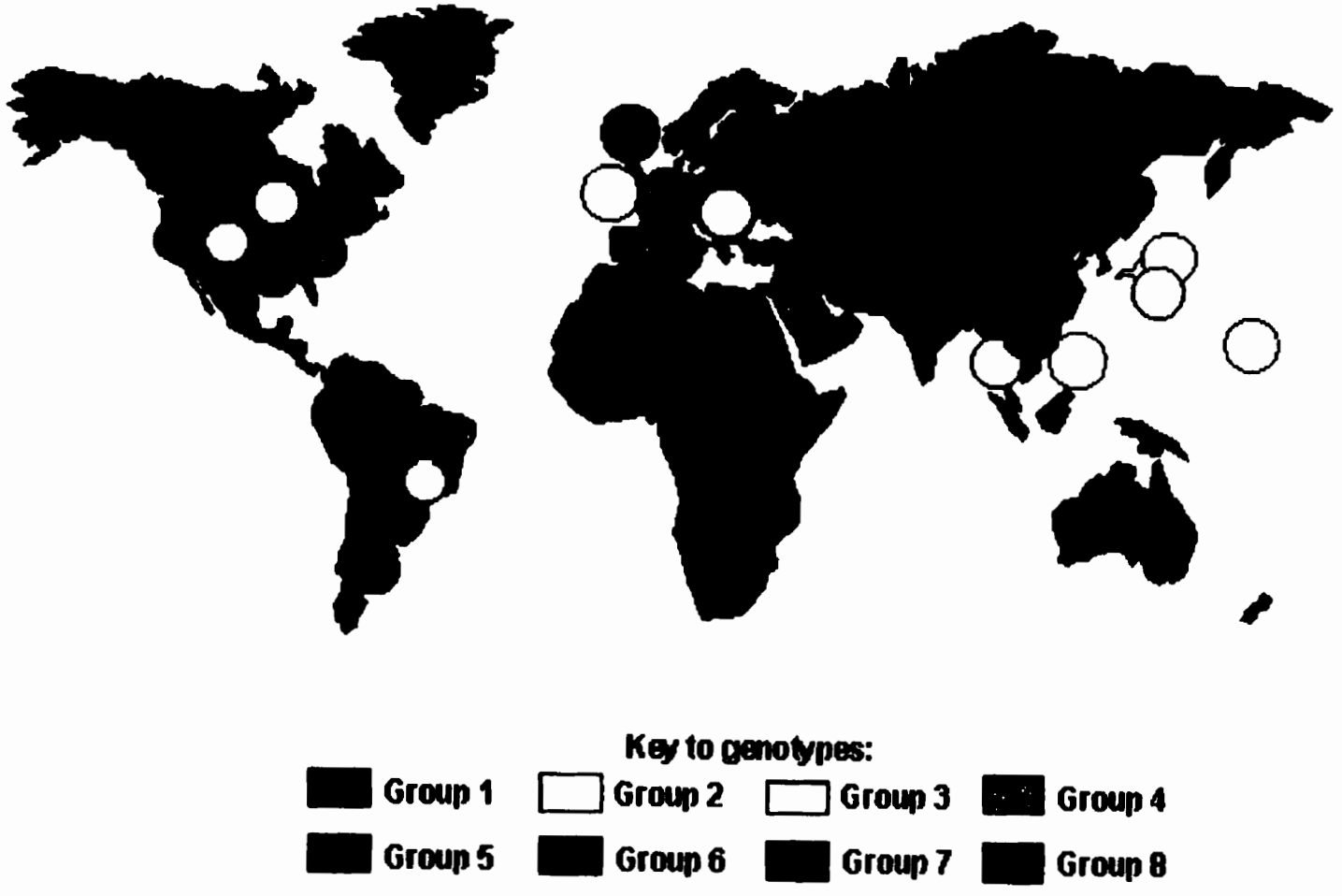


1.7 Genetic Diversity of Measles

Measles virus strains have recently been divided into eight distinct genotypes based on the gene sequences of the hemagglutinin (H) or nucleocapsid proteins (N) (Figure 1.3). These two genes are the most variable of the six measles virus genes. The carboxy terminus of the N protein can vary up to 12% at the nucleotide level. Since the extent of diversity among the measles strains still remain an enigma, the number of genetic groups will probably increase in the future. Group 1 of measles virus contains both the wild-type isolate and the vaccine strain of viruses. This group includes the original Edmonston strain of measles isolated in 1954 and the vaccine strain that was subsequently derived from the original Edmonston isolate. Most of the wild-type measles strains isolated from different regions of the world in the 1950s and 1960s are all part of group 1 (10). The group 1 measles virus was widely distrubuted prior to the administration of the measles vaccine. Currently, group 1 measles viruses are still circulating around the world. However, group 2 measles viruses were first isolated in Japan in the early 1980s. This group of viruses was responsible for an outbreak of measles that occured in the United States during 1989 to 1991. Representatives of group 2 include the Illinois-1 (Chicago-1) strain of measles virus, whose genome has almost been completely sequenced (10). Group 3 measles viruses were first isolated from Japan and Thailand, and were later transmitted to North America and Europe. This group has recently been shown to include the most predominant strains of measles. Groups 4 and 5 of measles are mostly found circulating in the western European nations. These viruses have caused sporadic outbreaks in North America as well. The viruses among group 6 appear to have a high level of genetic variability in comparison to the other groups of measles. However, specific nucleotides sequences found in group 6 of measles are indicative of an African lineage. Viruses from this group have been imported to North America from African nations such as

Figure 1.3 Global distribution of measles genetic groups

This figure is taken from **Bellini *et al.*, 1998.** Measles virus strains have recently been divided into eight distinct genotypes based on the gene sequences of the hemagglutinin (H) or nucleocapsid proteins (N). Colored circles indicate areas where measles viruses from various genetic groups have been isolated. Viruses not assigned to one of the eight groups are labeled in brown.


Americas, 1988-1997
Europe and Asia, 1988-1997
Key to genotypes:
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

Cameroon, Gambia and Kenya. Group 7 of measles was first isolated in Montreal, Canada in 1988. Recently, measles viruses from this group have also been identified in South Africa and Pakistan. Finally, group 8 of measles virus was isolated from China in the early 1990s. Currently, measles virus from this group is also circulating in other parts of Asia.

1.8 Measles Virus Structure and Functions

Measles virus is a member of the morbillivirus genus, a subgroup of the paramyxovirus family. Under the electron microscope, measles virus appears roughly spherical, but pleomorphic particles may vary from 300 nm to 1000 nm in diameter (98). It is an enveloped virus with a single-stranded negative sense RNA genome encoding 6 consecutive non-overlapping gene products. These polypeptides include the nucleocapsid protein (N) for viral RNA encapsidation, the matrix protein (M) for virion assembly, the fusion protein (F) for fusion between the viral membrane and the host cell membrane, the hemagglutinin protein (H) for cell attachment and the phosphoprotein (P) along with the large protein (L) for viral transcription and replication. The 4.5 X 10^6 kDa MV RNA genome (Figure 1.4) has been completely sequenced and its length is approximately 16000 nucleotides. This sequence may vary slightly depending on the strain of the measles (3, 6, 9, 11, 98, 135, 140). The viral envelope, a lipid bilayer, is derived from the host membrane, and contains two different integral glycoproteins, the F protein cleavage products (F1-40 kDa and F2-20 kDa) and the 78 kDa H protein (16, 135) (Figure 1.5). Immediately beneath the envelope lies a nonglycosylated 37 kDa M protein (53). The M protein is important for virion assembly at the cell membrane and subsequent viral budding. In measles infected cells, the M protein is thought to interact with the nucleocapsid and the inner surface of the cellular membrane, as well as the cytoplasmic tails of the transmembrane proteins (61, 163). In the

Figure 1.4 Measles virus genome

The measles virus genome is organized into 6 nonoverlapping genes that encode the 6 structural proteins, the nucleocapsid protein (N), the matrix protein (M), the fusion protein (F), the hemagglutinin protein (H), the phospho-protein (P) and the large protein (L). In addition, the P gene also encodes two nonstructural proteins V and C, which may mediate the transition between viral transcription and replication.

Gene size (ncts)

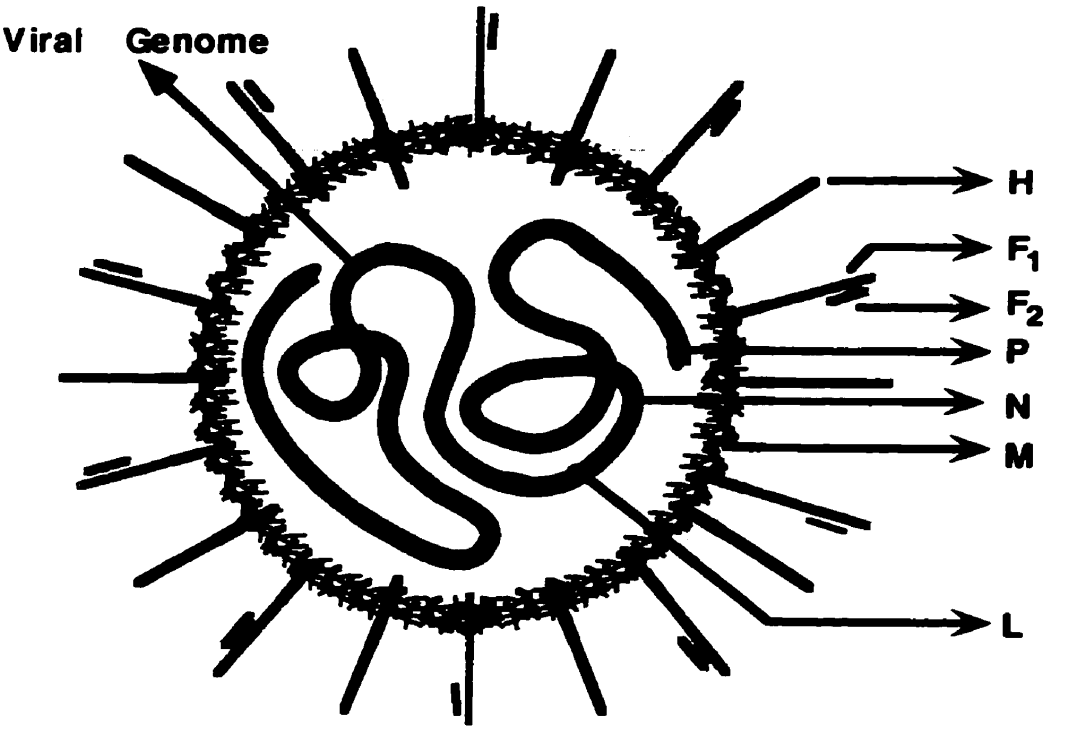
1688	1656	1468	2375	1960	6645

Protein size (kDa)

60	P-72 C-21 V-40	36	60	78	200

Figure 1.5 Structure of measles virus

Measles virus appears roughly spherical, but pleomorphic particles vary from 300 nm to 1000 nm in diameter. Measels virion consists of 6 different viral proteins: Nucleocapsid (N) is responsible for viral RNA protection, the matrix protein (M) is for virion assembly, fusion protein (F) is responsible for fusion between viral envelope and host cell membrane, hemagglutinin (H) is for cell attachment, phospohoprotein (P) and large protein (L) are for viral transcription and replication.


Viral Genome
H
F1
F2
P
N
M
L

virion, the viral RNA associates with the 60 kDa N protein, the 72 kDa P protein and the 250 kDa L protein (9, 11). The N protein has two distinct functions: The N protein associates with the viral RNA genome to protect the viral RNA from nuclease digestion. Encapsidation of the viral genome by the N protein is especially significant since experimental evidence has demonstrated that naked full length viral RNA cannot be used as an infectious agent (92). Next, an association between the viral genome, viral mRNA, with the N proteins forms a nucleocapsid complex, which is essential for the transcription of viral proteins and replication of the viral genome (164, 165). Measles virus transcription and replication also require the presence of the P and the L proteins, which form the viral polymerase (134). The P protein is probably involved in the regulation of transcription and replication while the L protein possesses the catalytic domain of the RNA dependent RNA polymerase (92). In addition to these six structural proteins, there are two nonstructural proteins that are only found in infected cells. These are the 21 kDa C protein and 40 kDa V protein which are both expressed from the P gene using overlapping reading frames. The C protein is translated from the same mRNA as the P protein but uses an AUG start codon 19 nucleotides downstream (9). The V protein shares the first 231 amino acids with the amino terminus of the P protein. However, an extra non-template directed guanosine residue inserted into the mRNA at position 751 results in a frame shift mutation and the addition of 68 amino acids at the carboxyl terminus of the V protein (23). The function of these two proteins is still unclear, but they may also be involved in the regulation of transcription and replication. In addition, recent evidence suggests that the V protein may interact with the cellular DNA repair protein, DDB1/XPE (94). Future studies are required to elucidate the functions associated with the V protein.

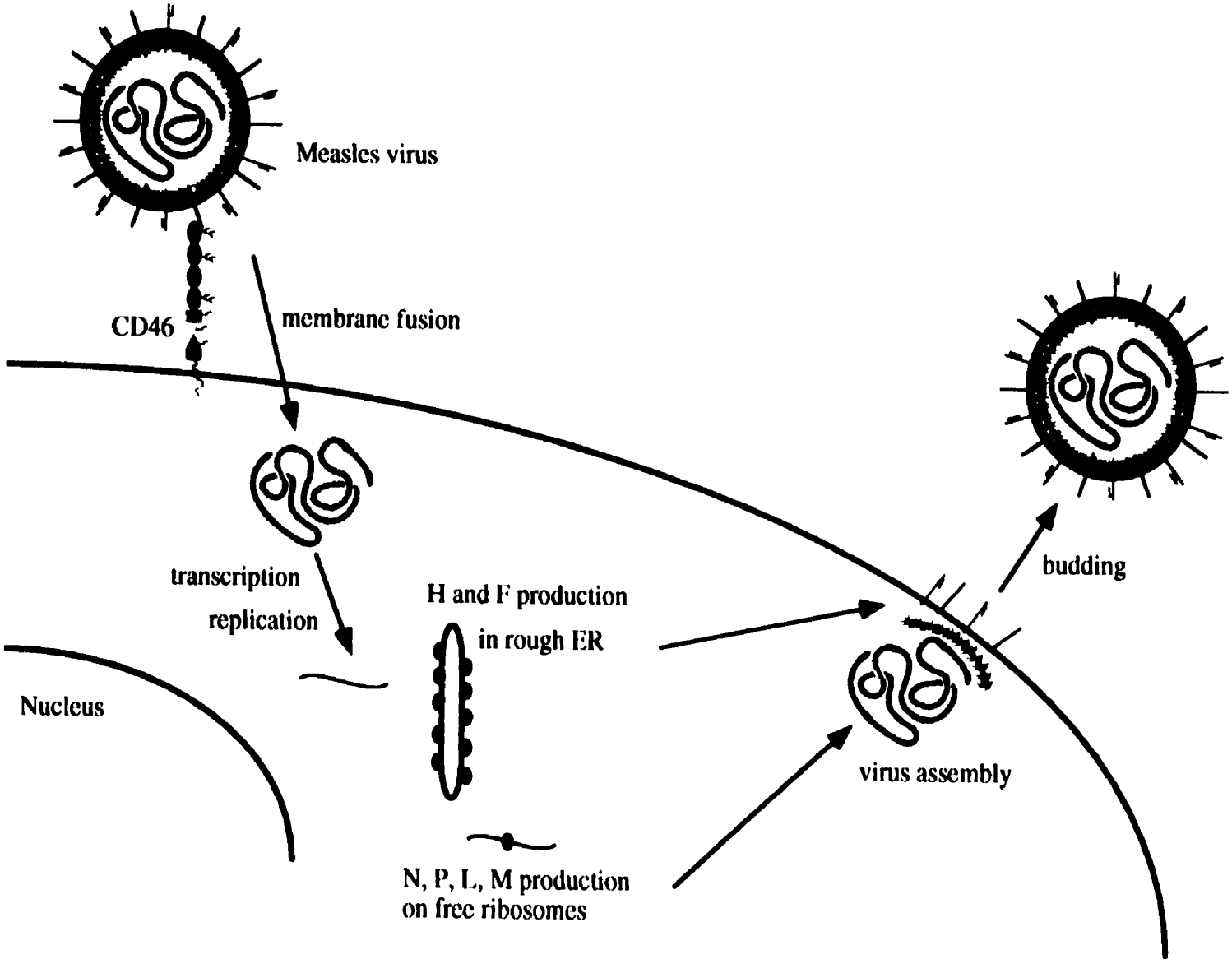
1.9 Measles Virus Life Cycle

1.9.1 Overview

Measles virus attaches to a specific receptor on the cell membrane of host cells via the H protein (Figure 1.6). This is followed by viral penetration of the cell membrane, a step which is mediated by the F protein. The F protein also mediates syncytia formation between infected and non-infected adjacent cells. Although over-expression of F protein alone is sufficient to form syncytia (167), recent evidence suggests that the presence of both H and F proteins in close proximity are required for efficient fusion to occur (100, 173). Following fusion of the viral and cell membranes, the viral genome along with its associated proteins, are injected into the cytoplasm of the host cell where viral transcription and replication take place. In the infected cell, viral transcription occurs first, since synthesis of viral proteins is required for replication of the genome. The components required for transcription are present either in the nucleocapsid core or are provided by the host cell cytoplasm. The primary transcripts are mainly monocistronic and are not encapsidated by the N protein. Most of the structural and nonstructural proteins are produced on free ribosomes found in the cytoplasm. However, the H and F proteins are translated and modified in the ER and Golgi, and are subsequently transported to and inserted into the plasma membrane. The replication of the viral genome in the cytoplasm immediately follows the transcription and translation of the viral proteins. In replication, synthesis of full-length positive sense and negative sense viral genome occurs by using machinery similar to that in transcription. The only difference between transcription and replication is that the encapsidation of the viral genome by the N proteins occurs immediately after the replication process. The encapsidation of the viral genome is essential for the subsequent viral assembly. Virion assembly occurs in two steps: First, the N

Figure 1.6 The measles virus life cycle

MV enters the host cells via interaction between the H protein and the cellular receptor, CD46. Subsequently, fusion occurs between the two membranes. Transcrition of the viral proteins and replication of the viral genome occur in the cytoplasm. Two glycoproteins, H and F, are translated in the rough endoplasmic reticulum (ER) and transported to the cell membrane. The virus is assembleed at the cell membrane and buds out of the infected cells.


Measles virus
CD46
membrane fusion
transcription
replication
H and F production
in rough ER
Nucleus
budding
virus assembly
N, P, L, M production
on free ribosomes

proteins encapsidate the RNA genome, and the P and L proteins are assembled into the nucleocapsid core. Second, the nucleocapsid cores interact with the M proteins and acquire the H and F glycoproteins from the cell membrane during the process of budding.

1.9.2 Initial Steps of Measles Infection

Measles virus infects a wide range of human cell types including lymphocytes, macrophages, and epithelial and endothelial cells of the lung, skin, kidney, liver, and gastrointestinal tract (104). In addition, it is well established that several species of monkey erythrocytes, but not human erythrocytes, can be agglutinated by measles virus (31, 114). Rodent cells, on the other hand, appear to be resistant to measles virus infection. Therefore, it appears that the viral receptor for measles virus is widely distributed over different human tissues with the exception of erythrocytes. Our lab and another group have identified CD46 (membrane cofactor protein) as the primary high affinity receptor for the vaccine strain of measles virus (31, 114). Two measles glycoproteins, H and F, are involved in specific host recognition and viral entry. The H protein is a type II glycoprotein with a molecular weight of 78 kDa. The H protein contains 4 to 5 different N-glycosylation sites and is expressed as a homotetramer on the viral envelope or on the surface of the infected cell (100, 121). Glycosylation of the H protein is important for proper folding, antigenicity, oligomerization, and transport from the Golgi to the cell membrane (75). Unlike other viruses from the *Paramyxoviridae* family, the H protein of measles does not have neuraminidase activity. Therefore, the primary role of the H protein is to bind to specific cell surface proteins and to initiate viral entry. Thus, the H protein may be important in determining the host tropism of measles virus. The precise receptor binding sites of

the H protein are still unknown although antibody inhibition studies and site-directed mutagenesis studies have indicated specific regions comprised of amino acids 126-135, 309-318, 491-552, and 587-596 may be important for this interaction (49, 76, 141, 156).

The fusion protein is expressed as an inactive precursor protein (F_0) with a molecular weight of 60 kDa. Similar to the F proteins of other paramyxoviruses, the measles virus F protein is a type I glycoprotein which is translated and modified in the rough endoplasmic reticulum and the Golgi. The F protein is expressed as a homodimer on the infected cell surface. In the Golgi, the F_0 precursor is cleaved by furin, a host cell protease, into its active, disulfide-linked F_1 (40 kDa) and F_2 (20 kDa) forms. Cleavage occurs within the sequence: NH_2-Arg-Arg-His-Lys-Arg/Phe-Ala-Gly-COOH. The new amino terminus of the F_1 contains a stretch of highly hydrophobic region, the fusion peptide, which is conserved among the F proteins of the paramyxoviruses (135). This cleavage is essential for producing a functional F protein and initiating membrane fusion and syncytia formation. Lymphoblast cell lines devoid of the cellular protease fail to cleave F_0 and do not produce syncytia upon measles virus infection (44). However, absence of F protein cleavage does not appear to inhibit incorporation of the F protein into the mature measles virion, although this virus appears to be non-infectious. The F protein also possesses a cysteine-rich region that is important for the interaction between the H and F proteins as demonstrated through studies with chimeric F proteins prepared from canine distemper and measles viruses (172). It also has an amphipathic α helix, or a leucine zipper, which may play a role in fusion process. This is supported by studies showing inhibition of the fusogenic activity when mutations are introduced at the leucine zipper region (17). The cytoplasmic tail of the F protein is another region that is highly conserved among morbillivirus F proteins. The cytoplasmic tail of the F protein may be involved in interactions with

the matrix protein since mutations or deletions of the tail can prevent budding of measles virions (22).

Furthermore, in vitro studies using an over-expression system such as the vaccinia system, appear to indicate that the presence of both H and F proteins are required for effective fusion to occur (159-161, 173). Similar experiments with other paramyxoviruses such as CDV and Sendai virus have confirmed the importance of specific interactions between both glycoproteins for the process of fusion (36, 72, 108, 145). Nevertheless, our laboratory has shown that over-expression of the F proteins alone using either the adenovirus, vaccinia or baculovirus systems, can also cause fusion and syncytia formation, although the process occurs at a much slower rate (data not shown)(5, 167).

1.9.3 Measles Virus Transcription and Replication

Measles virus transcription and replication occurs in the cytoplasm of the infected cells utilizing an RNA polymerase which co-purifies with the virion (66, 67). The viral polymerase is believed to be composed of the L and P proteins. The L protein is a very large protein with a molecular weight of 200 kDa, and may contain the catalytic domain of the RNA polymerase complex. Although there is no direct evidence defining its precise function, the L protein of measles virus has homology with the L protein of other negative strand RNA viruses and by analogy appears to contain mRNA methyltransferase and polyadenylation activities (11, 129). In addition, the presence of only a small amount of L protein in the measles nucleocapsid core is consistent with the situation found in other viruses (92). Another protein which is present in the measles RNA polymerase complex is the 72 kDa phosphoprotein, denoted as P protein. The functional significance of the P protein has also not been clearly defined. However, studies of P proteins from other paramyxoviruses indicate that phosphorylation at specific sites of the P

protein is required in order for transcription to occur (7). Furthermore, phosphorylation of the P protein does not depend on other viral proteins since P proteins expressed independently in cell lines are phosphorylated effciently (2, 4). Additional studies with either Sendai virus or NDV have demonstrated that viral transcription can not be performed by L protein or P protein alone. Rather, the presence of both proteins is required for this specific task (57, 65).

1.9.3.1 Viral Transcription

Transcription of measles virus starts at the 3' terminus, with the synthesis of a 55 bp (+) strand of leader sequence by the measles RNA polymerase (67). The initial steps of measles viral transcription appear to be similar to other non-segmented negative strand RNA viruses (134). Following synthesis of the leader, the polymerase transcribes the remaining genes sequentially from the 3' end to the 5' end of the viral genome. The polymerase appears to detach itself from the nucleocapsid at the intergenic regions, and the frequency of this detachment appears to correlate with the increased of distance from the promoter. This detachment occurs at each gene junction and tightly controls the levels of mRNA production. Therefore, less and less mRNA is produced as the polymerase transcribes to the 5' end of the genome (92). The amounts of viral mRNA transcripts present in the measles virus infected cells relative to the frequency of the N mRNA are as follows: N (100%), P (81%), M (67%), F (49%), and H (39%) (20, 21, 150). Viral mRNAs are capped at the 5' ends with 7mGpppAm by an unknown mechanism (177). The 3' ends of the viral mRNA are polyadenylated through a mechanism of polymerase slipping or stuttering. In the process of slipping, the polymerase repeatedly copies 4 to 7 U's following the polyadenylation signal (87). However, the polymerase can continue this process of reinitiation, transcription, and termination until the last viral gene, L, is transcribed.

Transcription of the measles viral genome can sometimes result in production of polycistronic mRNA. Complementary studies with the polycistronic mRNA indicate that the intergenic regions of the mRNA are identical to the genomic intergenic sequences (20, 33, 34, 109, 175). In addition, mRNA editing of the P gene generates a second mRNA transcript (23, 87, 162). In infected cells, half of the P mRNA is identical to the P gene and the other half contains an extra nucleotide (G) which is inserted into a conserved sequence during transcription (23). The extra G causes a shift in the reading frame allowing for the inclusion of 68 amino acids which forms a cysteine rich domain with Zn^{+2} binding properties (95). This altered mRNA translates into a protein with the same 231 amino acids as the N-terminus of the P protein and a completely different carboxy terminus. Different paramyxoviruses contain this conserved sequence which supports the mRNA editing mechanism (23).

1.9.3.2 Viral Replication

Most of the information regarding the transition from transcription to replication of measles virus has been derived from experiments conducted with other nonsegmented viruses such as Sendai virus and VSV. The shift from transcription to replication depends largely on viral protein synthesis. In the case of Sendai virus, infected cells treated with drugs that inhibit protein synthesis were able to synthesize viral mRNA but viral replication appeared to be inhibited (138, 139). The enzyme used for viral replication is essentially the same polymerase complex used for viral transcription with the addition of phosphorylated N protein (7, 12, 13).

The first step of viral genome (-ve sense) replication requires the formation of a (+ve sense) antigenome. The amino terminus of the N protein interacts with the P protein and forms a soluble complex, which is utilized for the encapsidation of

the leader sequence of the genome (51, 66). Accumulation of N/P complexes causes the polymerase complex to ignore the intergenic sequences and to produce a full length antigenome (92). Conversely, when insufficient amounts of the N/P complex are present, the viral polymerase complex transcribes either monocistronic or polycistronic mRNAs which are used for the production of viral proteins. Finally, production of the (-) viral genome is accomplished through a similar process as the synthesis of the antigenome using antigenome RNA as the template for the polymerase (92). The negative strand viral genome of measles virus is subsequently packaged into infectious virions.

1.10 Measles Virus Receptor

Many approaches have been used to identify the cellular receptor for measles virus. However, most attempts have resulted in failure. The binding between measles virus and the cellular receptor was found to occur at a neutral pH and at 37° C. One of the early attempts to isolate the receptor involved a measles inhibition assay using soluble protein obtained from monkey erythrocytes (42). Although 2 glycoproteins, 20 kDa and 30.6 kDa, were identified from Vero cells as possible candidates for the viral receptor, subsequent experiments have yielded inconclusive results (89). Another candidate that has been suggested to be a measles virus receptor is the substance P-binding protein (58, 59). However, limited expression of this protein in neuronal tissues indicated that it was probably not the high affinity receptor for measles virus since this pathogen can infect a wide variety of different human tissues and cell types (54). Finally in 1992, a specific monoclonal antibody directed against a human cell surface glycoprotein was identified that can inhibit measles virus infection. (115). This antibody immunoprecipitated two proteins from a HeLa cell lysate with molecular weights of

57 and 67 kDa. Similar results were obtained when measles virus bound to a radiolabelled HeLa cell lysate were immunoprecipitated with anti-H antibodies (32). CD46 has been subsequently identified as the measles virus high affinity receptor by two independent groups (31, 113). Naniche *et al.* immunoprecipitated the receptor using the monoclonal antibody and microsequenced the amino terminus of the receptor protein (113). Simultaneously, Dörig *et al.* observed that measles virus cannot bind to rodent cells (31). Using rodent-human somatic hybrid cell lines in measles virus binding assays, mouse cells containing the q arm of human chromosome 1 were found to support measles virus binding. Since CD46 is the only gene on chromosome 1 coding for proteins of 57 and 67 kDa and is expressed in most human cells (except erythrocytes), it was considered as a likely candidate for the measles virus receptor. The CD46 gene was expressed in Chinese hamster ovary (CHO) cells and its presence on the cell surface was confirmed by flow cytometry using an antibody against CD46. The rodent cells expressing human CD46 supported measles virus binding and infection and exhibited virus-mediated cytopathic effects (113). The distribution of CD46 molecules in human tissues is almost ubiquitous. CD46 is located on all of the major organs as well as cells endothelial, epithelial cells, fibroblasts, T and B cells, monocytes, granulocytes and platelets. However, CD46 is not present on the surface of human erythrocytes. CD46 is also expressed on the surface of primate cells lines, such as HeLa, Vero, CV-1, T cell leukemia and B cell lymphoma cell lines. Even though measles does not cause systemic disease in rodents due to lack of CD46 molecules, neurotropic strains of measles virus have been shown to induce a neuronal infection in the brains of hamsters, mice and rats, suggesting that these strains of virus may use another receptor in the brain of these animals (18, 93, 123).

1.10.1 Cellular Function of CD46

Membrane cofactor protein (MCP) or CD46 is a member of the gene cluster coding for regulators of complement activation found on the human chromosome 1 (96, 97). The complement system is a part of the immune response used by vertebrates to defend themselves against pathogens. The complement system consists of two different pathways: the classical and alternative pathways. In both pathways, activation of the complement cascade involves several proteolytic factors such as C3b and C5b and the formation of the membrane attack complex (MAC) on the surface of foreign cells. The final consequence of complement activation is osmotic lysis or opsonin-mediated phagocytosis of foreign particles (1). In addition, complement components can also form immune complexes and act as opsonins, which result in phagocytosis of foreign cells by macrophages. Unfortunately, unchecked complement activation or nonspecific deposition of complement complexes such as C3b or C4b on host cells can also lead to autoimmune destruction. In order to prevent this effect, the complement system is self-regulated by a number of serum and membrane bound complement regulatory proteins which block self-destruction by complement activation. There are several regulators of the complement cascade including decay accelerating factor (CD55), complement receptor 1 (CD35), complement receptor 2 (CD21), inhibitor of reactive lysis (CD59), C4b binding protein, Factor H and MCP (CD46). CD46 normally serves as a cofactor for the serine protease, factor I. Interaction between CD46 and complement components C3b and C4b on the host cells permits factor I to cleave these factors, inhibiting formation of the attack complex. Therefore, CD46 plays a critical role in protecting host-tissue from complement mediated damage.

1.10.2 Structure of CD46

The CD46 gene consists of 14 exons and 13 introns and spans more than 43 kb of genomic DNA (Figure 1.7a) (96, 97). CD46 is a type I membrane bound glycoprotein with a 34 amino acid signal peptide at the amino terminus which is subsequently removed during protein maturation in the endoplasmic reticulum. The extracellular portion of the protein consists of 4 domains of short consensus regions (SCR1-4), ser/thr/pro rich sequences (STPA, STPB and STPC), and a protein segment of undefined function (U). CD46 also has a hydrophobic transmembrane domain and a cytoplasmic carboxy-terminal tail (Figure 1.7b). There are N-glycosylation sites on SCR1, 2, and 4 while the STP region contains O-glycosylation sites. Differential glycosylation and alternative splicing of the STP region as well as the cytoplasmic tail gives rise to 4 different isoforms of CD46 consisting of 2 populations of the glycoprotein with molecular weights ranging between 51-58 kDa and 59-68 kDa. Specific isoforms of CD46 have been identified in the salivary gland, kidney and brain. Nevertheless, most tissues express all isoforms. Recently, the 3-D crystal structure of the SCR1and 2 domains of CD46 protein was solved (Figure 1.8) (19). The structure of each domain folds into β-barrels, which consists of antiparallel β-sheets joined together by 2 disulfide bonds. The hinge region of the SCR1 and 2 domains creates a concave groove and provides limited perpendicular movement between the two domains.

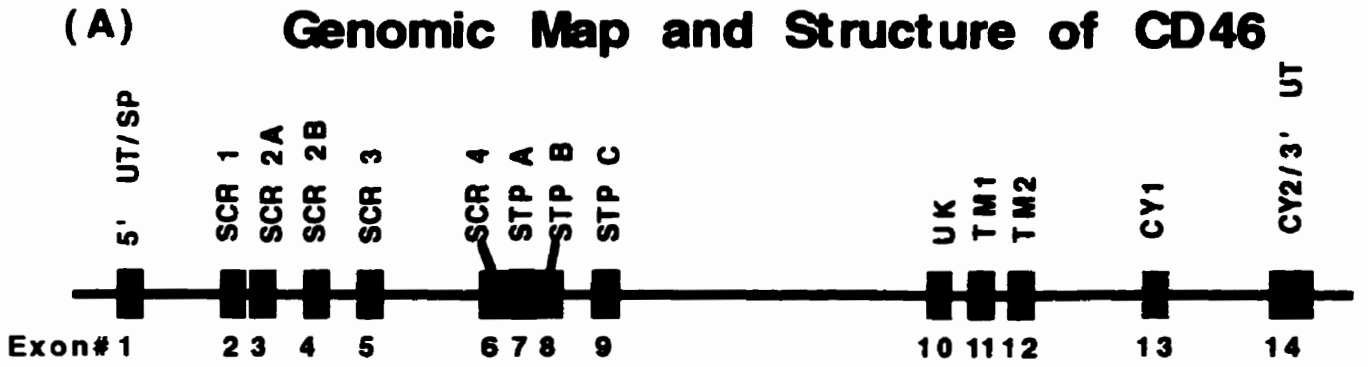
1.10.3 Interaction Analysis of CD46 and Hemagglutinin Protein

Since the discovery that CD46 is the receptor for the measles virus, extensive studies have been conducted investigating the nature of the interaction between CD46 and the hemagglutinin protein of measles. Early experimental evidence has indicated that all the isoforms of CD46 can be utilized as the receptor

Figure 1.7 Genomic Map and Sturcture of CD46

(A). CD46 gene consists of 14 exons and 13 introns and spans more than 43 kb on the genomic DNA.

(B). CD46 is a type I glycoprotein with 34 amino acid signal peptide at the amino terminus. The signal peptide is subsequently removed in the ER as the protein matures. The extracellular portion of the protein consists of 4 domains of short consensus repeats (SCRI-IV) a ser/thr/pro rich sequence (STP). and region of undefined function (U). CD46 also has a transmembrane hydrohobic domain and cytoplasmic tail.


(A)
Genomic Map and Structure of CD46
5' UT/SP
SCR 1
SCR 2A
SCR 2B
SCR 3
SCR 4
STP A
STP B
STP C
UK
TM1
TM2
CY1
CY2/3' UT
Exon# 1 2 3 4 5 6 7 8 9 10 11 12 13 14

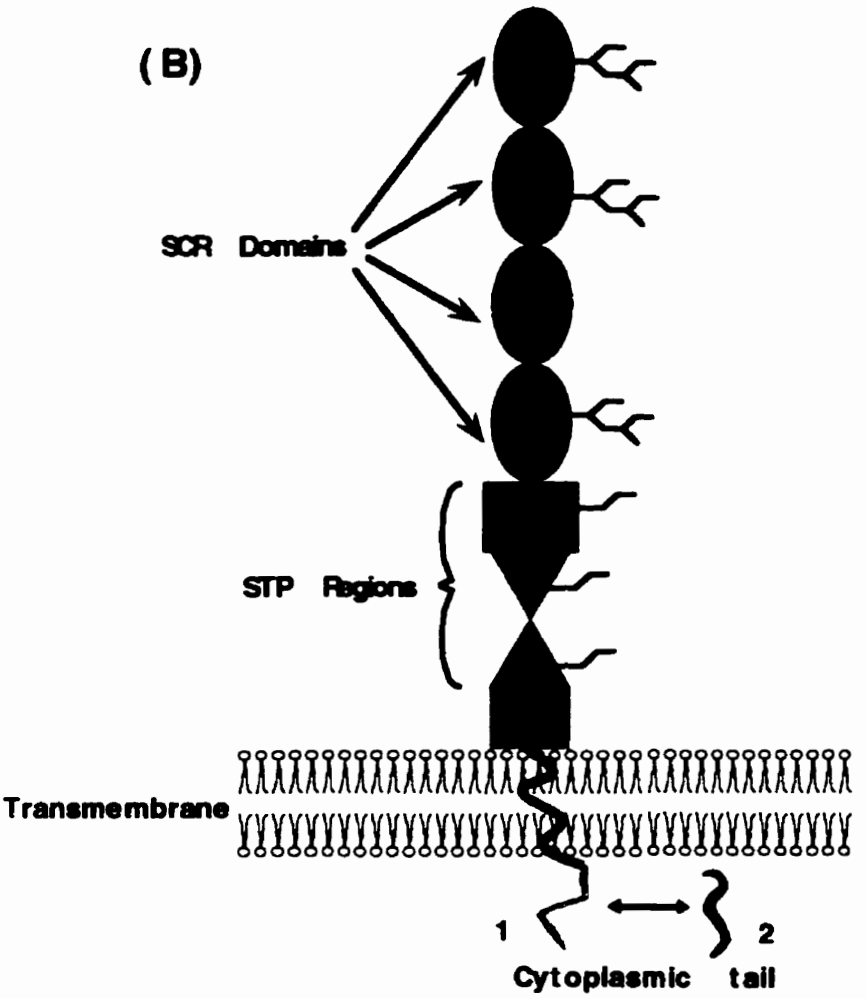
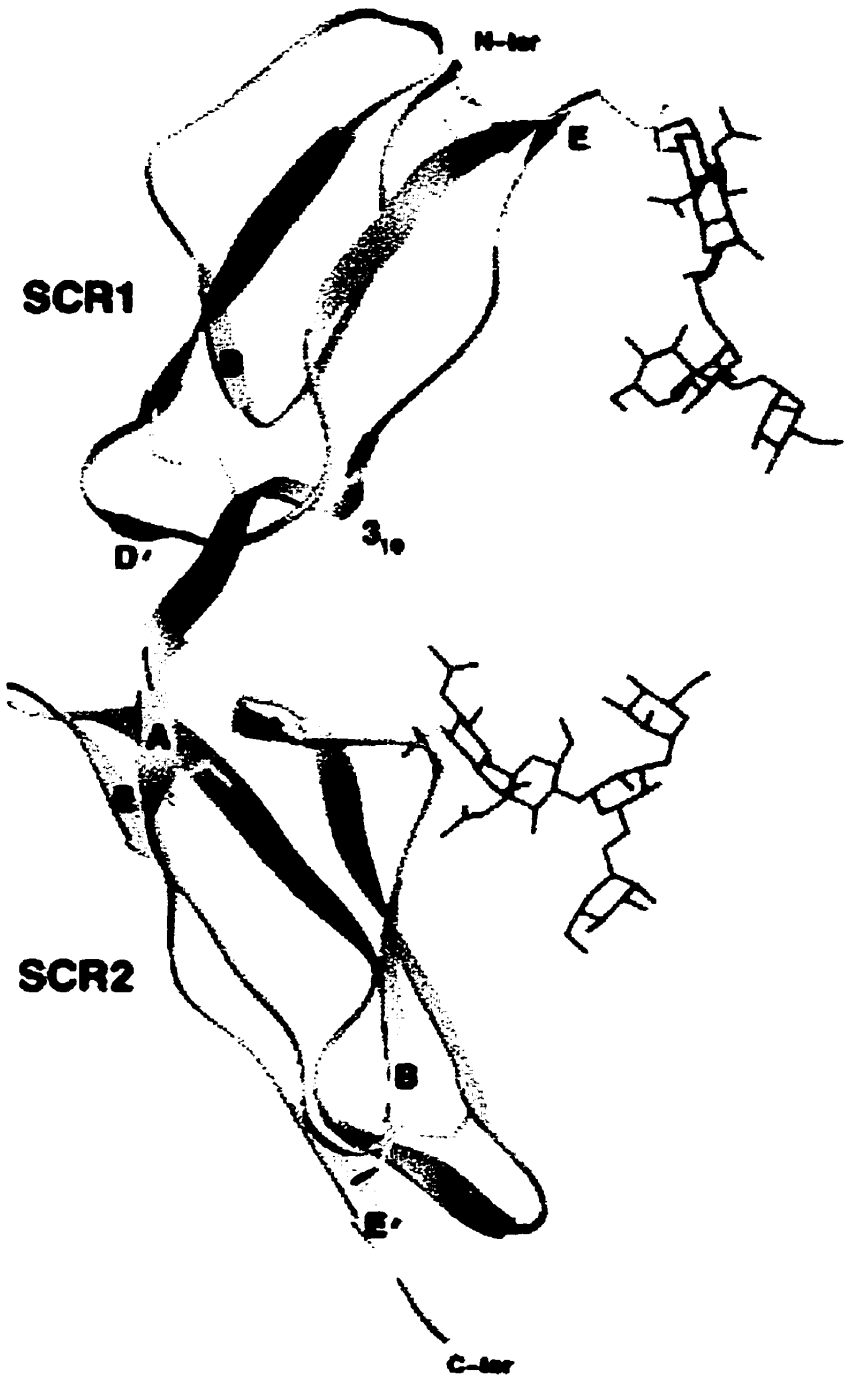

(B)
SCR Domains
STP Regions
Transmembrane
1
2
Cytoplasmic tail

Figure 1.8 Crystal structure of the N-terminal two short consensus repeats SCR1 and SCR2 of CD46

This figure is taken from **Casasnovas *et al.*, 1999.** The overall structure of each domain folds into a β-barrels, which consists of antiparallel β-sheets joined together by 2 disulfide bonds. The hinge region of the SCR1 and 2 domains creates a concave groove and provides limited perpendicular movement between the two domains. Disulfide bonds and carbohydrate residues are shown in yellow and red, respectively.


N-ter
E
SCR1
D'
3_{10}
A
B
SCR2
B
E'
C-ter

for measles and can sustain viral infections and syncytia formation (48, 101). Furthermore, the cytoplasmic tail and the transmembrane domain of CD46 can be replaced by a glycosyphosphatidylinositol anchor sequence without hampering its ability to function as the measles virus receptor. Soluble CD46 molecules composed of only the extracellular regions can partially inhibit measles virus infection of CHO cells expressing human CD46 as well as rosette formation with green monkey erythrocytes (29, 153, 166). These results imply that the regions of CD46 responsible for attachment to the measles H protein are most likely located within the SCR domains. Additional evidence obtained from a virus overlay protein binding assay (VOPBA) and functional studies of CD46 expressed in the baculovirus system has revealed that disulfide bonding and N-linked glycosylation of CD46 molecules are crucial for measles virus binding (73, 99). These results indicate that a successful measles virus infection requires conformational integrity of the CD46 SCR domains. Subsequently, studies obtained using either CD46/CD55 hybrid proteins or CD46 deletion mutants have mapped the binding regions to the SCR1 and SCR2 domains (77, 102). In addition, some but not all of the monoclonal antibodies specific for either the SCR1 or 2 domains can inhibit virus binding and infection (73, 74, 77, 102). The binding regions for measles virus to CD46 appears to be distinct from the complement proteins C3b and C4b, which bind to the SCR2, 3 and 4 domains of CD46. The interaction between CD46 and measles H protein has also been depicted as essential for the subsequent fusion event. Using a vaccinia expression system, Nussbaum *et al.* showed that co-expression of CD46, H and F were required in order to render effective syncytia formation (120). Furthermore, Buchholz et al. demonstrated that the distance between the SCR1 and 2 domains of CD46 and the cell membrane can affect its binding to the measles H protein and fusion efficiency (15). Using CD46 and CD4 hybrid proteins, Buchholz *et al.* showed that increasing the distance between the

MV binding sites and the cell membrane enhanced virus binding but reduced fusion activity (15). Recently, Seya *et al.* demonstrated, through the use of CD46 and CD55 chimeric molecules, that the transmembrane domain of CD46 is essential for efficient syncytia formation (153). However, our data suggest that measles fusion does not necessarily require the presence of CD46. Fusion can also be observed by over-expressing measles H and F proteins using a vaccinia expression system in 3T3 mouse fibroblasts which do not synthesize CD46 (data not shown). Althrough syncytia formation is less efficient in these cells it implies that another mouse protein can substitute for CD46 when large amounts of H and F are present in some rodent cells. It may also be possible that measles virus can gradually adapt to use other receptors on cultured cells, a hypothesis which is supported by virus which has been propagated in chicken embryonic fibroblast cell line (38).

1.11 Additional Studies Involving CD46 and Measles

1.11.1 Is Moesin a Co-Receptor for Measles?

A year after the discovery of CD46 as the cellular receptor for measles virus, another cellular molecule, moesin, was also implicated as part of the receptor complex for measles virus. Moesin is a member of the ezrin, radixin, and moesin (ERM) family that forms part of the cytoskeleton network. The majority of moesin is located in the cytosol of the cell associated with the plasma membrane. However, a very small portion of moesin is exposed to the cell surface. Similar to CD46, moesin is widely distributed in all tissues. In contrast to CD46, moesin is highly conserved among different mammalian species. Using specific monoclonal antibodies directed against moesin, Dunster *et al.* showed that measles virus binding and fusion are inhibited (35). In addition, moesin and CD46 were co-immunoprecipitated when chemical cross-linkers were used (149). The two

molecules were also shown to be in close proximity using the electron microscope. All this evidence seemed to stipulate the involvement of both proteins as a measles receptor complex (149). However, Doi *et al.* have recently put the significance of moesin as part of the measles receptor into question (30, 38). Using moesin-negative mouse ES cells, this group demonstrated that cells with or without moesin, could not sustain a measles infection without the presence of CD46. The only difference between the moesin positive and the moesin negative mouse ES cells was the features of syncytia formation. CD46 positive and moesin negative ES cells infected with measles virus appeared to have fragmented syncytia formation. This was probably due to the lack of proper cytoskeletal organization (30).

1.11.2 CD46 Transgenic Mice

Small animal models permit researchers to better understand different aspects of a normal viral infection such as viral infectious cycle, host range and tropism, viral interactions with the immune system, and a potential screening mechanism for antiviral treatment. Receptors for other human viruses, including poliovirus and HIV have been identified and subsequently introduced into small animals that were normally not susceptible to the virus. In the case of measles virus, rodents, including rats, mice, and hamsters, are not normally susceptible to MV infection. Following the discovery of CD46 as the measles receptor, many laboratories, including ours, engineered transgenic mice, to express the human CD46 protein. Our transgenic mice ubiquitously expressed CD46 under the control of a β-actin promoter. RT-PCR and FACS analysis performed on different tissues and the lymphocytes of the transgenic mice have confirmed the expression of CD46. However, two attempts to infect these mice did not result in a normal MV infection (Richardson, unpublished). Other CD46 transgenic mice generated by different laboratories have also yielded similar results (71, 110, 132). In addition,

CD46 transgenic rat also demonstrated similar results upon measles virus infection (118). Nonetheless, Horvat *et al.* were able to demonstrate limited measles viral replication of CD46 positive primary cell lines derived from the transgenic mice. The failure of the CD46 transgenic mice to sustain a measles virus infection indicates that additional host restrictions, such as a block in viral replication may be present in rodents.

1.11.3 Immunosuppression associated with measles and CD46

In 1908, a physician, von Pirquet, recognized that measles infection resulted in the loss of skin test reactivity to tuberculosis, and that delayed-type hypersensitivity (DTH) is inhibited for several weeks (von Pirquet, 1908). Although different aspects of measles virus induced immunosuppression have been studied, the nature of immunosuppression caused by measles still remains an enigma to the immunologists. These investigations have included mitogen induced T cell proliferation studies and direct infection of lymphoid cells such as nature killer cells (55, 168). The discovery of CD46 as the measles virus receptor has led to the speculation that CD46 may be involved in the mechanism of measles induced immunosuppression. Karp *et al.* has proposed that interleukin-12 (IL-12), a potent activator of cell-mediated immunity, expressed in monocytes and macrophages, is inhibited during measles infection (81). Furthermore, they have demonstrated that the mechanism associated with inhibition of IL-12 production is mediated through CD46 since both measles virus and monoclonal antibody cross-linking of CD46 can result in a decrease of IL-12 production *in vitro* (81). Finally, Karp *et al.* speculate that the measles virus interaction with CD46 may be a plausible mechanism for measles induced immunosuppression. In addition, Yant *et al.* have identified a specific amino acid sequence, Tyr-X-X-Leu, located in the cytoplasmic domain of

CD46 molecules, which is critical for the down-regulation of CD46 in measles virus infected cells (176). They also speculated on potential signal-transduction and immunosuppression mechanisms associated with MV-induced CD46 down-regulation (62, 176). However, a recent publication by Galbraith et al. showed that other morbilliviruses, such as rinderpest virus (RPV) can also down regulate CD46 in infected monocytes although RPV does not utilize CD46 as the receptor for viral entry (47). This finding may suggest that downregulation of CD46 is not directly related to the measles virus interaction with CD46.

1.12 Research Objectives

During the initial stages of infection, many similarities can be observed between measles virus and other deadly viruses such as HIV and EBV (116, 117). Measles virus interaction with its cellular receptor, CD46, appears to be a good model for studying the early events of virus infection, attachment to, and penetration of the host cells. In addition, CD46 is also an interesting molecule in its own right. CD46 expression is up regulated on tumor cells, and it has been suggested to have other cellular functions separate from its role as a complement regulator and a viral receptor. In addition, a mouse CD46 homologue was recently identified in testicular tissue. The discovery of this homologous molecule may help to elucidate other functional roles of CD46 in normal and tumor cells. In the following chapters, characterization of the specific regions of CD46 responsible for its interaction with measles virus H protein was investigated using site-directed mutagenesis and a novel pseudovirus-binding assay. In chapter 2, we demonstrated that the CD46 molecules from the lymphocytes and erythrocytes of the New World monkeys all lacked the SCR1 coding region adjacent to the amino terminal signal peptide, which prevented Edmonston strain of measles virus infection

in these New World monkeys. In chapter 2 and 4, we also generated artificial mutations by replacing either charged or hydrophobic amino acids with alanine or serine residues into the SCR1 and SCR2 domains of CD46 and mapped the antigenic epitopes of 5 monoclonal antibodies which are known to inhibit the binding of measles virus H protein to CD46. These results were used to model the SCR1 and SCR2 domains of CD46 from an analogous region in another complement regulatory protein, Factor H, whose three dimensional structure has been previously reported. In chapter 3, experimental evidence was presented implicating the existence of a new receptor for the wild-type strain of measles virus. The understanding of these infectious mechanisms of measles virus is critical to the design of viral inhibitors.

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Chapter 2: Artificial Mutations and Natural Variations In The CD46 Molecules From Human and Monkey Cells Map Regions Important For Measles Virus Binding[1]

[1] The results from this chapter have been published in the *Journal of Virology* 71: 6144-6154 (1997). I have done the experiments in Figures 2.2, 2.3, 2.4 and Table 2.2. Table 2.1 in this publication was a joint effort from Dr. Ruth Dörig, Anne Marcil and myself. Sequence alignment of SCR1 to SCR4 domains CD46 molecules from lymphocytes of Old World and New World monkeys was performed by Farida Sarangi and Dr. Chris Richardson (Figure 2.1).

2.1 Introduction

CD46, also known as membrane cofactor protein (MCP), was recently shown to be a primate-specific receptor for the laboratory adapted Edmonston strain of measles virus (15, 16, 22, 50). This surface glycoprotein is expressed on most types of cells and normally functions as a regulator of complement activity and helps to protect self tissue from autoimmune destruction (40, 41). CD46 is composed of 4 extracellular short concensus domains (SCR1, SCR2, SCR3, SCR4), followed by a region rich in serine, threonine, and proline (called STP), a transmembrane region and a short cytoplasmic domain at its carboxyl terminus. Variations in splicing of exons encoding STP cassettes, and cytoplasmic regions yield glycoproteins which vary in size from 57-67 kDa and consist of all 4 SCR domains, a combination of STP regions, and one of 2 different possible carboxy termini (62, 63, 71). Each of the known splicing variants of human CD46 is capable of binding measles virus (21, 46).

The CD46 binding domains for the laboratory adapted Edmonston strain of measles virus have recently been mapped to SCR1 and SCR2 (47) using chimeric recombinant molecules composed of parts of CD46 and a related molecule called CD55 or decay accelerating factor (DAF). Another group has done similar experiments with chimeras of CD46 and CD4 (11) while others expressed CD46 deletion mutants in Chinese hamster ovary cells (30) and confirmed the role of SCR1 and SCR2 in virus binding. Monoclonal antibodies specific for SCR1and SCR2 also block measles virus binding to the host cell (30, 47). The role of carbohydrate linked to the asparagine residue in the SCR1 domain of CD46 appears to be minimal while that associated with asparagine in SCR2 appears to be critical for binding measles virus (42, 43).

Measles virus (25, 78) is a paramyxovirus which consists of a negative strand RNA genome and an envelope which contains two surface glycoproteins known as hemagglutinin protein (H) and membrane fusion protein (F). A non-glycosylated membrane protein (M) lies on the inner side of the envelope and mediates an association of the glycoproteins with the nucleocapsid of the virus. H protein is responsible for virus attachment and interacts with CD46 (4, 14, 42) while F mediates penetration and syncytia formation (45, 65, 67, 82). Interaction of H with its receptor has been shown to downregulate surface CD46 in cultured cells (28, 35, 73-75) and 4 amino acids on the virus attachment protein have recently been implicated in this interaction (8). In addition, virus grown in cultured cells also binds to the receptors on many, but not all, species of monkey red blood cells to yield hemagglutination (20, 53-55, 57-59, 69). Hemagglutination assays with erythrocytes from cercopithecus and rhesus monkeys have traditionally been used to titer laboratory strains of measles virus indicating that the red blood cells from many Old World monkeys do possess the receptor for this virus. However, the erythrocytes of human, chimpanzee, and gorilla do not hemagglutinate in the presence of measles virus which correlates with the absence of CD46 on the surface of red blood cells from these higher primates (19, 52).

Since a number of species of monkey red blood cells vary in their ability to bind measles virus and subsequently hemagglutinate (18-20), we proposed to use the receptors on these erythrocytes as "natural mutant proteins" in an attempt to further define the binding regions of CD46. In this manuscript, we first studied the ability of New World and Old World monkey red blood cells to hemagglutinate in the presence of measles virus. Surprisingly, none of the New World monkey erythrocytes hemagglutinated in the presence of measles virus although it was evident that they expressed CD46 on their cell surface. The CD46 molecules from the lymphocytes and red blood cells of New World monkeys all lacked the SCR1

coding region adjacent to the amino terminal signal peptide. A specific mutation present within the CD46 molecules of baboons was noted which could account for diminished hemagglutination activity in the presence of measles virus. In addition, the effects of certain artificial mutations introduced into CD46 by site specific mutagenesis is also reported.

2.2 Materials and Methods

2.2.1 Cell lines and virus

HeLa, Vero, and HuTK$^-$143B cells were purchased from American Type Culture Collection (Rockville, MD). Sf9 insect cells were supplied by Invitrogen (San Diego, CA) and were grown in Grace's media. Hela, Vero and HuTK-143B cells were propagated in Dulbecco's minimum essential medium (GIBCO/BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum. In addition, HuTK-143B cells were cultivated in the presence of 12.5 μg/ml of bromodeoxyuridine. Monkey (cercopithecus, cynomologus and rhesus) red blood cells were purchased from Connaught Laboratories (Toronto, Canada) and baboon erythrocytes came from Lampire Biological Laboratories (Pipersville, PA). Other monkey red blood cells were obtained from Granby Zoo (Granby, Canada), the Montreal Biodome (Montreal, Canada), Quebec City Zoo (Quebec City, Canada), Toronto Metropolitan Zoo (Toronto, Canada), the NIH primate centre (Poolesville, MD), Merck-Frosst Pharmaceuticals (Montreal, Canada) and the New England Primate Center (Boston, MA). The Edmonston strain of measles virus was originally obtained from Dr. Erling Norrby (Karolinska Institute, Sweden) and was propagated in Vero monkey kidney cells as previously described (24).

2.2.2 CD46 polypeptide expression and production of antibodies

Polyclonal antibodies (CD46-333) directed against entire CD46 protein and antisera directed against the SCR1domain (∝SCR1) of CD46 protein were prepared by immunization of rabbits with purified recombinant protein. The entire CD46 coding region (isotype C2) was synthesized by polymerase chain reaction (PCR) with oligonucleotide primers as previously described (15). The DNA fragment was cloned into the Nhe I site of the baculovirus expression vector BlueBac 2 (pETL)

and recombinant baculovirus was generated (36, 68, 80). Recombinant CD46 protein was expressed in Sf9 insect cells, resolved on SDS polyacrylamide gels, electroeluted, and injected into rabbits to generate a polyclonal antibody using routine methods (66, 81). A DNA fragment containing SCR1 was synthesized by polymerase chain reaction (PCR) with oligonucleotide primers corresponding to the 5' end (5'-AACGGATCCT-GTGAGGAGCCACCAACA-3') and 3' end (5'-TGGTGTTCGAAAATTGTCCGCAGTAGA-CTCTG-3') of the SCR1 coding sequence. The PCR product was digested with BamHI and HindIII then cloned into the bacterial expression plasmid pT7-7His cut with the same enzymes (77). The resulting plasmid was transformed into *Escherichia coli* BL21 (DE3) by electroporation, and the recombinant protein was expressed by induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The SCR1 polypeptide fused to (His)6 was purified using metal chelation chromotography with Ni-NTA agarose (Qiagen) under denaturing conditions (61). Purified SCR1 polypeptide was injected into rabbits for the production of polyclonal antibodies (27). In addition, the monoclonal antibody E4.3 (specific for SCR1) was purchased from Bio/Can Scientific (Mississauga, Canada) and monclonal M75 (specific for SCR2) came from Seikugaku Inc. (Tokyo, Japan).

2.2.3 Hemagglutination of different primate red blood cells by measles virus

Primate blood samples were collected from animals in vacutainer tubes containing heparin/EDTA (Beckton Dickinson, Franklin Lakes, NJ) and stored in Alsevier's solution (GIBCO/BRL) for no more than 2 days. Cells were washed 2 times with PBS and a 1% (v/v) solution was used in the hemagglutination assays. A standard sample of concentrated measles virus (10^7 PFU/ml) was diluted serially (1:1) with PBS and 50 µl of each dilution was added to individual wells of a 96

well microtiter plate with round bottom chambers (Costar, Cambridge, MA). A 50 µl sample of the red blood cells was added to each well. Hemagglutination was allowed to proceed for 2 hours at 4° C. Erythrocytes settled to the bottom of the chambers when the assay was negative but formed a suspended matrix when positive. The assay titer was the highest dilution of virus which produced hemagglutination.

2.2.4 Isolation of monkey lymphocytes and synthesis of cDNAs from CD46 mRNA

Lymphocytes from different monkey species were prepared using a Ficoll-Paque (Pharmacia, Upsala, Sweden) discontinuous gradient. Fresh monkey blood (2-3 ml) was diluted with an equal volume of balanced salt solution (0.01% anhydrous D-glucose, 5 µM $CaCl_2$, 98 µM $MgCl_2$, 0.545 mM KCl, 0.126 M NaCl, and 14.5 mM TrisHCl pH7.4) and layered on to 3 ml of Ficoll-Paque. The sample was centrifuged at 700g for 30 min. at 20°C. The lymphocyte layer at the balanced salt solution/Ficoll-Paque interface was collected and dilulted with 3 volumes of balanced salt solution. Following centrifugation at 700g for 10 min at 20°, the lymphocytes pellet was washed once with 1ml of balanced salt solution, then resuspended in 1ml of TRIzol (GIBCO/BRL). Chloroform (0.2ml) was added and the TRIzol lysate was vortexed and left for 3 min at room temperature. The denatured extract was centrifuged at 10,000g for 10 min at 4°C. RNA in the upper aqueous layer was then precipitated with 0.5 ml of isopropanol at room temperature for 10 min, then concentrated by centrifugation at 10,000g for 10 min. Finally, the pellet was washed once with 75% EtOH, dried in the air, and resuspended in 50 µl H_20. Synthesis of cDNA was performed from mRNA using the First Strand Synthesis kit (Pharmacia) and a specific CD46 primer (5'-GGGACAACACAAATTACTGC-3'). Double-stranded DNA fragments were

generated using nested polymerase chain reactions (PCR). Primers derived from the human CD46 sequence corresponding to 5'-GCAACTCCAACAACTATGGC-3' and 5'-AGTGTCCCTTTCCTTCCTG-3' were used for the first 30 rounds of PCR amplification. Interior or "nested" primers corresponding to 5'-ACAATCACAGCAATGACCCA-3' and 5'-CGCTTTCCTGGGTTGCTTC-3' of human CD46 were used for the following 30 rounds of PCR reaction.

2.2.5 Determination of the 5' terminal sequences of CD46 mRNA

The 5' terminal coding regions of different monkey CD46 molecules were determined using the Marathon™ cDNA Amplification Kit (Clontech, Palo Alto, CA) as described in the reference manual. Poly A+ RNA was first isolated from total RNA using a QuickPrep mRNA purification kit (Pharmacia). Reverse transcriptase and oligo dT primer were used to synthesize the first strand cDNA from 1ug of poly A^+ RNA. The second strand DNA was synthesized using a mixture of RNAse H, DNA pol I, DNA ligase, and then T4 DNA polymerase (26). The Marathon cDNA adaptor was ligated to the dsDNA after synthesis of second strand cDNA. Monkey CD46 fragment containing the 5' coding region was generated using nested PCR with human CD46 specific primer (5'TAAGACACTTTGGAACTGGG-3') and Marathon cDNA Adaptor specific primer AP1 (5'-CCATCCTAATACGACTCACTATAGGGC-3') for the first 30 rounds of PCR reaction and human CD46 specific nested primer (5'--3') and Marathon cDNA Adaptor specific nested primer AP2 (5'-ACTCACTATAGGGCTCGAGCGGC-3') for the next 30 rounds of PCR amplification. CD46 DNA fragments from the different monkeys were rendered blunt with PFU polymerase (Stratagene, San Diego, CA), then cloned into the Srf I site of the PCR Script Amp (SK+) vector (Stratagene, La Jolla, CA).

2.2.6 SDS-Polyacrylamide gel electrophoresis and immunoblot analysis

SDS polyacrylamide gel electrophoresis and Western immunoblot analysis were performed as previously described (81). E4.3 and M75 monoclonal antibodies (1:500 dilution) were incubated with nitrocellulose paper blotted with total proteins from OST-7 cells infected with mutant CD46 recombinant virus. Binding of monoclonal antibody was detected by ECL chemiluminescence (Amersham, Arlington Heights, IL).

2.2.7 DNA sequencing

DNA fragments contained in PCR-Script AmP SK(+) vectors were sequenced using an Applied Biosystems 430I automated sequencer located at the Amgen DNA sequencing facility (Amgen, Thousand Oaks, CA). Sequence analysis and alignments were performed using Lasergene DNASTAR software (Madison, WI).

2.2.8 Flow cytometry analysis of monkey red blood cells and lymphocytes

Monkey blood (50µl) was centrifuged at 1500g for 5 min, washed twice with 5 ml of FACS buffer (PBS containing 1% of BSA, 5 mM EDTA, and 0.1% sodium azide). The red blood cells were resuspended in 100 µl of the same buffer containing 1:100 dilution of either preimmune antibody, CD46-333 antibody or SCR1 antibody for 1 hour on ice. Following the incubation, cells were washed twice with 3 ml of FACS buffer by centrifugation at 1500g for 5 min. The cells were resuspended in 100 µl of the same buffer contating 1:100 dilution of fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG(H+L) for 1 hour on ice. After washing twice with the 3 ml FACS buffer, the blood cells were suspended in

0.5 ml of FACS buffer and were subsequently analyzed on a Beckton Dickinson analyzer equiped with 15 mW argon laser at 488 nm. The data were collected and analyzed using CellQuest software.

Monkey lymphocytes were also analyzed by flow cytometry. The monkey blood samples were prepared and labeled as described above. Following the incubation with FITC-labeled anti-rabbit IgG, the cells were washed and resuspended in 100 µl of FACS buffer. Red blood cells were lysed by the addition of 1 ml of FACS lysis buffer (Beckton Dickinson) to each sample for 1 min. The lymphocytes were washed washed twice with 3 ml FACS buffer and monkey lymphocyte fluorescence data were collected as described in the previous paragraph. Mouse OST7 cells (2 x 10^6 cells) which expressed mutant forms of CD46 were suspended in 1 ml of Cell Dissociation Buffer (Sigma, St. Louis, MO) and washed 2 times by centrifugation with FACS buffer. The final pellet was suspended in 100 µl FACS buffer prior to analysis by fluorescence cytometry.

2.2.9 Site-specific mutagenesis of CD46 and expression of mutants using vaccinia recombinants

Portions of SCR1 and SCR2 domains were replaced with point mutations or 4 - 6 alanine residues using gel-purified oligonucleotides consisting of 30-40 nucleotides. Mutations were introduced into the CD46 molecule using the QuickChange™ site-directed mutagenesis kit from Stratagene. The CD46 coding region (isotype C2) was cloned into PCR-Script Amp SK(+) plasmid, denatured and annealed with 2 complementary oligonucleotides containing the desired mutation, the mutagenized DNA strands were extended with *Pfu* polymerase, the methylated nonmutated parental DNA template was digested with Dpn I, and the mutated plasmid was used to transform XL2-Blue ultracompetent cells. Mutant plasmids were isolated and the CD46 inserts were completely sequenced. The

mutagenized CD46 coding regions were excised from the PCR-Script plasmid following digestion with BspHI (5'end) and Bgl II (3'end) and ligated into the vaccinia vector pTM1 (containing the T7 promoter) which had been digested with the compatible restriction enzymes, Nco I and BamH I. Vaccinia virus recombinants were prepared and titered by plaque assays as previously described using HuTK$^-$ 143B cells and bromodeoxyuridine selection (17). CD46 mutants were expressed in mouse OST-7 cells which contained the T7 polymerase and protein synthesis and surface expression was monitored by Western immunoblot and FACS scan analysis.

2.2.10 Direct binding assays between CD46 mutants and insect cells expressing measles virus H protein

Mutant CD46 coding sequences were cloned into the pTM1 vaccinia expression vector which uses the T7 promoter to direct transcription of the foreign gene. Recombinant vaccinia virus was prepared as previously described (17) and mutant CD46 molecules were expressed in mouse OST7 cells which contain the T7 polymerase. Sf9 insect cells were infected for 48 hr with a recombinant baculovirus (81) which had been generated with the BlueBac2 expression vector (Invitrogen, San Diego, CA; 36, 68) and synthesized both the measles virus H protein and ß-galactosidase. The insect cells were colored blue by adding Bluogal (GIBCO/BRL, Grand Island, NY) at 36 hr infection from a stock solution (50mg/ml in dimethylformamide) to give a 0.05% (w/v) final concentration. Infections with recombinant vaccinia virus were allowed to proceed 12 hrs, Sf9 insect cells expressing the measles H protein and ß-galactosidase were incubated for 1/2 hour with the vaccinia-infected mouse OST7 cells in the presence of PBS containing 5% fetal calf serum. Non-adsorbed insect cells were eluted by washing the mouse cells 2 times with PBS. Binding in 25 cm^2 tissue culture flasks could be

quantitated visually under the microscope or quantitatively by the hydrolysis of o-nitrophenylgalactoside (ONPG) using a ß-galactosidase assay kit (Stratagene, La Jolla, CA). Cells were lysed in 0.5 ml of 100mM sodium phosphate buffer (pH 7.5) containing 0.2% Triton-X 100 and 1% NP-40. Aliquots of lysate (50 µl) were added to a 96-well microtitration plate and freshly prepared buffer (100 mM sodium phosphate, 10 mM KCl, 1mM $MgSO_4$, 50mM ß-mercaptoethanol, pH7.5) was mixed with each aliquot to give a final volume of 160 µl. A 50µl volume of ONPG solution (4 mg/ml in 100 mM sodium phosphate, pH 7.5) was added to each well and incubated at 37° until a yellow color was evident after about 30 min. The reaction was terminated by addition of 90µl of 1M Na_2CO_3 and color intensity was measured at 420 nm with a SpectraMax 250 ELISA plate reader purchased from Molecular Devices (Sunnyvale, CA). Results were linear over time for an absorbance range of 0.1-1A420 units.

2.2.11 Nucleotide sequence accession numbers

The nucleotide sequences coding for the extracellular domains of monkey CD46 molecules were submitted to GenBank (NCIB) and have the following accession numbers: *Aotus trivirgatus* (U87914), *Papio hamadryas* (U87915), *Callimico goeldii* (U87916), *Callithrix jacchus* (U87917), *Saguinus mystax* (U87918), *Saimiri scireus* (U87919), *Cercopithecus aethiops* (U87920), *Macaca fascicularis* (U87921), *Macaca mulatta* (U87922), and *Pithecia pithecia* (U87923).

2.3 Results

2.3.1 Analysis of CD46 surface expression and hemagglutination of primate red blood cells in the presence of Edmonston measles virus

Certain monkey red blood cells are known to bind measles virus and have been routinely used to titer measles virus in hemagglutination assays (19, 56). However, it is known that human and chimpanzee erythrocytes do not hemagglutinate in the presence of measles virus (19, 54). This can now be attributed to the fact that the red blood cells of humans, gorillas, and chimpanzees do not have CD46, the receptor for measles virus, on their cell surface (52). African green monkey (*Cercopithecus aethiops*) erythrocytes are known to be most sensitive to measles virus-induced agglutination (18, 20, 69). We collected red blood cells from a number of Old World and New World monkeys and assayed their ability to hemagglutinate in the presence of Edmonston measles virus which had been propagated in Vero monkey kidney cells (Table 2.1). The presence of CD46 on the surface of these monkey red blood cells was first verified by fluorescence analysis using a polyclonal antibody specific for human CD46. All primate red blood cells which were tested, with the exception of human and chimpanzee erythrocytes, bound the CD46 antibody. Levels of immune recognition were probably less in *Lemur macacao* due to species specific variation of its CD46 protein. Hemagglutination assays mediated by measles virus were performed and it was obvious that erythrocytes from South American marmosets and tamarins (*Callimico goeldii*, *Callithrix jacchus*, *Pithecia pithecia*, *Saimiri scireus*, and *Leontopithecus rosalia*) and Madagascar lemur failed to bind measles virus (Table 2.1). Old World monkey red blood cells varied in their ability to hemagglutinate in the presence of virus. As expected, human and chimpanzee red blood cells did not

Table 2.1 CD46 FACS ANALYSIS AND HEMAGGLUTINATION OF PRIMATE RED BLOOD CELLS BY MEASLES VIRUS

Primate Species	Pre-Immune[a]	Anti-CD46[a]	Hemagglutination Titer[b]
Homo sapiens (human)	0.710	0.359 (0)	1/1
Pan troglodytes (chimpanzee)	0.611	0.389 (0)	1/1
Cercopithecus aethiops (African green monkey)	0.309	6-8 (100++)	1/64
Cercopithecus diana	0.220	8.188 (100++)	1/40
Papio anubis (baboon)	0.280	6.894 (100++)	1/8
Papio hamadryas (baboon)	0.263	7.825 (100++)	1/8
Macaca fascicularis (cynomolgus)	0.403	6.289 (100++)	1/32
Macaca mulatta (rhesus)	0.354	8.059 (100++)	1/32
Erythrocebus patas	0.298	7.014 (100++)	1/32
Cercocebus torquatus lunulatus (mangaby)	0.477	8.38 (100++)	1/32
Callimico goeldi (Goeldii's marmoset)	0.295	13.68 (100++)	1/2
Callithrix jacchus (common marmoset)	0.279	5.257 (100++)	1/2
Pithecia pithecia (white-faced saki)	0.190	9.848 (100++)	1/1
Saimiri scireus (squirrel monkey)	0.383	3.886 (100+)	1/1
Leontopithecus (lion-tamarin)	0.203	4.731 (100++)	1/1
Lemur macaco (black lemur)	0.250	0.39 (100+)	1/1

[a]Expression of CD46 was monitored by FACS using a rabbit polyclonal antibody specific for the human cell surface antigen. The antibody specific fluorescence intensity is represented as a number. The number in parentheses indicates the percentage of the cells that were fluorescent. a plus sign indicates that the mean fluorescence was 2-10 times that of the pre-immune control. two plus signs indicate the signal was 10-100 times the mean of the pre-immune control.

[b]Hemagglutination titers were measured in microtiter plates with non-concentrated measles virus from culture media diluted serially by 1/2 in PBS buffer; an equal volume of 1% (v/v) suspension of monkey RBC was added to each well and the assay was allowed to incubate for 6 hrs at 4°. The larger dilution indicated an increased ability of the RBC to hemagglutinate in the presence of measles virus- less virus was required to make the RBC's clump. Assays with Old World monkeys were performed 4 times and averaged.

hemagglutinate while *Cercopithecus aethiops*, *Cercopithecus diana*, *Macaca fascicularis*, *Macaca mulatta*, *Erythrocebus patas*, *Cercocebus torquatus lunulatus* yielded high hemagglutination titers. Cercopithecus monkeys consistently yielded slightly higher hemagglutination titers than the rest of the monkeys, as previously reported (18, 20). On the other hand, red cells from baboons (*Papio anubis* and *Papio hamadryas*) always produced much lower titers in our assays. We proposed to isolate mRNA from the lymphocytes of these monkeys, synthesize cDNA, and sequence the regions coding for the extracellular domains of CD46 in an attempt to explain the species specific variation in hemagglutination titers exhibited by these different red blood cells.

2.3.2 Sequence comparisons of CD46 extracellular domains from different primates

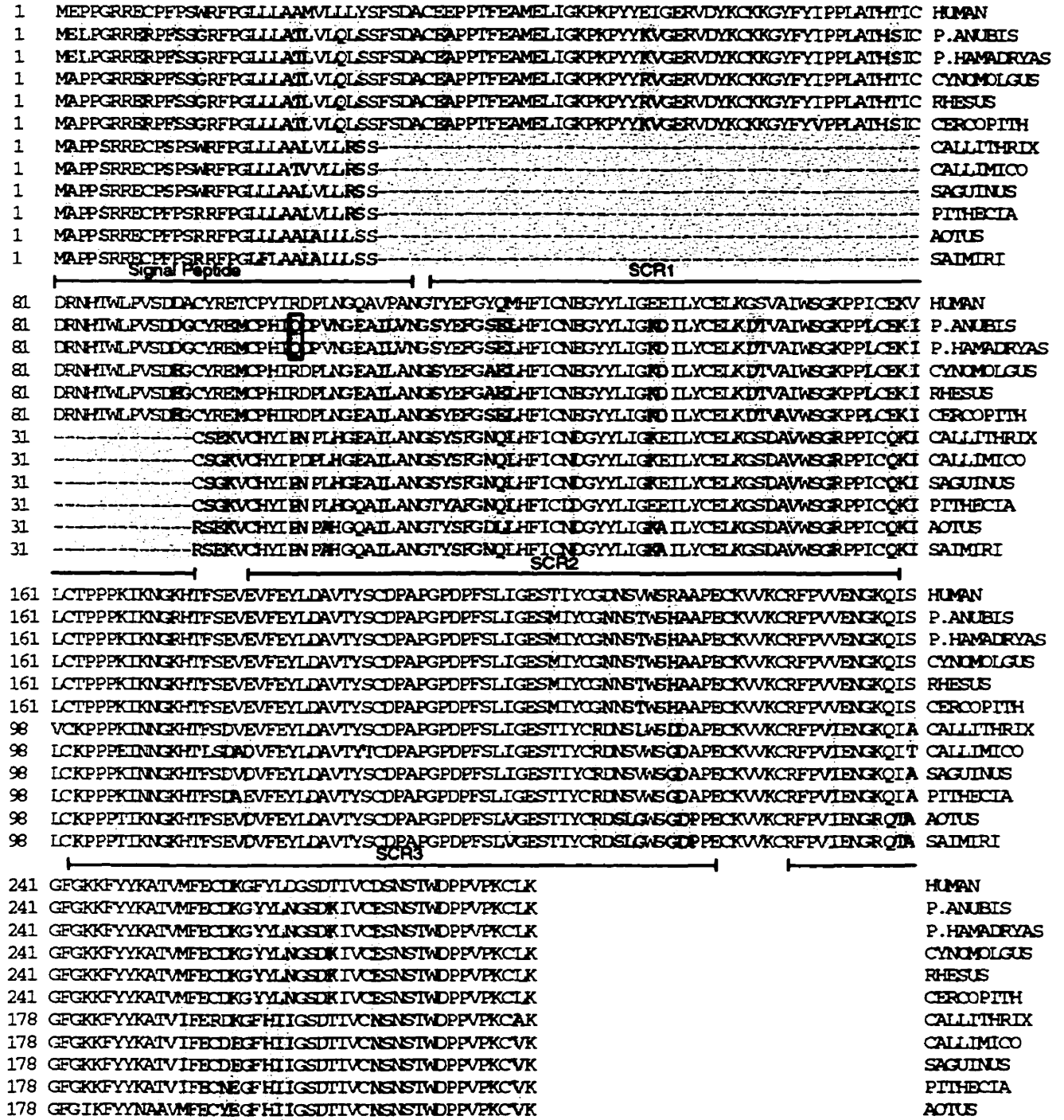
Lymphocytes were isolated from the blood of different monkeys and cDNA's were prepared using specific primers and reverse transcriptase, followed by polymerase chain reaction (RT-PCR). The predicted amino acid sequence was deduced from at least 3 separate PCR reactions for each type of monkey. Subsequently, signal peptide and short consensus regions (SCR1-SCR4) were aligned using the Clustal program from Lasergene (Figure 2.1). Overall, the CD46 molecules from the different primates were highly conserved and the protein sequences of the SCR3 and SCR4 regions from all the primates were extremely similar. SCR3 and SCR4 domains of Old World macaques exhibited 93% identity to the human sequence while those of New World monkeys were 80% identical. This conservation would seem consistent with the role of these regions of CD46 in complement regulation (40, 41). The conserved amino terminal signal peptides, STP, and membrane spanning regions facilitated RT-PCR across the extracellular domain of CD46. It was immediately evident that the SCR1 coding region

(consisting of 63 amino acids) was missing from the cDNA's derived from the mRNA of New World monkeys (*Callithrix jacchus, Pithecia pithecia, Callimico goeldii, Aotus trivirgatus, Saimiri scireus*). The observation that the SCR1 coding region was missing from mRNA of lymphocytes and erythrocyte precursors of New World monkeys would explain why the erythrocytes of New World monkeys did not hemagglutinate in the presence of measles virus since this region has previously been implicated in measles virus binding (30, 47). The SCR2 region varied most from the human sequence for both Old World and New World monkeys. This region was 73% identical to the human SCR2 for Old World monkeys and 68% identical for New World monkeys. Conserved regions of identity might be expected to participate in binding to the measles virus H protein.

The CD46 sequences for different classes of Old and New World monkeys resembled each other and could clearly be used to classify the primates into different groups. Baboons, macaques and African green monkeys belong to the family Cercopithecidae (51) and all have similar changes at 28 positions in the signal peptide and SCR regions when compared to the same extracellular portion of human CD46. Bands of amino acid identity at over 22 positions could easily differentiate whether the CD46 sequence belonged to an Old or New World monkey. South American monkeys could be further subclassified on the basis of 10 additional variations in the sequence shown in Figure 2.1. Marmosets (*Callithrix jacchus, Callimico goeldii*) and tamarins (*Saguinus mystax, Saguinus oedipus,* and *Leontipithecus rosalia*) belong to the Callithrichidae family (51). On the other hand, squirrel monkeys (*Saimiri scireus*), owl monkeys (*Aotus trivirgatus*), and white-faced sakis (*Pithecia pithecia*) are members of the Cebidae family (51). These family affiliations are reflected in amino acid variations found in the external domains of CD46 (Figure 2.1).

Figure 2.1 Amino acid sequence alignment of SCR1 to SCR4 domains cd46 molecules from lymphocytes of old world and new world monkeys

Blood samples were obtained from a variety of primates originating from either Africa or South America. Lymphocytes were isolated by gradient centrifugation, total RNA was extracted and cDNA was prepared with reverse transcriptase using specific oligonucleotide primers from the conserved STP and transmembrane domains. DNA sequences were amplified using conserved oligonucleotides upstream of the signal peptide region and regions adjacent to the SCR4 regions. The 5' ends of New World monkeys were also cloned by RACE using a conserved primer from the SCR3 region. At least 3 independent cDNA clones were sequenced, translated to yield a predicted protein, and aligned using the Clustal method in the Lasergene DNA analysis package. Residues which differ from the human consensus are shaded. Old world primates include humans (*Homo sapiens*), cynomolgus monkeys (*Macaca fascicularis*), rhesus monkeys (*Macaca mulatta*), baboon (*Papio anubis* and *Papio hamadryas*), and African green monkey (*Cercopithecus aethiops*). New world primates which we tested were the common marmoset (*Callithrix jacchus*), Goeldii's marmoset (*Callimico goeldii*), moustached marmoset (*Saguinus mystax*), the white-faced saki (*Pithecia pithecia*), owl monkey (*Aotus trivirgatus*), and squirrel monkey (*Saimiri scireus*). Signal peptide and SRC1, SCR2, SCR3, and SCR4 regions are indicated by solid lines beneath the sequences. The mutation found in baboon CD46 at amino acid 103 is highlighted by a solid box.


Signal Peptide
SCR1
SCR2
SCR3
SCR4

CD46 sequences from baboons contained only 7 amino acid changes when compared to the cercopithecus monkey sequence - 6 of these changes (positions 71, 92, 106, 113, 149, 172) were conservative but the other change at postion 103 produced an Arg to Gln substitution. This change might account for the reduced hemagglutination properties of baboon red blood cells and was considered in subsequent studies. Cynomolgus (*Macaca fascicularis*) and rhesus (*Macaca mulatta*) monkey CD46 sequences were almost identical to the sequence of *Cercopithecus aethiops* and all changes were minor and conservative. A slightly greater level of CD46 cell surface expression on the erythrocytes of *Cercopithecus aethiops* and *Cercopithecus diana* could explain the consistent difference in hemagglutination between cercopithecus monkeys and macaques (Table 2.1). It should also be noted that the Edmonston strain of measles virus has been routinely propagated in Vero and CV-1 cells, which are derived from the kidneys of an African green monkey. Propagation of this laboratory strain in culture, would ultimately favor the binding of measles virus to the *Cercophithecus aethiops* CD46 receptor over that of other monkeys.

Convserved blocks of amino acids in the SCR1and SCR2 regions could give some clues as to which regions are most important in binding to measles virus. SCR1 regions between amino acids 37-54 and 56-91 seem to be almost identical in the Old World monkeys. In addition, SCR2 regions consisting of residues 96-99, 103-109, 113-117, 124-135, 137-144, and 146-162 are almost identical in CD46 molecules from the Old World monkeys and could contribute to virus binding. Glycosylation sites at Asn83 and Asn114 are absolutely retained, 6 out of 8 cysteine residues are conserved, and the 13 prolines are maintained at all positions with the exception of residues 100, 104, and 112. It was interesting to note that baboons, macaques, and African green monkeys contained another potential glycosylation site in their SCR3 domain at residue 213. However, this should not

effect binding to H since this region of CD46 does not appear to interact with the virus during attachment.

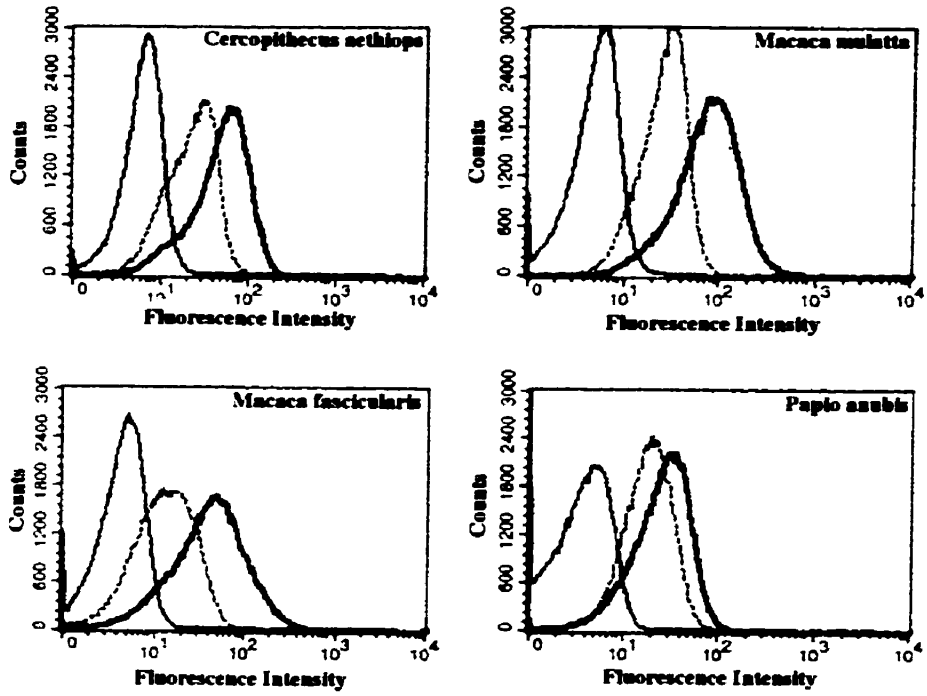
2.3.3 Polyclonal antibodies confirm that the SCR1 region is absent from CD46 molecules expressed on the red blood cells and lymphocytes from New World monkeys

SCR1 polypeptides (amino acids 30-91) derived from CD46 were expressed in *Escherichia coli*, recombinant protein was purified and injected into rabbits in order to generate polyclonal antibodies. These antibodies were used to study surface expression of SCR1 on red blood cells, lymphocytes, and a number of cell lines from Old and New World monkeys using fluorescent antibody analysis. A polyclonal antibody directed against the entire CD46 molecule indicated that the erythrocytes from most Old and all New World monkeys express CD46 on their cell surface (Figure 2.2). However, SCR1-specific antibodies clearly confirmed that the erythrocytes of Old World monkeys (*Cercopithecus aethiops, Macaca mulatta, Macaca fascicularis,* and *Papio anubis)* expressed the SCR1 domain of CD46 on their surface (Figure 2.2A), while analysis of New World monkeys (*Aotus trivirgatus, Saimri scireus, Callithrix jacchus, Saguinus oedipus*) showed that it was absent (Figure 2.2B). Analysis of lymphocytes, following lysis of red blood cells, gave identical results (data not shown). This result confirmed our RT-PCR analysis of mRNA derived from lymphocytes and explained why New World monkey red blood cells did not hemagglutinate in the presence of the laboratory strain of Edmonston measles virus since the SCR1 domain has been implicated in measles virus binding (30, 47).

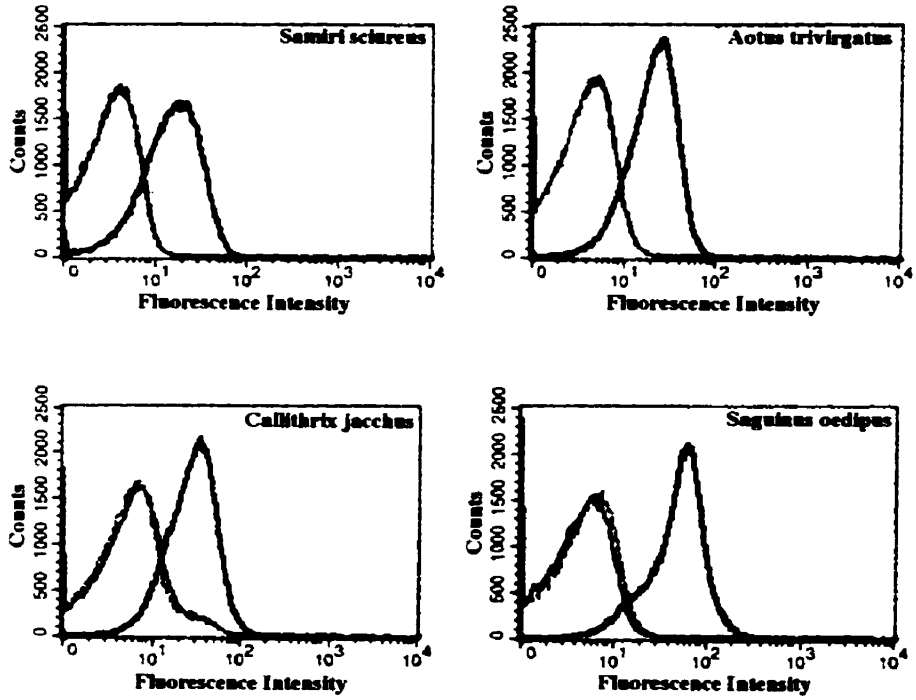
Figure 2.2 Analysis of SCR1 domains and CD46 molecules from red blood cells of Old World and New World monkeys using fluorescence cytometry

Erythrocytes from monkeys were incubated with either pre-immune, SCR1-specific, or polyclonal antisera directed against human CD46. Red blood cells were subsequently washed twice with FACS buffer using low speed centrifugation. Following resuspension of the pellet, the cells were incubated with FITC-labeled goat anti-rabbit IgG and again washed twice with FACS buffer using low speed centrifugation. The labeled erythrocyte pellet was resuspended in FACS buffer and analyzed using a Beckton Dickinson analyzer equiped with a 15 mW argon laser at 488 nm. The data were collected and analyzed using CellQuest software. Cell counts are indicated on the y-axis and the logarithm of the fluorescence intensity is represented on the x-axis. Analysis of some Old World monkey (*Cercopithecus aethiops, Macaca mulatta, Macaca fascicularis, Papio anubis*) red blood cells is presented in Panel A while that of 4 different species of New World monkeys (*Saimiri scireus, Aotus trivirgatus, Callithrix jacchus, Saguinus oedipus*) is shown in Panel B. (Red line) polyclonal antisera directed against entire human CD46 molecule; (Blue line) polyclonal antisera directed against SCR1 ; (Green line) rabbit pre-immune antisera.

A. Old World Monkeys



B. New World Monkeys

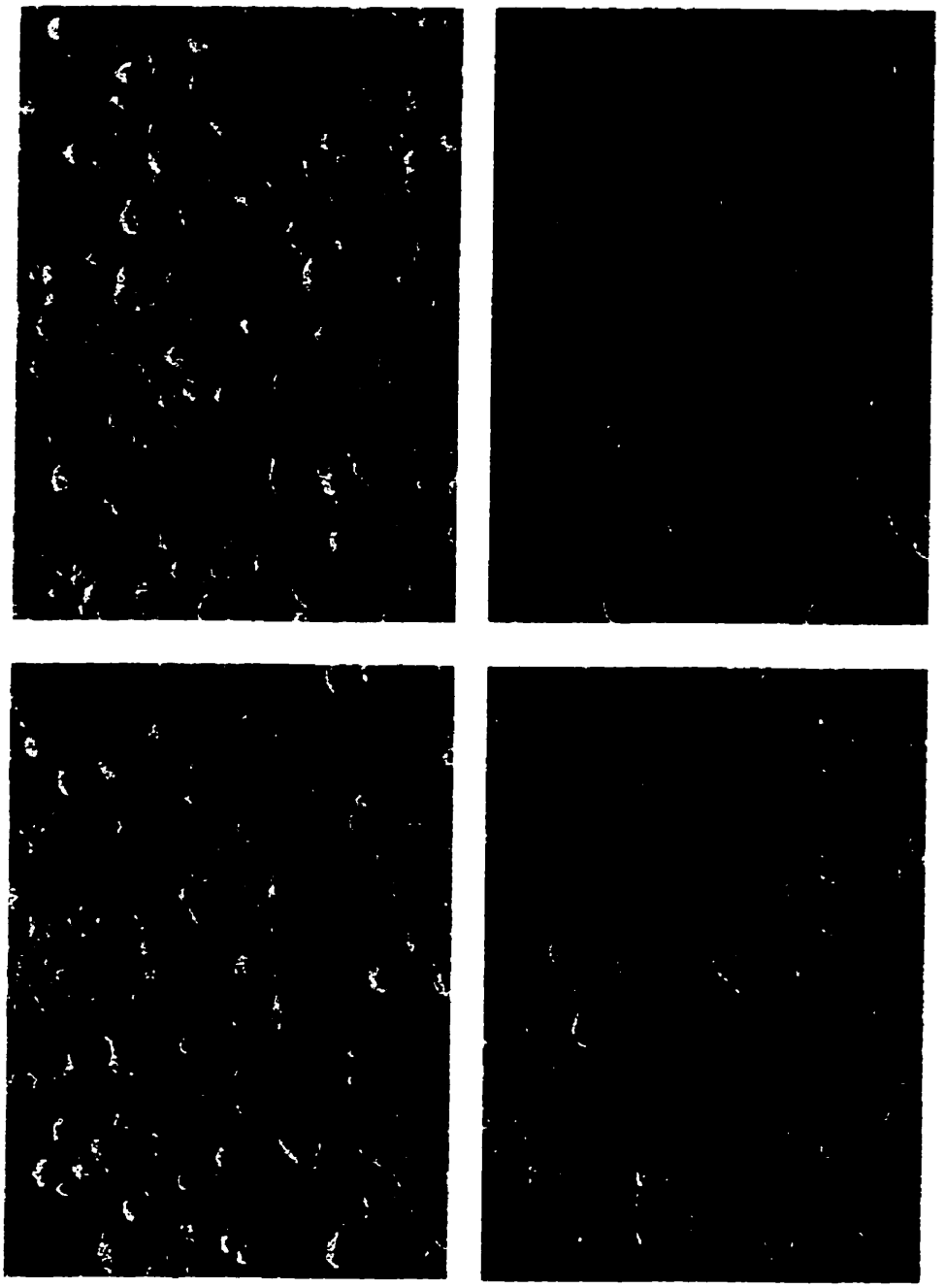


2.3.4 A novel binding assay using Sf9 insect cells expressing measles virus H protein and ß-galactosidase can be used to study the binding domains of CD46

Our laboratory previously demonstrated that Sf9 insect cells infected with a recombinant baculovirus expressing the measles virus H protein (Sf9-H) were capable of binding to red blood cells from African green monkeys (80). Since recombinant baculovirus was derived using the BlueBac II (pETL) expression vector (36, 68), the insect cells expressed ß-galactosidase as well as measles virus H protein. We reasoned that these infected Sf9 cells grown in suspension should be capable of binding to mammalian cells with CD46 at their cell surface. Preliminary studies demonstrated that Sf9-H cells could indeed bind to HeLa cells and in addition could be stained blue in the presence of Bluogal, a substrate for ß-galactosidase (29). Control Sf9 insect cell infected with wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV) did not adhere to target cells and could be washed away. A mouse cell line contaning the T7 polymerase gene (OST-7) does not normally bind measles virus. When infected with a recombinant vaccinia virus expressing human CD46 under control of the T7 promoter, these rodent cells could bind the Sf9-H cells (Figure 2.3D). Mouse cells infected with wild type vaccinia virus or a recombinant vaccinia virus expressing the SCR1 , STP, and transmembrane regions of CD46 could not bind the Sf9-H cells (Figure 2.3A, 2.3B). However, mouse OST-7 cells infected with a vaccinia recombinant expressing SCR1 , SCR2, STP, and transmembrane regions (Figure 2.3C) could bind Sf9-H cells just as well as if infected with vaccinia synthesizing the whole CD46 molecule (Figure 2.3D). These results confirm previous findings (11, 30, 47) which show that both the SCR1 and SCR2 domains of CD46 are required for measles virus binding. The results presented in Figure 2.3 attest to the credibility of this assay and establish a simple direct binding assay for interaction between

Figure 2.3 Binding assay for mutant CD46 molecules using insect cells expressing the H protein of measles virus

Mouse OST-7 cells were infected with wild type vaccinia virus (A), vaccinia virus expressing the SCR1 domain, STP, and CD46 transmembrane region (B), vaccinia recombinant expressing SCR1, SCR2, STP, and CD46 transmembrane domains (C), or with a recombinant virus expressing the entire human CD46 molecule (D). OST-7 cells, alone, normally do not bind measles virus. Sf9 insect cells infected with a baculovirus expressing measles H protein and ß-galactosidase were incubated with Bluogal substrate, washed with PBS, and added to the vaccinia-infected cells. Binding of blue-colored insect cells to mouse cells which expressed either SCR1/SCR2 (C) or the entire human CD46 molecule (D) was apparent.



measles virus H protein and the human CD46 molecule. Previous investigators monitored this binding indirectly through the ability of measles virus to initiate infection in rodent cells expressing CD46 variants (47), fluorescence microscopy with measles antibodies (47), fluoresence cytometry measurements using H monoclonal antibody (30, 50), the ability to form rosettes between African green monkey erythrocytes and the CD46-bearing target cell (15) or semi-quantitatively with a biotinylated H protein overlay binding assay using nitrocellulose blots containing CD46 (42). Our new binding assay with Sf9-H cells offers the advantage of being quick, sensitive, and easy to quantitate with either Bluogal or o-nitrophenylgalactoside (ONPG) substrates.

2.3.5 Site-specific mutagenesis can be used to map regions of CD46 important for interaction of the H protein

Binding of measles virus to African green monkey red blood cells has previously been reported to be reversed by incubation with 1M arginine and consequently electrostatic interactions appeared to be important in virus attachment (37). With this result in mind, we decided to initiate our mutagenesis studies by systematically substituting charged amino acids in the SCR1 and SCR2 domains with alanine. Larger stretches of the CD46 binding domains were also replaced with tracts of 4 to 6 alanine residues. Mutant CD46 molecules were expressed in mouse OST-7 cells using recombinant vaccinia virus and binding assays were performed with Sf9-H cells. Expression of all CD46 mutants on the surface of OST-7 cells was analyzed by fluorescence cytometry and similar amounts of recombinant protein were expressed on all cells. Levels of mutant CD46 surface expression is presented in Table 2.2. Binding of Sf9-H cells to OST-7 cells expressing mutant CD46 molecules was measured colorimetrically with an ONPG assay and the results relative to normal CD46 are presented in Figure 2.4.

Table 2.2 SITE-SPECIFIC MUTAGENESIS, CELL SURFACE EXPRESSION, AND REACTIVITY OF CD46 MUTANT PROTEINS WITH SPECIFIC ANTIBODIES

Mutation[a]	Surface Expression[b] (Mean Fluorescence)	E4.3[c] Monoclonal	M75[c] Monoclonal
CD46 C2	8.777 (100++)	+++	+++
EE36/37AA	8.140 (100++)	-	+++
E42A	9.150 (100++)	+	+++
E45A	8.445 (100++)	+	+++
KK49/51AA	8.774 (100++)	-	+++
E55A	9.490 (100++)	+	+++
ER58/59AA	9.880 (100++)	+	+++
DK61/63AA	8.564 (100++)	+++	+++
KK65/66AA	8.313 (100++)	+++	+++
H77A	8.140 (100++)	+++	+++
DR81/82AA	9.215 (100++)	+++	+++
N83A	7.894 (100++)	+++	+++
H84A	9.787 (100++)	+++	+++
DD91/92AA	8.737 (100++)	+++	+++
RD103/104AA	8.677 (100++)	+++	-
N114A	8.995 (100++)	+++	-
E118A	7.757 (100++)	+++	+
H124A	10.111(100++)	+++	+++
E129A	9.950 (100++)	+++	+++
E136A	7.825 (100++)	+++	+++
E137A	8.901 (100++)	+++	+++
E142A	10.405(100++)	+++	+++
E144A	8.499 (100++)	+++	+++
K153A	9.789 (100++)	+++	+++
Multiple 1 (E42-G48)	7.000 (100++)	-	+++
Multiple 2 (K49-E55)	10.216(100++)	-	+++
Multiple 3 (E58-C64)	8.978 (100++)	-	+++
Multiple 4 (Y70-A75)	7.557 (100++)	+++	+++
Multiple 5 (D81-W86)	7.002 (100++)	+++	+++
Multiple 6 (Y95-T98)	8.561 (100++)	+++	+++
Multiple 7 (N128-L133)	11.433(100++)	+++	+++
Multiple 8 (I149-K153)	9.358 (100++)	+++	+++

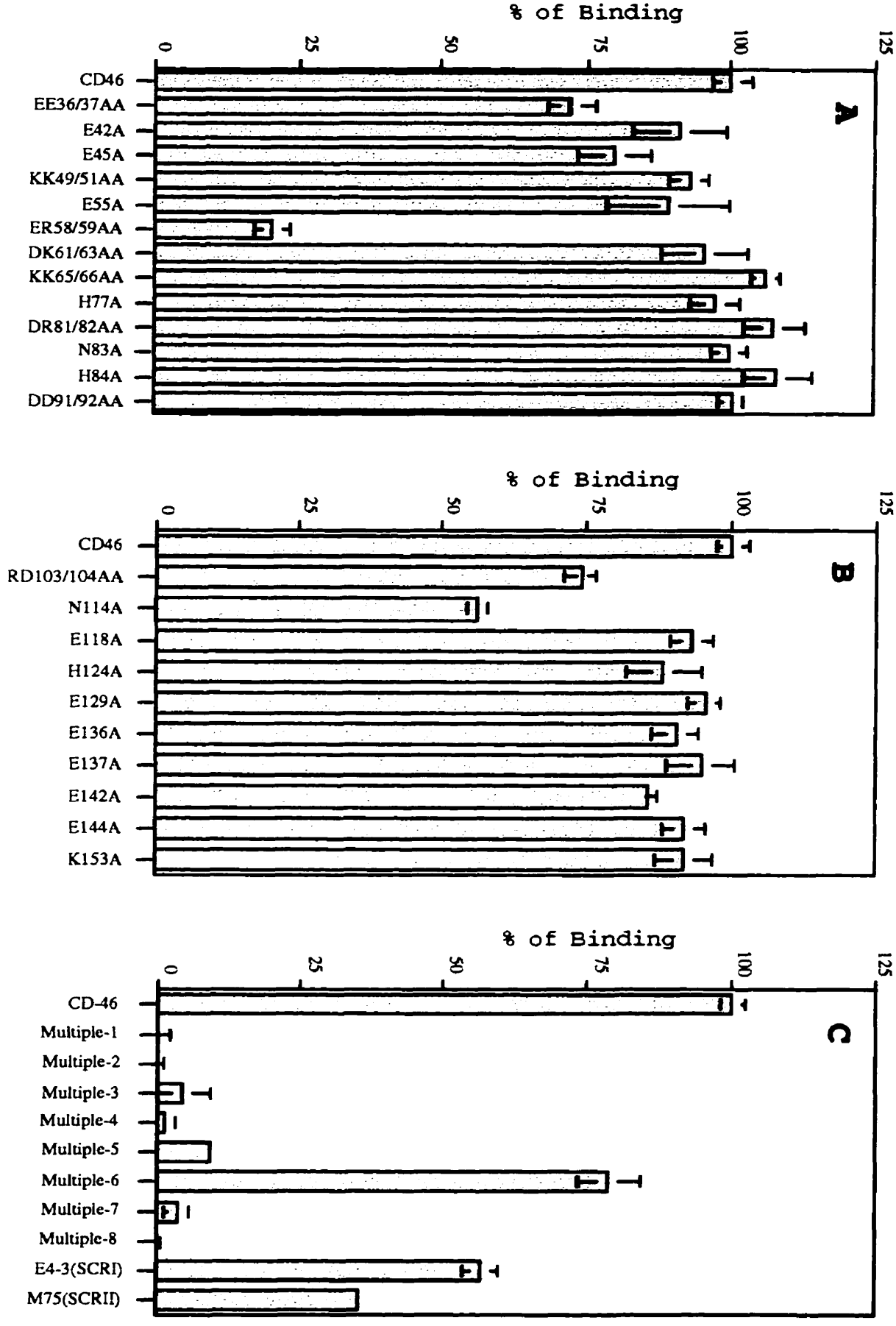
[a]The CD46 molecule (isotype C2) was subjected to site-specific mutagenesis as described in Materials and Methods. Position of the mutation is indicated by the amino acid residue numbered from the amino terminus of the polypeptide and includes the signal peptide prior to post-translational cleavage. The letter to the left of the number indicates the original amino acid, while the letter to the right indicates the change. Multiple mutants 1-8 consist of alanine tract substitutions between the indicated positions.

[b]Mean flluorescence of CD46 mutant proteins which were expressed on the surface of mouse OST-7 cells was determined by FACS analysis using a rabbit polyclonal antibody directed against CD46. Analysis was performed 3 times with an error of ±15%. The antibody specific fluorescence intensity is represented as a number. The number in parentheses indicates the percentage of the cells that were fluorescent, a plus sign indicates that the mean fluorescence was 2-10 times that of the pre-immune control, two plus signs indicate the signal was 10-100 times the mean of the pre-immune control.

[c]Reactiivity of the mutant CD46 molecules with monoclonal antibodies E4.3 and M75 was determined by immunoblot analysis of proteins from a mutant CD46 recombinant vaccinia virus infected cell lysate. (-) indicates that no band appeared on the blot, (+) indicates the presence of a faint band, and (+++) indicates that the monoclonal antibody recognized the mutant CD46 protein.

Figure 2.4 Effect of CD46 site-specific mutations and monoclonal antibodies directed against SCR1 and SCR2 on binding to insect cells expressing the measles virus H protein

Mouse OST-7 cells were infected with recombinant vaccinia virus expressing the mutant CD46 molecules described in Table 2.2. Sf9 insect cells which expressed both measles virus H protein and ß-galactosidase were added to the mouse cells for 1/2 hr and subsequently washed with PBS. Binding was quantitated by a colorimetric assay for ß-galactosidase using ONPG as a substrate and was compared to a standard value produced by cells expressing the normal CD46 molecule. This binding relative to normal human CD46 is represented on the y-axis of the histogram, while the various mutations are listed on the x-axis. OST-7 cells infected with wild type vaccinia virus served as a negative control, and exhibited no binding to Sf9-H cells. Mutations introduced in SCR1 are shown in Panel A, those placed in SCR2 are presented in Panel B, and multiple alanine substitutions in both SCR1 and SCR2 appear in Panel C. The inhibitory effects of monoclonal antibodies directed against SCR1 (E4.3) and SCR2 (M75) are also shown in Panel C. These two commercial antibodies were diluted 1:20 and added to binding assays and inhibition was noted.


% of Binding
0
25
50
75
100
125
CD46
EE36/37AA
E42A
E45A
KK49/51AA
E55A
ER58/59AA
DK61/63AA
KK65/66AA
H77A
DR81/82AA
N83A
H84A
DD91/92AA
A
SCRI Mutations
% of Binding
0
25
50
75
100
125
CD46
RD103/104AA
N114A
E118A
H124A
E129A
E136A
E137A
E142A
E144A
K153A
B
SCRII Mutations
% of Binding
0
25
50
75
100
125
CD-46
Multiple-1
Multiple-2
Multiple-3
Multiple-4
Multiple-5
Multiple-6
Multiple-7
Multiple-8
E4-3(SCRI)
M75(SCRII)
C
Multiple Mutations

Substitution of charged amino acids at the SCR1 amino terminus (EE36/37AA and E45A) appeared to moderately affect binding and led to 25-30% inhibition of binding. Mutation of the glycosylation site in SCR1 at Asn83 had no effect on binding nor did mutations of the charged residues surrounding this site. However, the mutation ER58/59AA dramatically inhibited binding up to 80%. Mutation of RD103/104AA and another N-glycosylation site (N114A) in the SCR2 domain caused moderate but reproducible inhibition of binding, 25% and 50% respectively. As previously stated, the Arg residue at position 103 has been replaced by a Gln in baboons (*Papio anubis* and *Papio hamadryas*) and we and others have noted that baboon erythrocytes are not nearly as effective in hemagglutination assays mediated by measles virus. Changes at amino acid 103 appear to diminish measles virus binding but not abolish it. Another laboratory also recently reported that the mutation at Asn114 destroyed this glycosylation site and reduced infections and binding by measles virus (44). It has not been shown whether this carbohydrate chain participates directly or indirectly in binding. For the most part, single amino acid mutations do not completely abolish binding of mutant forms of CD46 to Sf9-H cells. This implies that several distinct regions in SCR1 and SCR2 cooperate in binding in a conformational rather than linear manner, and that no one region of SCR1 and SCR2 is critical for virus interaction.

We were surprised to observe that multiple substitutions of 4-6 alanine residues at 7 locations over the SCR1 and SCR2 domains (E42-G48, K49-E55, E58-C64, Y70-A75, D81-W86, N128-L133, I149-K153) all totally abolished binding to Sf9-H cells. However, substitution of residues in the hinge region between SCR1 and SCR2 domains (Y95-T98) had little inhibitory effect. These changes seem to indicate that major distortions of the SCR1 and SCR2 domains cannot be tolerated in binding assays. Again the mutliple substitutions did not affect display of the mutant CD46 molecules on the surface of the mouse OST-7

cells (Table 2.2). It is quite possible that multiple alanine mutations might disrupt intramolecular disulfide bond formation and alter the native conformation of the virus binding domains.

2.2.6 Monoclonal antibodies E4.3 and M75 inhibit binding of H protein by interacting with specific regions in SCR1 and SCR2

Two monoclonals which react with CD46 were previously reported to block measles virus infections and prevent attachment of the measles virus H protein to the target cell (15, 30). We were able to map the regions on CD46 which interact with monoclonals E4.3 and M75 using vaccinia recombinant virus expressing different CD46 mutants (Table 2.2) and show that they inhibited binding of Sf9-H cells (Figure 2.4). E4.3 inhibited binding of normal CD46 to Sf9-H cells by 45% while M75 yielded 67% inhibition. In order to map the monoclonal antibody epitopes, OST-7 cells were infected with vaccinia recombinants and infected cell lysates were analyzed using immunoblots with the 2 monoclonals and diminished reactivity of the monoclonal with mutant CD46 molecules was noted (Table 2.2). E4.3 antibodies appeared to recognize residues 36-59 in the amino terminal domain of SCR1 while M75 mapped to amino acids 103-114. These epitopes coincided with the regions where site specific mutations had the most effect on virus binding. It will be interesting to note the inhibitory effects of additional monoclonals, when they become available, which recognize other regions of SCR1 and SCR2.

2.4 Discussion

CD46 was previously shown to be the receptor for the Edmonston strain of measles virus (15, 50). This paper expands upon the results of 2 other laboratories (30, 47) which claimed that both the SCR1 and SCR2 domains of CD46 are sufficient for virus binding. We proved that this process occurs via direct interaction of H protein with SCR1 and SCR2 using a novel binding assay with Sf9 insect cells expressing the viral surface protein, H. In our study we were surprised to note that all New World monkeys which were analyzed did not express the SCR1 domain in CD46 molecules present on their red blood cells and lymphocytes. This observation accounted for their inability to bind measles virus and hemagglutinate. The SCR1 deletion corresponds to a missing second exon which is not retained in the CD46 mRNA during the processing of precursor RNA; however, the exon still appears to be present in chromosomal DNA (29) which implies some irregularity in the splicing process. The composition of STP and cytoplasmic domains of CD46 are known to vary through a process of alternate splicing, but exons correspoding to the SCR domains have never been reported to be deleted through mRNA processing (62, 63, 71). No functional role has yet been assigned to the SCR1 domain of this molecule since complement components, C3b and C4b, have previously been demonstrated to interact with SCR2, SCR3, and SCR4 (1, 12, 30). A role in signal transduction has recently been proposed for CD46 and it was shown that interaction of this receptor with measles virus or monoclonal antibodes specific for SCR1 could downregulate the production of IL-12 (32). This receptor interaction could partially account for the immunosuppressive effects of measles virus. It remains to be determined if other cellular factors besides measles virus interact with the SCR1 domain. The deletion

of SCR1 in most tissues of New World monkeys (29) may have interesting evolutionary implications in terms of disease and immune regulation.

Red blood cells from the baboon, *Papio cynocephalus*, have previously been reported to hemagglutinate in the presence of laboratory strains of measles virus (57, 59). However, researchers showed that many Old World monkey red blood cells, including those from *Papio cynocephalus*, *Macaca fascicularis*, *Macaca mulatta*, and *Erythrocebus patas*, were less efficient in these assays than erythrocytes from the African green monkey (*Cercopithecus aethiops*) (18-20). We confirmed this result using two other species of baboon (*Papio anubis* and *Papio hamadryas*) and found that this decreased ability to hemagglutinate corresponded to a change at amino acid residue 103, which implicates this region of CD46 in measles virus binding. We were able to imitate this natural change through site-specific mutagenesis and our experiments revealed decreased affinity for the mutant CD46 in an Sf9-H binding assay. The nearby carbohydrate attachment site at Asn114 was also shown to be important for optimal binding, since mutation of this residue to Ala also reduced interaction with Sf9-H cells. In addition, a monoclonal antibody, M75, was mapped to the same region (amino acids 103-118) using immunoblot analysis of our mutant CD46 molecules, and it was also shown to inhibit binding effectively. These preceding studies appear to implicate the region of CD46 consisting of amino acids 103-118 as at least one of the sites involved in virus attachment.

The artificial mutation in CD46 which changed GluArg at positions 58 and 59 to AlaAla, had a dramatic effect on Sf9-H cell binding. This is a region which could be involved in electrostatic interaction with the binding region of measles H protein. Arginine at concentrations of 1M has previously been shown to abrogate virus binding and hemagglutination (37). The region at amino acids 58 or 59 could contribute to salt-dependent or charged residue interaction during the attachment

process (23, 37). The monoclonal antibody E4.3 has previously been studied in virus attachment experiments (30, 47). There is some discrepancy between the two laboratories as to the effectiveness of this antibody in inhibiting the binding of H protein to target cells, which could be related to the concentration of the reagent used in their experiments. Our results support those of Manchester et al. and confirm that E4.3 was indeed a potent inhibitor of measles H protein attachment. We further demonstrated that the monoclonal antibody E4.3 mapped to a region of CD46 between amino acids 37-59 using immunoblot analysis of our mutant CD46 molecules. Taken together, the preceding data indicate that the region of CD46 consisting of amino acids 37-59 also participates in binding to the measles virus H protein.

The CD4 binding region (amino acids 39-59) which interacts with human immunodeficiency virus (HIV) glycoprotein (gp120) has previously been mapped by other investigators through a series of site-specific mutations and interactions with monoclonal antibodies (5, 6, 9, 10, 13, 31, 48, 60, 72, 79). Synthetic peptides were also employed by other laboratories in attempts to map binding regions on CD4 but led to contradictory results (31, 38, 39, 64, 83). Originally we also attempted to perform peptide studies with measles virus and CD46 using 25 amino acid peptides derived from all regions of SCR1 and SCR2. Peptides at concentrations as high as 200μM had no effect in Sf9-H binding and measles viral infectivity assays. We presumed that binding of measles virus depended on conformational epitopes rather than linear peptide epitopes. Subsequently a genetic approach using site-specific mutagenesis was adopted in our laboratory to map binding regions on CD46. Initially we found that large mutagenic changes involving multiple alanine substitutions could not be tolerated. These multiple mutations appeared to perturb binding of Sf9-H cells to CD46, no matter which region was targeted. Surface expression of the mutated CD46 molecule was not

effected, but intramolecular disulfide bond formation may have been altered. The only area where multiple alanine mutations did not dramatically affect binding was the hinge region between SCR1 and SCR2 indicating that this region was relatively refractile to mutagenesis and probably was not important in measles virus binding. Smaller point mutations were subsequently utilized in our experiments to map regions in SCR1 and SCR2 which are important for measles virus binding.

A molecular understanding of the exact interaction of measles virus H protein with CD46 will ultimately depend upon structural studies using nuclear magnetic resonance or X-ray crystallography to dissect the binding regions of H, SCR1, and SCR2. Serum factor H, like CD46, is another complement binding protein which is composed of 20 short consensus regions (SCRs). The SCR15 and SCR16 regions of factor H, whose 3-D structure has been solved (7), bears some homology to the two terminal domains of CD46. The polypeptide backbone for CD46-SCR1 and CD46-SCR2 can be crudely modeled and superimposed on that for the SCR15 and SCR16 domains from factor H. However, one can only approximate regions of interaction between the receptor and viral glycoprotein at this time. The fine structure of the intramolecular loops defined by the cysteine residues and the orientation of the SCR domains about the hinge region between SCR1 and SCR2 will likely determine the residues available in the binding site. Mutagenesis offers some clues as to which residues are important in virus binding, but these experiments are actually a lead into structural studies.

Based on the deletion of SCR1 regions in New World monkeys, we might predict that marmosets and tamarins from South America are resistant to infections by the Edmonston strain of measles virus. Studies in our laboratory indicate that owl monkey kidney cells (OMK) and marmoset kidney cells (NZP-60) also contain this deletion and cannot be infected with measles virus in the laboratory (29). However, we and others (33) can infect a marmoset B cell line which has been

immortalized with EBV (B95-8) and results indicate that these cells contain both the SCR1-deleted and and non-deleted forms of CD46 (29). Other researchers have reported measles virus infections in cynomolgus, marmosets, tamarins, and squirrel monkeys (2, 3, 34). The disease in moustached marmosets has been reported to be characterized by severe gastroenterocolitis and immunosuppression (2) while the symptoms presented by squirrel monkeys are similar to those in humans and consist of rash, Koplik's spots, and infection of lymphatic tissue (34). The receptor distribution in these animals is currently under study in our laboratory and the nature of the SCR1 deletion in different organs of monkeys may determine the tissue tropism and nature of the disease in these animals. Since the SCR1 measles virus binding domain appears to be missing in these infected New World monkeys, it will be interesting to determine if another receptor besides CD46 can be used by measles virus during the process of attachment.

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Chapter 3: A Single Amino Acid Change in the Hemagglutinin Protein of Measles Virus Determines Its Ability to Bind CD46 and Reveals Another Receptor on Marmoset B Cells[2]

[2] The results from this chapter have been published in the *Journal of Virology* 72: 2905-2916 (1998). This publication was a joint effort from our laboratory and Dr. William Bellini's laboratory. I have done the experiments in Figures 3.1, 3.2A, 3.2B, 3.3, 3.5, 3.6 and 3.7. Experiments from Table 3.1 and 3.2 were performed by researchers from Dr. William Bellini's Laboratory . Sequence alignment of CD46 from monkey cell lines was performed by Farida Sarangi (Figure 3.2C) and antibody inhibition assay was performed by Dr. Chris Richardson (Figure 3.4).

3.1 Introduction

Our laboratory and another group have previously demonstrated that CD46 (also known as membrane cofactor protein) could serve as a receptor for the laboratory adapted Edmonston strain of measles virus (13,14,17,39). The Edmonston virus has been grown successfully in the laboratory for over 30 years following adaptation of the original wild type isolate to Vero monkey kidney cells (16). Attenuated vaccine strains of measles virus have also been generated by serial passage of the original Edmonston wild type isolate in tissue culture using human kidney, human amnion, dog kidney, and chick embryo cells (19,47). However, wild type isolates of measles virus have only recently been propagated efficiently in marmoset and human B cell lines without the need for adaptation to tissue culture (25). Measles virus is a negative stranded RNA virus which possesses an envelope containing two glycoproteins - the hemagglutinin (H) and a membrane fusion protein (F). Attachment of the virus to a specific host cell receptor is mediated by H while membrane fusion and penetration of the cellular plasma membrane is controlled by F (reviewed in 47,60).

CD46 is composed of four extracellular short consensus domains (SCR1, SCR2, SCR3, and SCR4) followed by a region rich in serine, threonine, and proline (called STP), a transmembrane region, and a short cytoplasmic domain at its carboxy terminus (33,34). Variations in splicing of 14 exons encoding SCR domains, STP cassettes, and cytoplasmic regions yield glycoproteins which vary in size from 57 to 67 kDa (41,42,50). All four SCR domains are normally expressed in the higher primates but SCR1 appears to be deleted from CD46 in the lymphocytes of South American monkeys (22). Binding of laboratory strains of measles virus to the SCR1 and SCR2 domains of CD46 has been rigorously studied in recent years (8,9,22,35,36,55). In addition, several investigators

reported that infections by the Edmonston strain of virus possessed the ability to downregulate the surface expression of CD46 on the infected cell (4,20,27,40,52,54). However, wild type isolates of measles virus did not produce this phenomenon (53). In addition, it has been known for many years that wild type isolates did not have the ability to hemagglutinate African green monkey red blood cells while laboratory strains adapted to growth in Vero cells did (16,57,58). Recent reports suggest that wild type isolates do not use CD46 as a receptor but may instead interact with another receptor which is present on activated B cells (10,29,53). This hypothesis was based upon the inabilty of wild type stains of measles virus to downregulate CD46, elicit hemagglutination of monkey erythrocytes, replicate efficiently in Vero cells, and cause fusion in infected HeLa cells.

A great deal of time and effort has been spent in sequencing genes from wild type measles virus isolates from around the world and comparing them to those of existing vaccine strains (45-49). Based upon nucleocapsid protein and H protein sequences, year of isolation, and geographic isolation, various isolates were assigned to one of eight groups; the Edmonston wild type and vaccine strains belong to group 1. Following the alignment of H proteins from 12 different vaccine strains and comparing them to the same protein from over 59 different wild type viruses, several amino acids consistently differed between the two types of viruses (46-49). These corresponded to amino acids 243, 252, 276, and 481. Approximately a third of the more recent measles virus isolates also possessed an additional N-linked glycosylation site at amino acid 416. Although sporadic changes occurred throughout the H protein, the sequences were highly conserved with greater than 95% identity. Recently variations of two amino acids at positions 451 and 481 of the hemagglutinin molecule were proposed to account for

differences in hemadsorption, syncytia formation, and CD46 downregulation between vaccine and wild type measles viruses (29).

A recent publication from our laboratory indicated that SCR1 was missing from CD46 molecules on the surface of red blood cells and lymphocytes of New World monkeys. Based upon our findings and those of others (9,23,36,55), we were aware that this region was essential for binding to the laboratory strain of Edmonston measles virus. We predicted that marmosets and tamarins from South America should be resistant to infections by the Edmonston laboratory strain of measles virus. Indeed this appears to be the case since marmosets inoculated with Edmonston virus developed no symptoms (2). However, other researchers have reported that the common marmoset, moustached tamarin, and squirrel monkeys are susceptible to infections by wild type virus which presents symptoms including severe gastroenterocolitis, immunosuppression, respiratory congestion, and in some instances rash and Koplik's spots (1,2,26). This basic observation coupled with the reports that wild type strains of measles virus were unable to hemagglutinate African green monkey red blood cells, led us to suspect that natural isolates of the virus used a receptor other than CD46 in order to bind to the host cell.

In this publication, we provide direct evidence for the existence of a second receptor for measles virus through use of a binding assay which was previously developed in our laboratory (22). Insect cells which expressed the wild type H protein bound to marmoset B cells but did not adhere to Vero monkey kidney cells or rodent lines expressing CD46 on their surface. In addition polyclonal antibodies directed against CD46 did not inhibit infections of the B cells with wild type virus. Differences in hemagglutinin protein structure between vaccine/laboratory and wild type strains of measles virus are described in this paper and the results are discussed in terms of receptor usage and viral pathogenesis in the infected host.

3.2 Materials and Methods

3.2.1 Cell lines and virus

HeLa, Vero, OMK, NZP-60, BJAB, 1A2, B95-8, and SML cells were purchased from American Type Culture Collection (Rockville, MD). Sf9 insect cells were supplied by Invitrogen (San Diego, CA) and were grown in Grace's media containing 10% fetal calf serum. Hela, Vero, and OMK cells were propagated in Dulbecco's minimum essential medium (GIBCO/BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum. NZP-60 cells were propagated in Dulbecco's minimum essential medium/Ham's F12 medium (GIBCO/BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 10 ng/ml epidermal growth factor, 0.005 mg/ml insulin, 5 ng/ml selenium and 0.005mg/ml transferrin. SML, B95-8, BJAB, and 1A2 cells were propagated in RPMI-1640 medium (GIBCO/BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum. The Edmonston strain of measles virus was originally obtained from Dr. Erling Norrby (Karolinska Institute, Sweden) and was cultivated in Vero monkey kidney cells as previously described (18). The Montefiore 89 strain of measles virus (wild-type) was obtained from Drs. Ilya Spigland and Amy Fox (Montefiore Medical Center, Bronx, NY) and was amplified in B95-8 cells as previously described (25). Moraten, Zagreb, and Schwarz measles virus vaccine strains were purchased from Merck (West Point, PA) and SmithKline Beecham (King of Prussia, PA) and cultivated in Vero cells.

3.2.2 Antibodies

Monoclonal antibodies directed against H (2B1-3) and polyclonal antibodies (CD46-333) directed against entire CD46 protein were produced in our laboratory as described previously (13, 22, 43). A rabbit polyclonal antibody directed against

native human CD46 was also obtained from Dr. J.P. Atkinson (Washington University, St. Louis, MI). Monoclonal antibodies directed agains matrix and hemagglutinin proteins of measles virus were purchased from Chemicon (Temecula, CA). Polyclonal antibodies directed against the SCR1 domains of moustached tamarin (*Saguinus mystax*) and humans were prepared in rabbits as previously reported (22). In addition, horseradish-peroxidase conjugated goat anti-mouse (IgG/IgM) antibody and fluorescein isothiocyanate-conjugated rat anti-mouse IgG(H+L) antibody were purchased from Jackson Laboratories (West Grove, PA).

3.2.3 SDS-Polyacrylamide gel electrophoresis and immunoblot analysis

Adherent and suspension cell lines were infected with either Edmonston strain (vaccine/laboratory strain) or Montefiore 89 strain (wild-type strain) of measles virus at a multiplicity of infection of 5 pfu per cell. Cells were harvested for 72 hours postinfection and washed twice with phosphate-buffered saline (PBS) by centrifugation then resuspended in 200 µl of sample buffer. SDS polyacrylamide gel electrophoresis and Western immunoblot analysis were performed as previously described (22,62). Primary antibody binding was detected with horseradish peroxidase conjugated goat anti-mouse antibody (1:5000 dilution) using the ECL chemiluminescence detection method (Amersham, Arlington Heights, IL).

3.2.4 Preparation of CD46 cDNA's from monkey tissues or cell lines

Monkey tissues were completely homogenized in the presence of 5ml TRIzol (GIBCO/BRL) using a polytron homogenizer (Brinkmann). B95-8 and SML cells ($1x10^7$ cells) were washed twice with PBS by centrifugation and

resuspended in 1ml of TRIzol (GIBCO/BRL). Total RNA was isolated according to the manufacturer's instructions. Synthesis of cDNA was performed from RNA using the First Strand Synthesis kit (Pharmacia) with the supplied random primer or a specific CD46 primer (5'-GGGACAACACAAATTACTGC-3'). Double-stranded DNA fragments were generated using nested polymerase chain reactions (PCR) as previously published (22). Interior or "nested" primers corresponding to 5'-CTTCTGGCGGCCATGGTGTTG-3' and 5'-TTTATTTTTGGAGGTGGTGTACAC-3' were derived from *Saguinus mystax* or *Saimiri sciureus* CD46 cDNA sequences (22) and used for the final 30 rounds of PCR amplification. Double-stranded DNA fragments corresponding to CD46 cDNA's from B95-8 and SML cells were cloned into PCR Script AMP (SK+) and sequenced. The 5' terminal coding regions of CD46 molecules from B95-8 and SML cells were determined using the Marathon™ cDNA Amplification Kit (Clontech, Palo Alto, CA) as previously described (22).

3.2.5 Preparation of cDNA containing the coding sequence for the hemagglutinin protein from the Montefiore 89 strain of measles virus

RNA was extracted from B95-8 cells infected with the wild type Montefiore 89 strain of measles virus using TRIzol (GIBCO/BRL) and cDNA was prepared with the First Strand Synthesis kit (Pharmacia). Double-stranded DNA fragments of wild-type measles hemagglutinin were generated using 30 rounds of PCR amplification and primers derived from the hemagglutinin sequence (5'-GGCGGATCCACAATGTCACCACAACGAGACCGG-3' and 5'-GAAGGATCCCTATCTGCGATTGGTTCCATCTTC-3'). Hemagglutinin cDNA fragments from 3 independent PCR amplifications were cloned into the Srf I site of the PCR Script Amp (SK+) vector (Stratagene, La Jolla, CA) and sequenced.

3.2.6 Construction of a chimeric CD46 molecule containing SCR1 and SCR2 from *Saguinus mystax* fused to human SCR3 and SCR4 domains

The SCR1 and SCR2 domains from human CD46 were replaced with SCR1 and SCR2 domains from B95-8 cells. A vaccinia expression vector pTM1 containing the human CD46 coding sequence was digested with NcoI and BsrGI restriction enzymes. SCR1 and SCR2 sequences from B95-8 cells were synthesized by polymerase chain reaction (PCR) using the specific oligonucleotide primers described above. The amplified DNA product was digested with NcoI and BsrGI restriction enzymes and inserted into the digested pTM1-CD46 expression vector. Vaccinia recombinant virus was prepared as previously described (Hsu et al., 1997). Chimeric CD46 was expressed in mouse OST-7 cells which contained the T7 polymerase and protein synthesis and surface expression was verified by Western immunoblot and FACS scan analysis.

3.2.7 Site-specific mutagenesis of measles hemagglutinin protein and expression of mutants using baculovirus recombinants

Specific mutations were introduced into the wild-type measles hemagglutinin molecule using the QuickChange^{TM} site-directed mutagenesis kit from Stratagene (La Jolla, CA) as previously described (22). Mutant plasmids were isolated and the measles hemagglutinin inserts were completely sequenced. The mutagenized hemagglutinin reading frames were excised from the PCR-Script Amp (SK+) plasmid following digestion with BamHI and subsequently inserted into the baculovirus expression vector pETL(BlueBac2) which also contains the ß-galactosidase gene. Baculovirus virus recombinants were generated as previously described (28,44,61). Recombinant H protein was expressed in Sf9 insect cells and protein synthesis and surface expression were monitored by Western

immunoblot and FACS scan analysis using hemagglutinin specific monoclonal antibodies.

3.2.8 Flow cytometry analysis of CD46 molecules and measles hemagglutinin molecules

Mouse OST-7, B95-8, OMK, SML, and NZP-60 cells (2 x 10^6 cells) which expressed CD46 were suspended in 1 ml of Cell Dissociation Buffer (Sigma, St. Louis, MO) and washed 2 times by centrifugation with FACS buffer (PBS containing 1% of BSA, 5 mM EDTA, and 0.1% sodium azide). Incubations were performed with a 1:100 dilution of either preimmune, polyclonal CD46(#333), or polyclonal *Saguinus mystax* SCR1 antibodies for 1 hour on ice. Cells were washed and incubated with fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG(H+L) secondary antibodies as previously reported (Hsu et al., 1997). Just prior to analysis cells were washed and suspended in 0.5 ml of FACS buffer and assays were performed on a Beckton Dickinson analyzer.

3.2.9 Direct binding assays between CD46 cell lines and insect cells expressing different measles virus H recombinant protein

CD46 molecules were expressed in either mouse OST-7 cells or hamster CHO cells as previously described (13,22). Sf9 insect cells were infected for 48 hr with recombinant baculoviruses which expressed Edmonston vaccine, Montefiore 89 wild type, or mutant H proteins in addition to ß-galactosidase. Binding assays between infected insect cells and mouse or hamster cells were performed as described previously (22). Nonadherant insect cells were washed away and binding was either visualized under the microscope in the presence of Bluogal or quantitated with the enzyme substrate ONPG.

3.2.10 Nucleotide sequence accession numbers

The nucleotide sequences coding for the extracellular domains of B95-8 CD46 and SML CD46 molecules, which originate from moustached tamarin (*Saguinus mystax*) and squirrel monkey (*Saimiri sciureus*) respectively, were submitted to Genbank (NCIB) and have the following accession numbers: *Saguinus mystax* SCR1 (AF025482), *Saimiri sciureus* SCR1 (AF025483), *Saguinus mystax* SCR1-deleted ectodomain (U87918), and *Saimiri sciureus* SCR1-deleted ectodomain (U87919). The sequence for the cDNA coding for hemagglutinin protein of the Montefiore wild type measles virus was also submitted and has the accession number (AF025484).

3.3 Results

3.3.1 Most organs of the common marmoset contain CD46 molecules with a deletion of the SCR1 domain which blocks infections by the Edmonston laboratory but not wild type strains of measles virus

We have previously demonstrated that the CD46 molecules from lymphocytes and erythrocytes of New World monkeys contain a deletion of the SCR1 domain (22). Since this region of the receptor is critical for binding to the hemagglutinin protein of the Edmonston laboratory strain of measles virus (8,23,36) we proposed that cells and tissues from South American monkeys may be resistant to infections by measles virus. Experiments were performed to determine whether the SCR1 domain was deleted in CD46 proteins from other organs of the common marmoset (*Callithrix jacchus*). Brain, heart, liver, lung, kidney, small intestine, spleen, stomach were homogenized, mRNA was extracted, and cDNA was prepared. PCR was performed across the SCR1 region using oligonucleotide primers derived from the conserved signal peptide and SCR3 domains. A 300 bp product was indicative of a deleted SCR1 domain while a 522 bp product was produced from a complete copy of the CD46 cDNA. Most organs from the marmoset contained the deleted form of CD46 (Figure 3.1) but the brain and heart may contain small amounts of the undeleted species in addition to the major deleted mRNA. Marmosets inoculated with the Edmonston strain of measles virus, did not exhibit disease symptoms nor did the tissues of these monkeys contain measles virus based upon RT-PCR analysis for nucleocapsid protein (Table 3.1). However the animals did seroconvert which may indicate the existence of a subclinical infections caused by non-specific uptake of the virus. These results seem to confirm previous findings (2) where marmosets infected intracerebrally with Edmonston virus developed encephalitis but displayed no visceral symptoms.

Figure 3.1 Southern blot of PCR amplification spanning SCR1 region of the common marmoset (*Callithrix jacchus*)

Brain, heart, liver, lung, kidney, small intestine, spleen, and stomachs of a common marmoset were isolated, homogenized in TriZol, mRNA was extracted, and cDNA was prepared with reverse transcriptase. PCR was performed across the SCR1 region using oligonucleotide primers derived from the conserved signal peptide and SCR3 domains. A 300 bp product was indicative of a deleted SCR1 domain while a 522 bp product was produced from a complete copy of the CD46 cDNA. Most organs from the marmoset contained the deleted form of CD46 but the brain and heart may contain small amounts of the undeleted species in addition to the major deleted mRNA. B95-8 marmoset B cells were homogenized and mRNA was extracted and treated in a similar manner as that from the marmoset organs. Undeleted and deleted forms of CD46 were present in the B95-8 cells. PCR analysis was also performed on cDNA clones which had been prepared from mRNA isolated from B95-8 cells and inserted into the pCR-script vector. The PCR products from the deleted clone (CD46ΔSCR1) and the nondeleted clone (CD46) templates were also analyzed. PCR products were resolved by agarose gel electrophoresis, transferred to nitrocellulose, and probed with ^{32}P-labelled fragments derived from the SCR2 and SCR3 regions of CD46 and subjected to autoradiography with Royal X-OMAT film for 24 hrs.

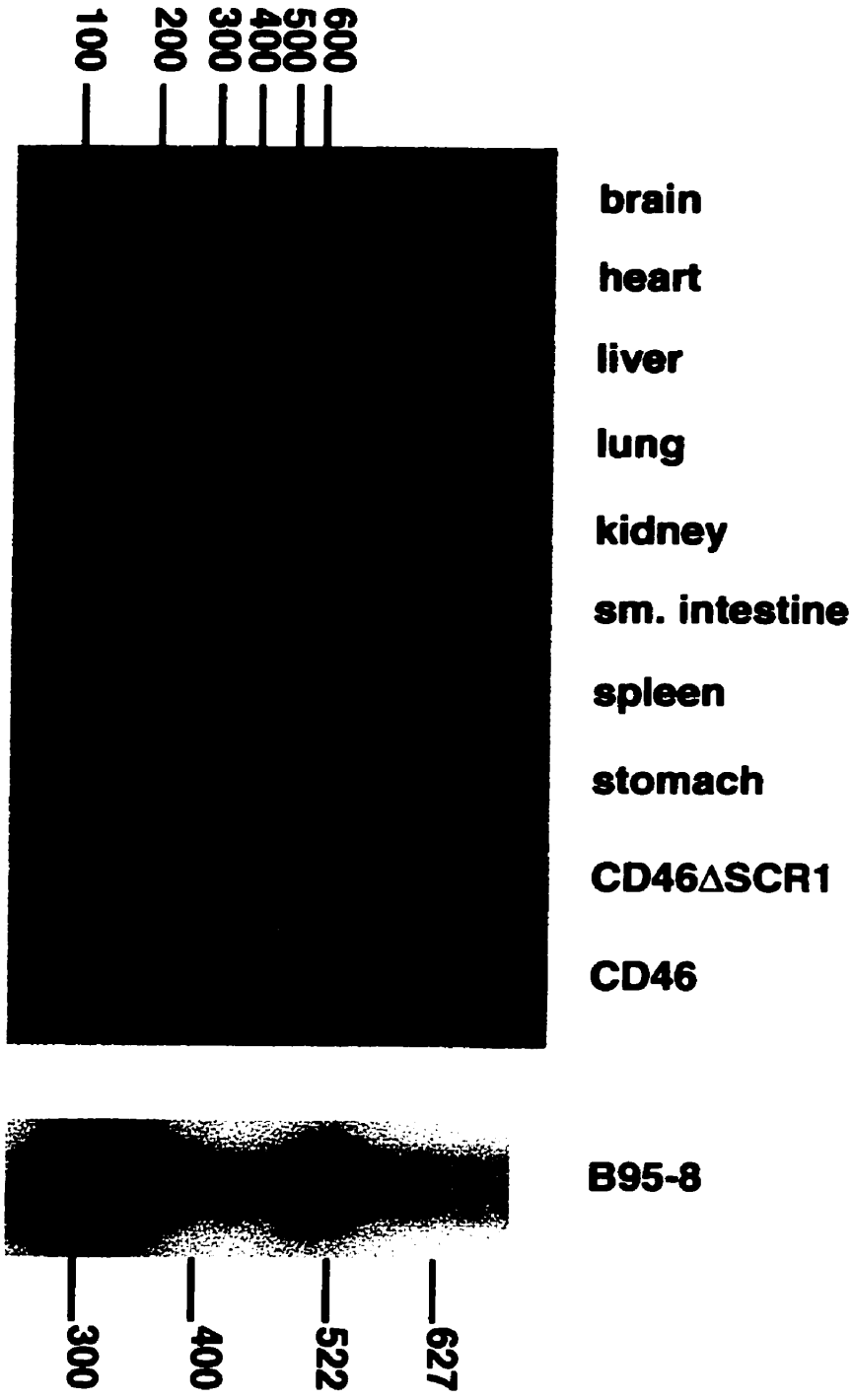

100
200
300
400
500
600
brain
heart
liver
lung
kidney
sm. intestine
spleen
stomach
CD46ΔSCR1
CD46
B95-8
300
400
522
627

TABLE 3.1 Infection of marmosets (*Callithrix jacchus*) with wild type and vaccine strains of measles virus

Group (n)[a]	MV-IgGpre[b]	MV-IgMpre[b]	MV-IgGpost[c]	MV-IgMpost	MV-PCR[d]	Virus Rescue[e]
MV Vaccine (3)	0/3	0/3	3/3	3/3	0/3	nd
MV wild type (3)	0/3	0/3	3/3	3/3	2/3	2/3
naive/exposed (3)	0/3	0/3	3/3	3/3	3/3	3/3

[a]Animals (n subjects) were anesthetized and inoculated intranasally with 10^4 $TCID_{50}$ of virus suspended in sterile saline. For the last group, naive animals were housed in cages adjoining the group inoculated with WT virus beginning on day of inoculation. The vaccine strain was Moraten/Edmsonston adapted to growth in Vero cells and the wild type strain was Pennsylvania-1 90 cultivated in B95-8 cells.

[b]ELISA specific for measles IgG or IgM were performed as previously described (37). Preinoculation serum samples were obtained 24 hours prior to inoculation or exposure.

[c]Post-inoculation serum samples were positive for MV IgG and IgM by 14-21 days after inoculation or exposue.

[d]RT-PCR to detect MV RNA was performed on RNA extracted from peripheral blood mononuclear cells and collected at weekly intervals from 7-60 days post inoculation or exposure using oligonucleotides specific for the nucleocapsid protein.

[e]Peripheral blood mononuclear cells collected at weekly intervals after inoculation were inoculated into B95-8 cultures.

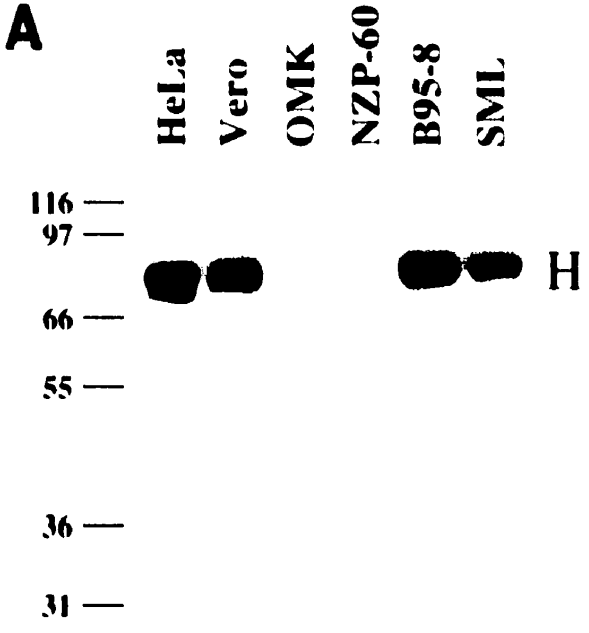
The small amounts of undeleted forms of CD46 which we found in the marmoset brain are consistent with the ability of Edmonston measles virus to cause encephalitis. However, we were at a loss to explain why marmosets, tamarins, and squirrel monkeys, which contained deletions in CD46, were still susceptible to infections by wild type strains of measles virus leading to severe gastroenterocolitis, immunosuppression, and respiratory distress (1,26,30). Productive infections with wild type measles virus were confirmed in one of our laboratories following inoculation of the common marmoset (*Callithrix jacchus*) with the Pennsylvania-1 90 strain of virus (Table 3.1). Wild type virus could be detected in peripheral blood cells of infected marmosets by RT-PCR but tests for the Edmonston strain in the other group of marmosets were negative. We began to suspect that wild type strains of measles virus may indeed use a receptor other than CD46 during the initial stages of infection.

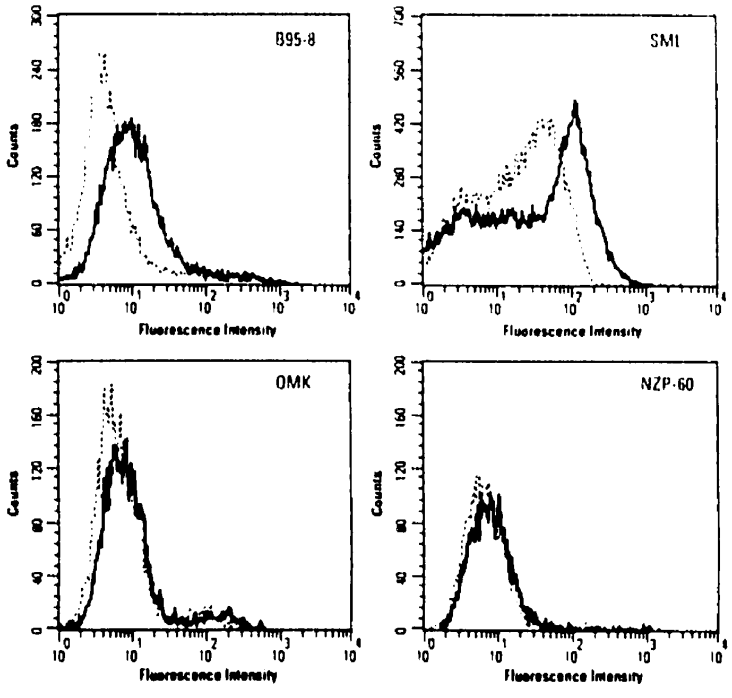
3.3.2 Marmoset B cells (B95-8) and squirrel monkey lung (SML) cells can be infected with the Edmonston strain of measles virus while owl monkey kidney (OMK) and marmoset kidney (NZP-60) cells are resistant to infection

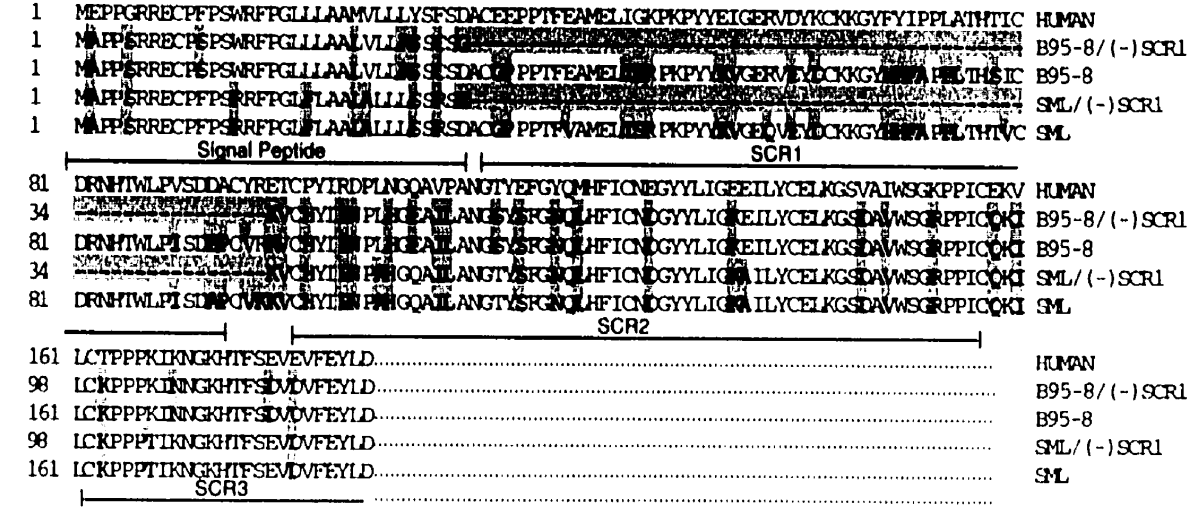
A B cell line (B95-8) transformed with Epstein Barr virus which was originally derived from the moustached tamarin (*Saguinus mystax*) (38). B95-8 cells were previously shown to support the growth of both wild type measles virus and virus which had been adapted to growth in Vero cells (25). Through RT-PCR we determined that B95-8 cells contain both the deleted and undeleted forms of CD46 mRNA (Figure 3.1). The SCR1 deletion corresponded to a missing exon2 in mRNA derived from the CD46 gene and the exon was previously shown to be present in marmoset chromosomal DNA (22,41,42,50). It is possible that under certain circumstances, such as viral transformation, exon 2 may be correctly spliced

Figure 3.2 Growth of the Edmonston strain of measles virus in New World monkey cell lines is impaired when SCR1 is deleted

Panel A: Human cervical carcinoma (HeLa), African green monkey kidney (Vero), owl monkey kidney (OMK), marmoset kidney (NZP-60), marmoset B (B95-8), and squirrel monkey lung (SML) cells were infected with the Edmonston strain of measles virus which had previously been adapted for growth in Vero cells. Cells were inoculated with 5 PFU/cell of virus and infections were allowed to proceed for 72 hrs after which the infected cells were subjected to immunoblot analysis with monoclonal antibodies directed against measles H protein. Viral protein synthesis was not observed in the OMK and NZP-60 cell lines but measles virus H protein was detected in B95-8 and SML cells. *Panel B*: FACS scan analysis was performed on B95-8, OMK, SML, and NZP-60 cells using an antibody which was directed against the SCR1 domain of the moustached tamarin (*Saguinus mystax*) and detected with goat antirabbit antibodies which had been conjugated to fluorescein (solid line). Cells were also tested with rabbit pre-immune antisera (dotted line). Shifts in fluorescence were observed in B95-8 and SML cells but not in OMK and NZP-60 cells. *Panel C* : mRNA was extracted from B95-8 and SML cells, cDNA was prepared, PCR products spanning the signal peptide, SCR1, SCR2, and SCR3 domains were prepared and sequenced. The predicted amino acid sequence is shown and was derived from 3 independent amplification reactions for each sequence. Both deleted and non-deleted forms of mRNA were present in the two cell lines.


A
HeLa
Vero
OMK
NZP-60
B95-8
SML
116
97
66
55
36
31
H


B
B95-8
SMI
OMK
NZP-60
Counts
Fluorescence Intensity


C
Signal Peptide
SCR1
SCR2
SCR3
HUMAN
B95-8/(-)SCR1
B95-8
SML/(-)SCR1
SML

to yield full length CD46 mRNA. We subsequently tested two Old World and four New World primate cell lines for their ability to support Edmonston measles virus infection. Viral protein synthesis was demonstrated through the presence of the measles hemagglutinin protein (H) following immunoblot analysis with a monoclonal antibody directed against H. As expected, the Old World primate cell lines, HeLa (human cervical carcinoma) and Vero (African Green monkey kidney), supported Edmonston measles virus infection as indicated by the presence of 79 kDa bands corresponding to the molecular weight of the H protein (Figure 3.2A). The New World monkey cell lines, OMK (owl monkey kidney) and NZP-60 (*Callithrix argentata* kidney), did not support Edmonston measles infection. On the other hand, the two other New World monkey cell lines, B95-8 and SML (squirrel monkey lung transformed with a simian retrovirus) did support Edmonston measles infection. Again mRNA from these cell lines was isolated, cDNA was prepared, and RT-PCR was performed across the SCR1 region using primers derived from the signal peptide of CD46 and SCR3. Southern blot hybridization of the PCR products revealed that the OMK and NZP-60 cell lines yielded only one band corresponding to the SCR1-deleted form of CD46 mRNA while the B95-8 and SML cell lines produced 2 products corresponding to deleted and non-deleted forms of CD46 mRNA (data not shown). Vero and HeLa cells yielded 1 PCR product which corresponded to the non-deleted form of CD46, as expected.

FACS scan analysis was also performed on B95-8, OMK, SML, and NZP-60 cells using an antibody which was directed against the SCR1 domain of *Saguinus mystax* . Cells were also tested with rabbit pre-immune antisera and a polyclonal antibody directed against the entire human CD46 molecule (data not shown). Each of the cell lines exhibited a shift in fluorescence due to the presence of CD46 on their surfaces, but only the B95-8 and SML cells possessed a fluorescent signal specific for the the SCR1 domain. (Figure 3.2B). Both SCR1

and SCR2 domains have previously been implicated in binding to measles virus which had been grown in Vero cells (8,9,22,36) and the fluorescence cytometry shown in Figure 3.2B supports the results of the viral infections shown in Figure 3.2A.

PCR products from the previous experiments with B95-8 and SML cells were cloned and sequenced using 3 independent amplification reactions. The deduced polypeptide sequences containing the signal peptide and short consensus regions (SCR1-SCR2) of CD46 were aligned using the Clustal program from Lasergene (Figure 3.2C). The larger cDNA's of B95-8 and SML CD46 molecules contained the SCR1 domain while the smaller isoform did not. The SCR1 regions from B95-8 cells (*Saguinus mystax*) and SML cells (*Saimiri sciureus*) were very similar and possessed 90% identity with the human sequence. Since the SCR1 coding region was present in the mRNA from both the B95-8 and SML cell lines, one might expect these two New World monkey cell lines should support infections by the laboratory strain of Edmonston measles virus, while cell lines lacking SCR1 (OMK and NZP-60) would not. These sequences corroborate our previous results with RT-PCR assays and FACS analysis, and provide one explanation as to why B95-8 and SML cells can support infection by the Edmonston laboratory strain of measles virus.

3.3.3 A wild type strain of measles virus (Montefiore 89) will infect B95-8 cells but cannot grow in other cell lines

Several different CD46 positive cell lines were inoculated with either Edmonston measles virus (adapted to growth in Vero cells) or the wild type Montefiore 89 strain of measles virus, in order to test their susceptibility to these viruses. Cells were incubated for 72 hours with either strain of virus and infection was monitored by the synthesis of the measles virus matrix protein (M) using

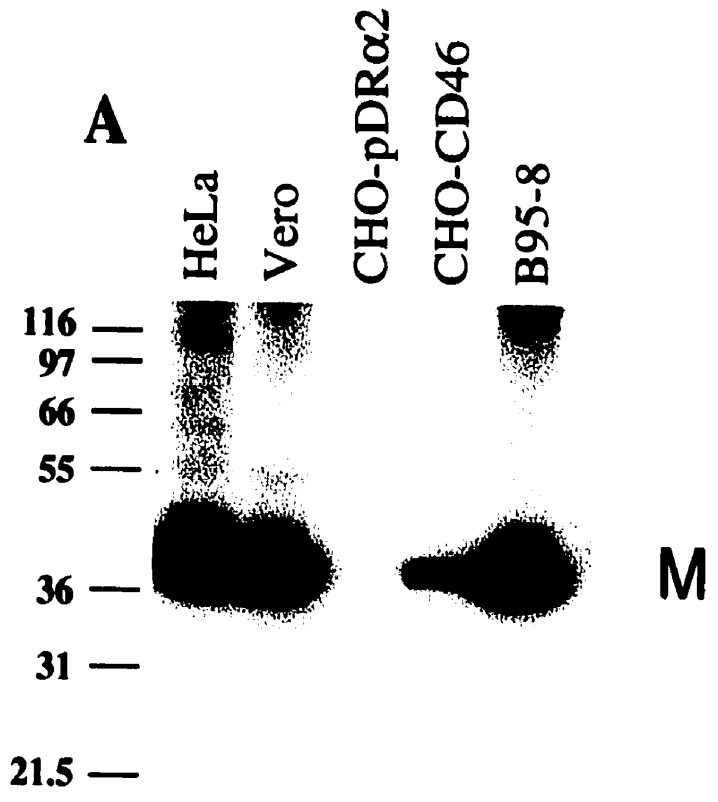
immunoblot analysis. As expected, HeLa, Vero, CHO-CD46, and B95-8 cells supported infection by the Edmonston strain of virus and 40 kDa bands corresponding to the M protein were present on immunoblots prepared from infected cell lysates (Figure 3.3A). CHO cells, which do not express CD46 on their surface, were transfected with the expression vector alone and did not support infection. HeLa, Vero, and CHO-CD46 cells which were inoculated with Montefiore 89 wild type measles virus, did not show any evidence of viral infection. However, B95-8 cells clearly supported infection by either the Montefiore 89 or Edmonston strains as indicated by the presence of viral M protein (Figure 3.3B). These results confirm the findings of others (25) and in addition suggest that wild type strains of measles virus may use a receptor other than CD46, which appears to be present on transformed marmoset B cells.

3.3.4 CD46 polyclonal antibody block infections by the Edmonston strain in Vero cells but does not inhibit infections by the Montefiore 89 strain of measles virus in B95-8 cells

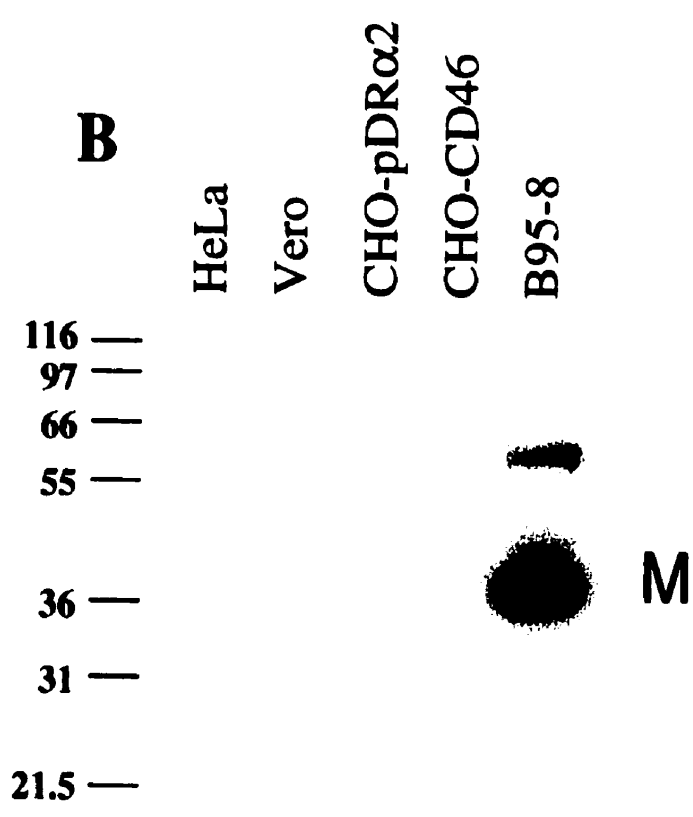
We previously demonstrated that a polyclonal antibody directed against the entire human CD46 molecule could both recognize CD46 proteins from different monkeys and inhibit infections by the Edmonston strain of measles virus in HeLa cells (13,22). In addition, the polyclonal antibody at dilutions as low as 1:400 effectively neutralized infections by the Edmonston strain in Vero monkey kidney cells (Figure 3.4E). Polyclonal antibody directed against CD46 and the marmoset SCR1 were also tested for there ability to inhibit infections by either the Edmonston laboratory strain or the wild type Montefiore strain of measles virus infection in marmoset B95-8 cells. The B95-8 cells were pre-incubated with dilutions of polyclonal anti-CD46/SCR1 serum or pre-immune antiserum ranging from 1:10 to 1:400 for a period of 1 hour; the Edmonston strain or Montefiore strain of measles

Figure 3.3 Infection of cell lines with Edmonston laboratory and Montefiore 89 wild type strains of measles virus

HeLa, Vero, Chinese hamster ovary cells transfected with an empty expression vector (CHO-pDRα2), Chinese hamter ovary cells expressing human CD46 (CHO-CD46), or a marmoset B cell line (B95-8) were inoculated with either Edmonston or wild type Montefiore 89 strains of measles virus. Cells were incubated for 72 hours with either strain of virus and infection was monitored by immunoblot analysis with a monoclonal antibody directed against the measles virus matrix protein (40 kDa). *Panel A* : HeLa, Vero, CHO-CD46, and B95-8 cells supported infection by the Edmonston strain of virus while CHO cells which were transfected with the expression vector alone (CHO-pDRα2) were not infected. *Panel B* : HeLa, Vero, CHO-pDRα2, and B95-8 cells were inoculated with the wild type Montefiore 89 strain of virus and infections were allowed to proceed for 72 hours. Only the B95-8 cells supported infection. Protein standards (kDa) are shown at the left of each panel.



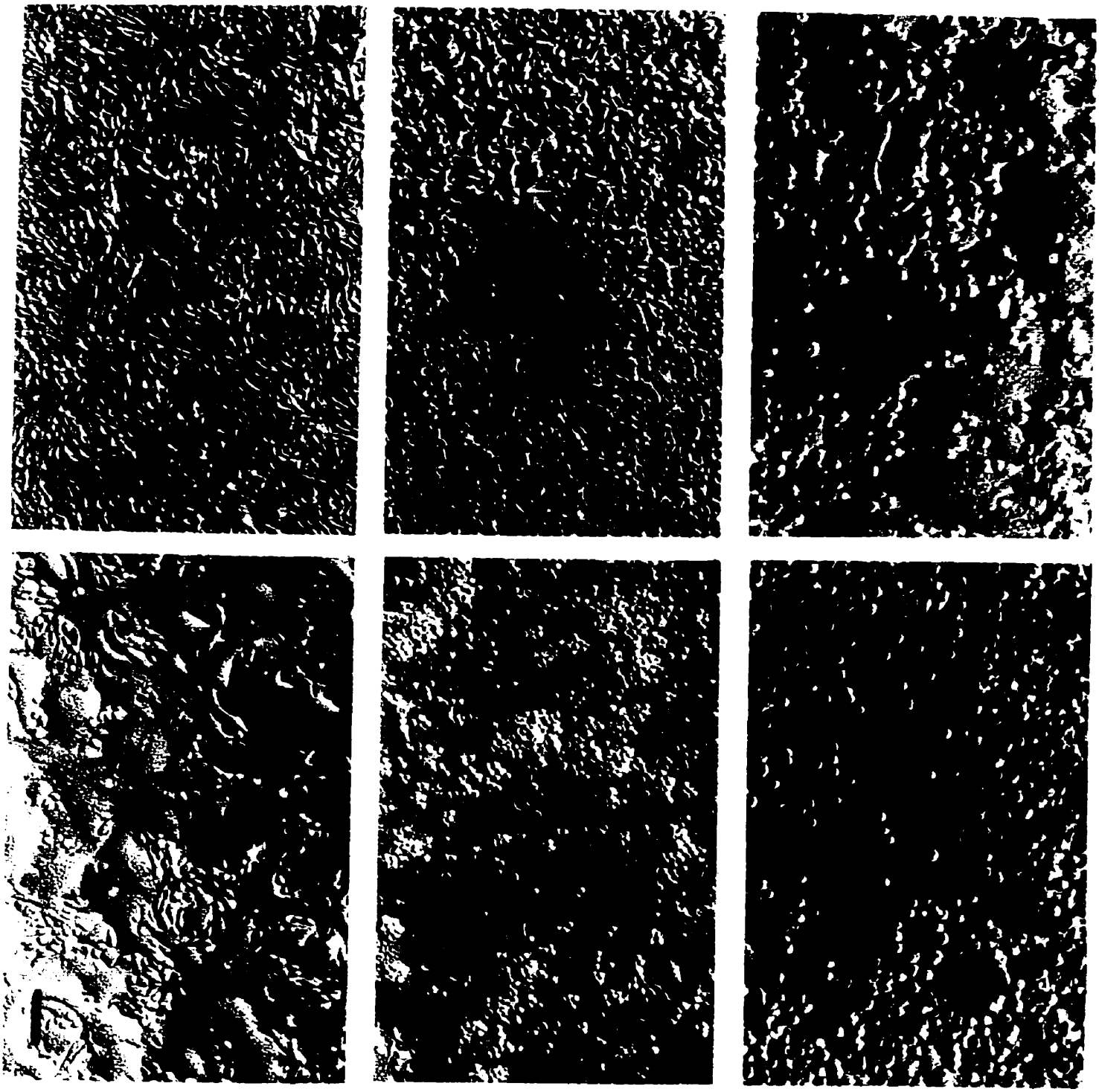
Edmonston



Wild Type

Figure 3.4 Polyclonal antibody directed against CD46 does not inhibit infections by the Montefiore 89 wild type strain of measles virus

Antibodies directed against CD46 and the marmoset SCR1 were combined and tested for their ability to inhibit infections by Montefiore 89 virus and Edmonston strain of measles virus in B95-8 and Vero cells. Cells were treated with CD46 and SCR1 immune antibodies (*Panels A,C,and E*) or preimmune serum (*Panels B, D, and F*). CD46 antibodies at dilutions of 1:10 had no effect upon infections of B95-8 cells by the wild type Montefiore 89 virus (Panel A) but partially inhibited infections of the same type of cells by the Edmonston strain of virus (*Panel C*). The same antibodies at dilutions as low as 1:400 completely inhibited infection of Vero cells by the Edmonston virus (*Panel E*). Infections were assessed by the formation of syncytia or multinucleated cells. The solid bar in Panel F represents 3 μm.



virus was added and allowed to adsorb for another hour, and virus was subsequently removed and replaced with fresh media. Cells were inoculated with measles virus at a m.o.i. of 1 PFU/cell, the infection was allowed to proceed for 36 hours in the presence of antibody, and the cytopathic effects found in infected cells were examined using the microscope. Virus dependent syncytia formation was clearly observed in B95-8 cells which were infected with either Edmonston and Montefiore 89 strains of measles virus in the presence of preimmune antibodies (Figures 3.4B and 3.4D). On the other hand, treatment of B95-8 cells with anti-CD46/SCR1 polyclonal antiserum at dilutions as low as 1:10 appeared to reduce infections by Edmonston measles virus, but failed to fully protect the cells from infection (Figure 3.4C). Infections of B95-8 cells by the Montefiore 89 measles virus were not inhibited by anti-CD46/SCR1 (Figure 3.4A). These qualitative observations were confirmed by immunoblot analysis and plaque assays (data not shown). The preceding data provide further evidence that CD46 may not function as a receptor for the wild-type strain of measles virus, since CD46-specific antibodies had no effect upon infections by the Montefiore 89 strain of virus. Wild type virus presumably binds to an as yet unidentified receptor which is present on marmoset B cells. In addition, the Edmonston strain of virus was only partially inhibited by CD46 antibodies during infections of B95-8 cells, and it too may use this new hypothetical receptor under certain circumstances.

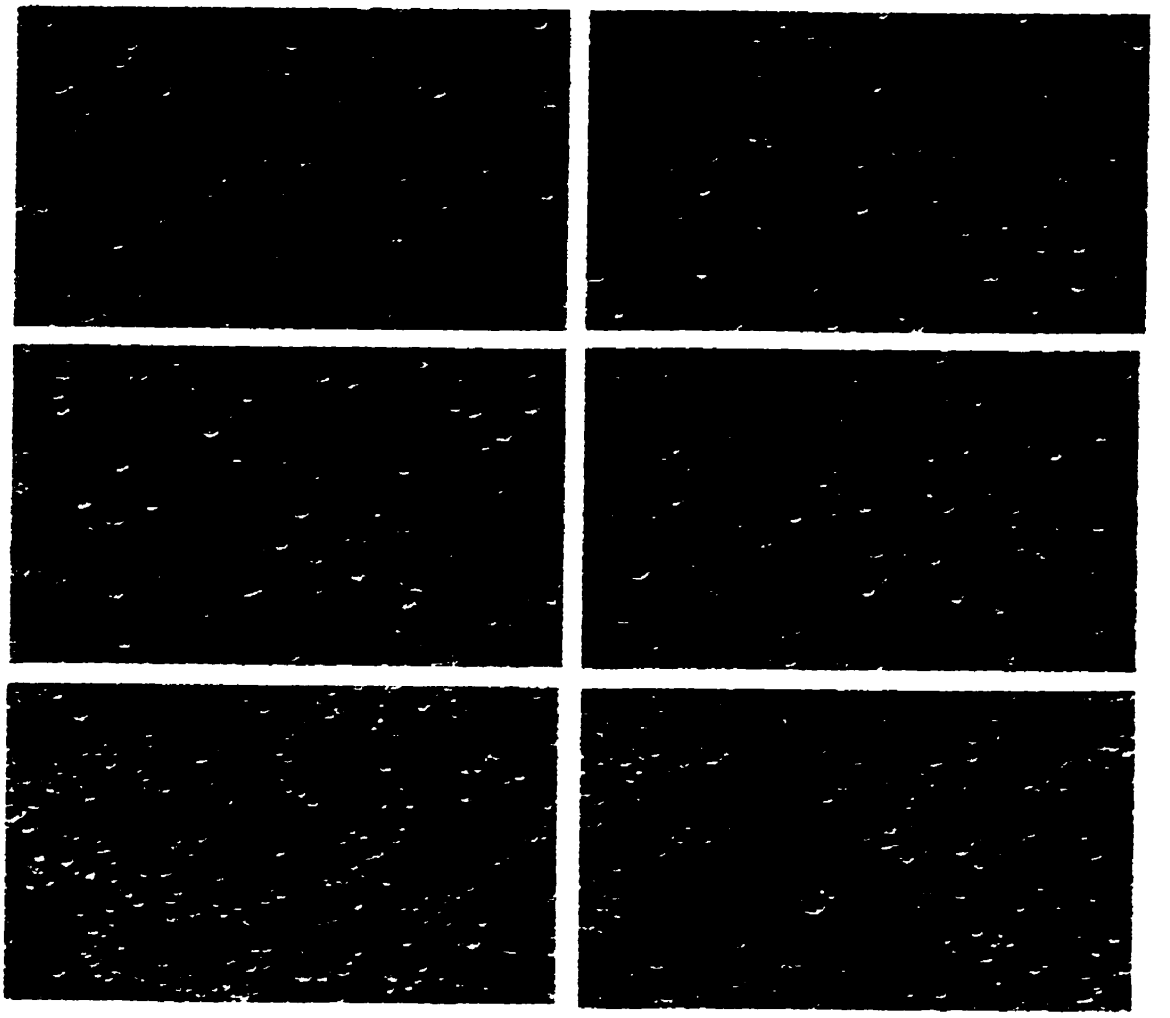
3.3.5 The hemagglutinin protein from the Montefiore 89 wild type strain of measles virus does not interact with CD46 in a direct binding assay

The ability of Montefiore 89 wild type and Edmonston hemagglutinin proteins to interact directly with CD46 or a receptor on immortalized B cells was measured in a binding assay that we have previously described (22). The cDNA

for the hemagglutinin protein of the Montefiore 89 strain was cloned and expressed on the surface of Sf9 insect cells using the recombinant baculovirus system. In addition, a marmoset/human CD46 chimeric molecule was constructed using the SCR1 and SCR2 domains from B95-8 cells and the SCR3, SCR4, STP, transmembrane, and cytoplasmic domains of human of CD46. The chimera was expressed in the mouse OST-7 cells using the recombinant vaccinia virus system in that same way that human CD46 was previously expressed in this mouse L cell line (22). Expression of chimeric CD46 on the surface of mouse OST-7 cells was confirmed by FACS scan analysis as previously described (22). The purpose of constructing the chimeric molecule was to determine whether subtle differences between human and marmoset CD46 molecules could account for the altered tropism of wild type virus for human and B95-8 cell lines. Sf9 insect cells expressing either Edmonston H or Montefiore 89 H, in addition to β-galactosidase, were stained blue by the addition of the enzyme substrate, Bluogal. The blue-colored insect cells were incubated with OST-7 cells expressing human CD46, marmoset/human chimeric CD46, or human CD21 molecules. Insect cells expressing H were also incubated with the marmoset B95-8 cell line. Sf9 cells which did not adhere to the target cells were washed away and binding of insect cells was first evaluated under the microscope (Figure 3.5). Insect cells which expressed the Edmonston H protein remained attached to mouse cells expressing human or chimeric CD46, as well as to the marmoset B95-8 cell line (Figure 3.5, panels A, C, and E). As we previously demonstrated (13,22), the Edmonston H protein did not bind to OST-7 cells, CHO cells, or to a mouse B cell line (data not shown). On the other hand, the wild type Montefiore H protein did not bind to mouse cells expressing human CD46 or chimeric CD46, but it did adhere to the marmoset B cell line (Figure 3.5, panels B, D, and F). Similar results were found with human lymphoma B cell lines grown in our laboratory where insect cells

Figure 3.5 Binding assays with H proteins from the Montefiore 89 and Edmonston strain of measles virus to mouse OST-7 cells expressing CD46 and marmoset B95-8 cells

Hemagglutinin proteins from the Montefiore 89 and Edmonston strains of measles virus were cloned and expressed on the surface of Sf9 insect cells using the recombinant baculovirus system. Sf9 cells also expressed β-galactosidase and were stained blue by the addition of Bluogal substrate. The blue-colored insect cells were incubated with mouse cells expressing human CD46 (*Panels A and B*), mouse cells expressing marmoset/human chimeric CD46 (*Panels C and D*) or B95-8 cells (*Panels E and F*) and loosely adsorbed Sf9 cells were washed away. Insect cells which expressed the Edmonston H protein remained attached to mouse cells expressing human or chimeric CD46 as well as to the marmoset B95-8 cell line (*Panels A, C, and E*). The wild type Montefiore H protein did not bind to mouse cells expressing human CD46 or chimeric CD46, but it did adhere to the marmoset B cell line (*Panels B, D, and E*). The solid bar in panel B represents 2.5 μm.



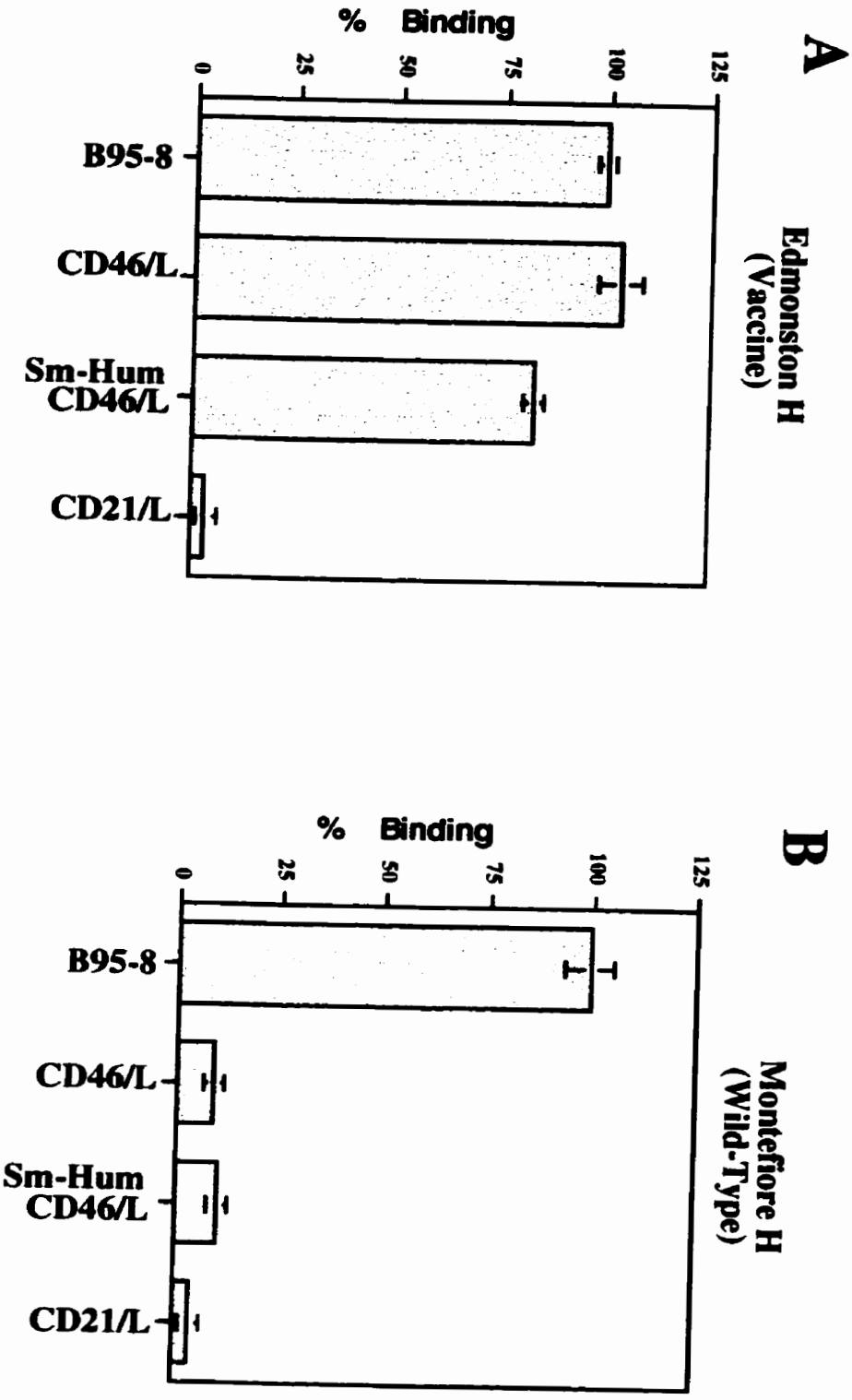
expressing wild type H or Edmonston H also bound to human BJAB and human 1A2 cells (data not shown). The binding results presented in the preceding experiments were quantitated and summarized in Figure 3.6 by measuring the hydrolysis of the β-glactosidase substrate, ONPG. We concluded that the H protein of the Edmonston vaccine strain of virus could bind to human CD46 or chimeric CD46 which was expressed on the OST-7 mouse L cells. The Edmonston H protein could also bind to receptors on the B95-8 cells (Figure 3.6A). On the other hand, the H protein of the Montefiore wild type virus did not bind to human CD46, chimeric CD46, or CD21, but did attach to some receptor which was present on B95-8 cells (Figure 3.6B). This receptor was not CD21, a molecule with similar properties to CD46, which is known to bind Epstein Barr virus. These direct binding assays clearly establish that wild type H protein interacts with some receptor other than CD46 which is present on the surface of marmoset B95-8 cells.

3.3.6 Amino acid residue 481 of the hemagglutinin molecule determines the viral protein's ability to bind to CD46 and reveals another receptor which is present on B95-8 cells

Measles virus isolates have been classified into 8 groups based upon nucleic acid sequence, year of isolation, and the country of isolation (45,48,49). Measles Montefiore 89 strain was isolated at the Montefiore Medical Center/Albert Einstein Medical College and appears to belong to Group 2 based upon its similarity (99.5% to 100% identity) to Chicago 1, San Diego 89, Illinois 89, Pennsylvania 90, Texas 89, and California 90 strains of measles viruses which were isolated between 1989-1990. The Montefiore 89 H sequence was 100% identical to that of California 90 measles virus. We aligned different hemagglutinin protein sequences from 59 wild type and 12 vaccine/laboratory strains of measles virus based upon data which had previously been entered in the NCIB Genbank. H proteins were aligned using the

Figure 3.6 Quantitation of Edmonston H and Montefiore H binding to mouse cells expressing human CD46, marmoset/human chimeric CD46, CD21 or to B95-8 marmoset cells

Sf9 insect cells expressing Edmonston or Montefiore 89 hemagglutinin proteins were incubated with B95-8 marmoset cells or mouse L cells expressing CD46, chimeric CD46, or CD21 as described in Figure 3.5. Loosely attached cells were washed away and binding was measured colorimetrically with the ONPG substrate for β-galactosidase. Edmonston H bound to marmoset B95-8 cells and mouse cells expressing human CD46 and chimeric CD46. It did not bind to mouse cells expressing CD21 (*Panel A*). Montefiore H bound to marmoset B95-8 cells but did not adhere to mouse cells expressing human CD46, chimeric CD46, or CD21 (*Panel B*). Binding is expressed as a percentage relative to either Edmonston H binding (*Panel A*) or Montefiore H binding (*Panel B*) to B95-8 cells.


A
% Binding
0
25
50
75
100
125
B95-8
CD46/L
Sm-Hum CD46/L
CD21/L
Edmonston H (Vaccine)
B
% Binding
0
25
50
75
100
125
B95-8
CD46/L
Sm-Hum CD46/L
CD21/L
Montefiore H (Wild-Type)

MegAlign Clustal program marketed by Lasergene (data not shown). The wild type strains had been propagated in marmoset B95-8 cells while the vaccine/laboratory strains were amplified in Vero monkey kidney cells. When the two different types of isolates were compared, differences were consistently observed at positions 211, 243, 276, and 481. Amino acids 451 and 481 had previously been implicated in determining hemadsorption, cell fusion and CD46 downregulation caused by vaccine strains of measles virus (29,57). Our alignments demonstrated that 97% of both wild type and vaccine strains contained a valine residue at residue 451 and only 5 wild type viruses conatained glutamic acid at this position. We concluded that this residue appeared to be irrelevant in determining the wild type virus phenotype. About a third of the wild type strains possessed an additional potential glycosylation site at position 416. The results of our alignments were related to the ability of individual isolates to infect either Vero or B95-8 cells. The results of these findings are summarized in Table 3.2. Wild type virus H proteins possessed an Asn resdidue at position 481 instead of the Tyr present in vaccine/laboratory viral H proteins. In all cases, viruses which contained a tyrosine at residue 481 were capable of infecting Vero cells, while those with an aspargine at this position grew in B95-8 cells but not the monkey kidney cell line. This change has previously been observed during successive passages of wild type measles isolates in Vero cells and may parallel the ability of virus to agglutinate monkey red blood cells (29,57). The gradual mutation of N481 to Y481 during adaptation of a Montefiore 83 wild type isolate to Vero cells over 10 passages was also observed by one of our laboratories (M.S. Sidhu, unpublished result). On the other hand, amino acid differences at positions 416 and 451 did not seem to affect the growth of wild type virus in Vero cells (Table 3.2). The N481Y mutation which occurs between the H proteins of wild type and vaccine/laboratory strains of measles virus

TABLE 3.2 Correlations of sequence variations in the H proteins from vaccine and wild type strains of measles virus and their ability to grow in Vero monkey kidney or B95-8 marmoset B cell lines

Virus	AA451[c]	AA481[c]	AA416[c]	B95-8[d]	Vero[d]
Vaccine Strains[a]	V	Y	D	+	+
Edmonston (Vero-cell adapted)	V	Y	D	+	+
Group 1 Wild Type[b] Edmonston, China 93-5	V	N	D	+	-
Group 2 Wild Type[b] Montefiore 89, Chicago-1	V	N	N	+	-
Group 3 Wild Type[b] Colorado 1994	V	N	N	+	-
Group 4 Wild Type[b] New Jersey 94	V	N	D	+	-
Group 5 Wild Type[b] Tennessee 94	K	N	D	+	-
Group 8 Wild Type[b] China 93-1	V	N	D	+	-

[a] Moraten, Rubeovax, Zagreb, Schwarz, and AIK-C vaccine strains were tested.

[b] All wild type viruses were initially isolated using B95-8 cells.

[c] Predicted amino acids in the H protein including potential glycosylation site at residue 416.

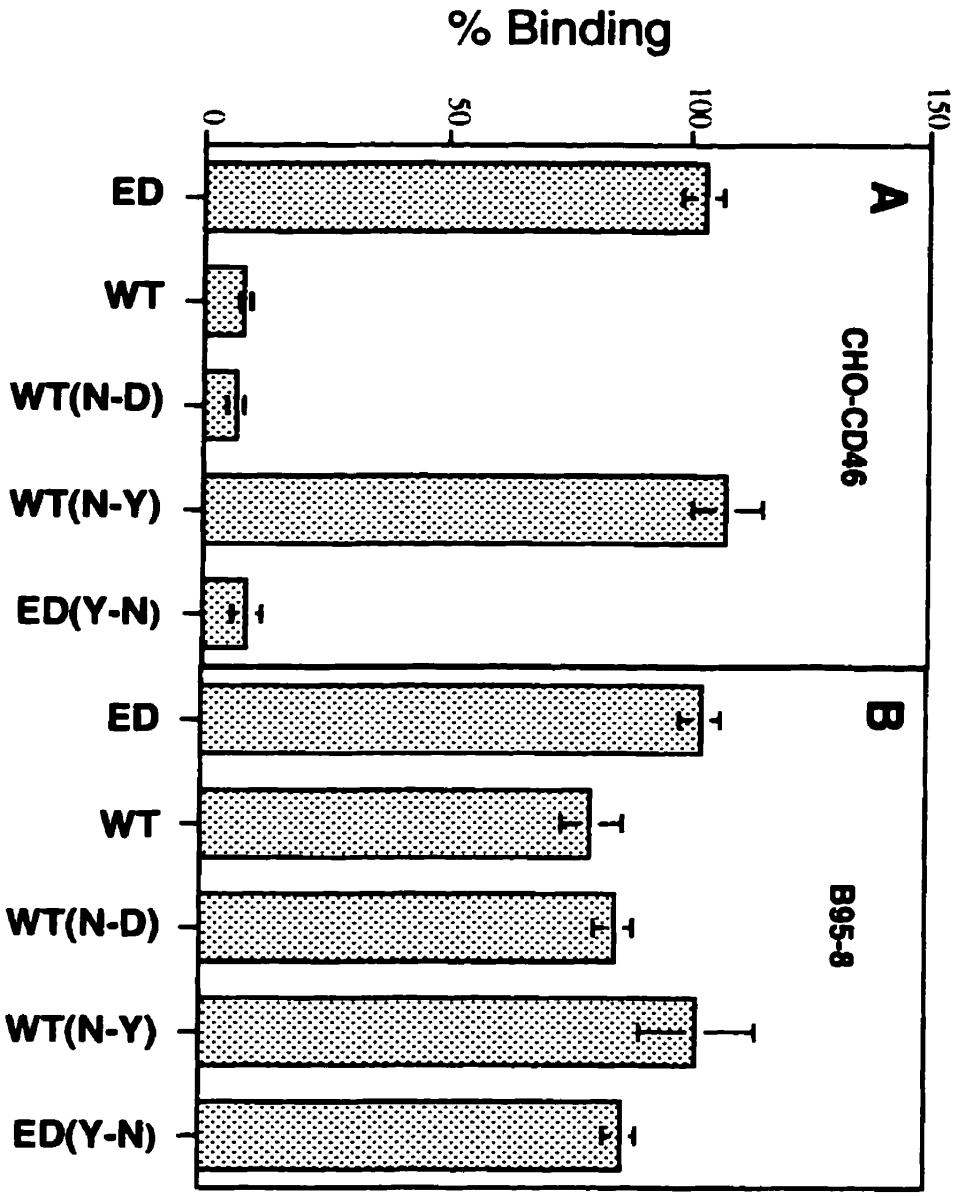
[d] B95-8 and Vero cells were infected and examined for the presence (+) or absence (-) of cytopathic effect and viral protein synthesis.

can beattributed to a single nucleotide change (AAC⇒ TAC). It is not yet known whether this change also occurs *in vivo* during natural infections.

In order to determine whether the N481Y change controls the ability of measles hemagglutinin protein to bind either to CD46 or to a new unidentified receptor which is present on B95-8 cells, mutations were introduced into the H protein at this position using site-specific mutagenesis. Mutated H proteins from wild type and Edmonston vaccine strains of measles virus were expressed in Sf9 insect cells and incubated with Vero, HeLa, CD46-CHO, or marmoset B95-8 cells. Binding of insect cells expressing the mutated H proteins, Montefiore 89 wild type H, or Edmonston H was assayed with CD46-CHO cells and the marmoset B cell line, and was subsequently quantitated with the ONPG assay for β-galactosidase (Figure 3.7). The Edmonston H protein bound to both the CD46-CHO and B95-8 cells while the wild type H protein attached only to the B95-8 cells. Mutations N416D, which abolished the potential glycosylation site, had little effect on binding of the wild type H to either CD46-CHO or B95-8 cells. However the N481Y mutation which was introduced into wild type H protein now permitted this glycoprotein to bind to CD46-CHO cells (Figure 3.7A). Similarily, when the Y481 was changed to N in the Edmonston H protein, attachment to CD46-CHO cells was abolished (Figure 3.7A). However, the Y481N change had no effect upon the binding of Edmonston H protein to B95-8 cells, and it would appear that this mutation did not perturb interaction with the putative receptor which is present on marmoset B95-8 cells (Figure 3.7B). Similar results were reproduced in mouse OST-7 cells which were infected with CD46 recombinant vaccinia virus, Vero cells, or HeLa cells following incubation with insect cells expressing mutant H proteins (data not shown). We concluded that the tyrosine residue at position 481 in the measles virus hemagglutinin molecule was a key determinant of H protein binding to the CD46 receptor. However, mutations at amino acid 481 did not impair

Figure 3.7 Tyr481Asn mutation inhibits the binding of Edmonston and Montefiore 89 hemagglutinin proteins to CD46

Edmonston H (*ED*), Montefiore H (*WT*), and mutated forms of these proteins were expressed in Sf9 insect cells and incubated with CHO cells containing human CD46 (*Panel A*) or marmoset B95-8 cells (*Panel B*). As expected, insect cells expressing Edmonston H (*ED*) bound to both CHO-CD46 and B95-8 cells, while wild type Montefiore 89 H (*WT*) protein only bound to B95-8 cells. The N416D mutation introduced into Montefiore 89 H (*WT N-D*) had no effect on binding to either cell line. However a N481Y mutation in the wild type H (*WT N-Y*)converted the protein to a CD46-binding phenotype. In addition when the Y481N mutation was placed in Edmonston H (*ED Y-N*), binding to CHO-CD46 cells was abolished. None of the mutations affected the binding of either Edmonston (*ED*) or wild type H (*WT*) proteins to marmoset B95-8 cells. Binding was measured by quantitating β-galactosidase activity and was expressed as a percentage relative to the binding observed for Edmonston H (*ED*) protein.


% Binding
0
50
100
150
A
CHO-CD46
ED
WT
WT(N-D)
WT(N-Y)
ED(Y-N)
B
B95-8
ED
WT
WT(N-D)
WT(N-Y)
ED(Y-N)

binding to the marmoset B cell receptor and these results suggest that another region of the measles H protein may interact with this as yet unidentified cell surface protein.

3.4 Discussion

This paper provides direct evidence for the existence of another receptor for measles virus, in addition to CD46, which is present on marmoset B cells. Through sequence analysis and binding assays with hemagglutinin molecules from wild type and Vero cell-adapted isolates of measles virus, it was apparent that Tyr481 of the H protein was critical in determining whether this protein bound to CD46. H proteins from attenuated vaccine strains and laboratory isolates of measles virus which had been propagated in Vero cells all contained this Tyr at position 481 while wild type isolates which had been propagated in marmoset B95-8 cells produced a protein with Asn481. Binding assays indicated that wild type H molecules did not interact with human or marmoset CD46 molecules but rather bound to another receptor which was present on B95-8 cells. Although polyclonal antibodies directed against CD46 and SCR1 reduced infections by the Edmonston laboratory strain of virus, they could not totally inhibit viral infections in B95-8 cells, even at very high concentrations. A decrease in infection by the Edmonston virus in these cells, when antibody is present, may reflect a smaller number of functional receptors and reduced binding affinity to the second receptor for the attenuated virus. Further studies will eventually clarify this situation. These same antibodies had no effect upon wild type viral infections in the B95-8 cell line. The fact that CD46 antibodies were very effective in blocking infections by the Edmonston virus in Vero monkey kidney cells coupled with the ability of the H protein containing the Y481N mutation to retain its binding properties for B95-8 cells, appeared to indicate that the Edmonston H protein may bind to either CD46 or the wild type virus receptor. There may be 2 separate sites on the Edmonston H protein which could bind to either CD46 or the unidentified receptor on marmoset B cells, but this remains to be confirmed.

Amino acids 211, 243, and 276 of the measles virus H protein consistently vary between attenuated laboratory and and wild type strains of virus. The role of these amino acids in binding to the host cell is still not apparent. One publication has suggested that amino acids 211-214 may contribute to the CD46 binding site (51) while amino acids 451-617 appear to constitute the primary receptor binding site which is involved in hemagglutination (51,57). Antibodies directed against regions spanning amino acids 185-195 have also been reported to inhibit hemagglutination (63) but may not be involved directly in binding to CD46. However, our evidence indicates that amino acids 211, 243, and 276, which differ between attenuated and wild type strains, may not be extremely important for binding to CD46, since wild type H containing the mutation N481Y binds just as efficiently as Edmonston H to cells expressing CD46 on their surface. The role these amino acids play in binding to the wild type viral receptor remains to be determined. Other investigators have suggested that the binding site for CD46 lies between residues 451 and 617 (12,17,51,57) and this seems to agree with our findings.

Results indicating the existence of a second receptor for measles virus which are presented in this paper partially explain why marmosets are susceptible to infections by measles virus in spite of the deleted SCR1 domain in their CD46 molecules. Our data also explain why wild type measles virus grown in B95-8 cells does not hemagglutinate African green monkey red blood cells in spite of these cells having CD46 on their surface. This second receptor obviously plays a critical role during infections initiated by wild type isolates of measles virus. The virus is normally spread as an aerosol to the nasopharynx. However, the primary cellular target for the virus is not known for certain. During the acute phase of measles virus infection the virus undergoes primary replication in the respiratory tract and disseminates throughout the body via the reticuloendothelial system. The virus has

been reported to undergo a round of secondary replication in lymphoid tissues, and monocytes are efficiently infected by the virus (15). Isolates of wild type virus were normally obtained as throat swabs containing mucosal epithelial cells and lymphocytes which were subsequently propagated in B95-8 cells. Tracheal and bronchial epithelial cells are thought to be the primary target cell for measles virus, but lymphocytes and monocytes in the local lymph nodes are also infected by the virus at very early stages. Virus spreads to the thymus, spleen, skin, conjunctivae, kidney, lung, gastrointestinal tract, respiratory mucosa, small blood vessels, and liver. Endothelial cells, epithelial cells, monocytes, macrophages, and lymphocytes are all target cells which support virus growth *in vivo* (19,37). The characteristic rash is caused by infiltration of macrophages to areas of skin endothelium that have been infected by the virus. The role that two different receptors play in natural measles virus infections remains to be determined. Since CD46 normally prevents complement lysis of the host cell (33,34) and has been shown to be down-regulated on the surface of the infected cell (4,20,27,40,52,53), it might not be advantageous for the virus to use this receptor under certain circumstances.

The shift in tropism which accompanies the N481Y mutation in wild type H molecules as they adapt to growth in culture requires a minor one nucleotide change (AAC⇒TAC). It is unclear whether this single base mutation also occurs *in vivo* during measles virus infections. Changes in the H protein could determine whether infections of lymphocytes, epithelial cells, or endothelial cells were favored. Precedent for a change in cellular tropism during an ongoing infection exists since it was recently shown that many HIV-1 infections are initially macrophage tropic, but through minor changes in the V3 loop of the envelope protein shift to become T cell tropic, and subsequently use the CXCR4 coreceptor for entry (reviewed in 7,31; 11). Minor changes in the viral attachment proteins of other viruses have also been associated with changes in receptor usage. Coxsackie B viruses can use either

CD55 (5) or CAR (6) as receptors depending upon whether they have been adapted to growth in a rhadomyosarcoma cell line or HeLa cells, respectively. Ross River virus is a member of the togavirus family and varies its tropism between small mammals, chicken fibroblasts, mosquitoes, and man with single amino acid changes in the E1 and E2 viral membrane proteins (24,59). Finally 2 amino acid changes in the S protein of transmissible gastroenteritis coronavirus of pigs were recently found to abolish enteric tropism to favor respiratory infections (3). Thus, small changes in viral glycoproteins routinely dictate what receptor can be utilized and influence the type of cell which can be infected. It is quite possible that wild type isolates of measles virus contain a mixture of virions which can use either CD46 or a new receptor found on B cells. In addition, small changes in the H protein are also associated with neurovirulent strains of measles virus in rats (32), which do not have a CD46 analogue, and this may reflect altered receptor usage in the brain. Since all wild type measles strains are currently being isolated with B95-8 marmoset B cells or activated human B cell lines, virions are now being selected in the laboratory which characteristically bind to the new unidentified B cell receptor.

The involvement of more than one cellular molecule in virus attachment and penetration in measles virus infections seems more than likely. The envelope glycoprotein of HIV-1 attaches to CD4 and a variety of chemokine receptors (reviewed in 7). This may also be the case with Coxsackie A viruses which were recently shown to tightly bind to ICAM-1 but also interact with the low affinity receptor CD55. Coimmunoprecipitation and chemical cross-linking studies seemed to indicate that CD55 and ICAM-1 are closely associated on the cell surface (56). Thus, one might expect other proteins to interact with CD46 which could act to pull the measles virus closer to the cell membrane in order to facilitate fusion with the cell membrane.

The new unidentified receptor for measles virus appears to be on marmoset B cells which have been immortalized and transformed by Epstein Barr virus (EBV). We tested immortalized human B cells and they too bound the H protein from Montefiore 89 wild type virus, while mouse B cells were less efficient in this process (data not shown). Previous investigators have claimed that measles virus can infect mouse B cells, which do not express CD46, and this suggests that the newly identified receptor may also be present on mouse lymphocytes (21). The identity of this new receptor for measles virus is unknown, but we are presently searching for and attempting to further characterize it in our laboratory. It also remains to be determined whether other coreceptors participate in the membrane fusion and internalization process of measles virus.

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Chapter 4: Use of Site-Specific Mutagenesis and Monoclonal Antibodies To Map Regions of CD46 Which Interact With Measles Virus H Protein[3]

[3] The results from this chapter have been published in the *Virology* 258: 314-326 (1999). I have performed all the experiments in Figures 4.1 to 4.4 and Tables 4.1 to 4.2. Sarah Sabatinos has assisted me in generating some of the hydrophobic mutations.

4.1 Introduction

Measles virus infects about 44 million individuals and is responsible for 1.5 million deaths each year. It is still the number one infectious killer of children in developing countries (9, 42). In spite of the success of immunization with an attenuated viral vaccine, numerous measles outbreaks are still reported in both Europe and North America (9). Measles virus is a member of the morbillivirus genus, a subgroup of the paramyxovirus family and the Edmonston isolate of measles has been used to generate the attenuated vaccines which are widely used today (13, 15, 37). It is an enveloped virus with a single-stranded negative RNA genome encoding 6 gene products: nucleocapsid protein (N) for viral RNA protection, matrix protein (M) which mediates virion assembly, phosphoprotein (P) and large polymerase protein (L) for viral transcription and replication, fusion protein (F) for membrane fusion and penetration of the host cell membrane, and hemagglutinin (H) for attachment to the host cell (11, 14).

Membrane cofactor protein (MCP) or CD46 was previously identified as the host cell receptor for the Edmonston laboratory strain of measles virus by two independent groups (10, 34). CD46 is a member of the regulators of complement activation (RCA) family of proteins, and its gene is located on human chromosome 1 (25, 26). Complement regulators such as CD46 (MCP) and decay accelerating factor (DAF), interfere with the formation of the complement attack complex on the membrane of normal cells and prevent deregulated complement cell lysis of the host cell. Therefore, CD46 plays a critical role in protecting uninfected self tissue from complement mediated damage. The gene coding for CD46 consists of 14 exons and 13 introns, and spans more than 43 kb of genomic DNA (25). It is a type I membrane glycoprotein and possesses a 34 amino acid signal peptide at its amino terminus which is subsequently removed during post-translational modification in

the endoplasmic reticulum. The extracellular portion of the protein consists of 4 domains or short consensus regions (SCR1-4) which characterize RCA proteins, followed by a ser/thr/pro rich sequence (STP) situated just above the membrane. In addition CD46 has a hydrophobic transmembrane domain and a cytoplasmic C-terminal tail specified by either exon 13 or exon 14. N-glycosylation occurs on SCR1, 2, and 4 while the STP region contains O-glycosylation sites. Differential glycosylation and alternative splicing of the STP region and cytoplasmic tails gives rise to different isoforms of CD46 with molecular weights ranging from 57 to 67 kDa.

In recent years, interaction between CD46 and measles virus H protein has been studied extensively. For example, monoclonal antibodies directed against SCR1 or SCR2 are capable of blocking viral infections in culture (6, 16, 18, 30, 39). In addition, CD46 deletion mutants and recombinant chimeric molecules composed of CD46 and DAF or CD46 and CD4 revealed that both the SCR1 and the SCR2 domains of CD46 were required for binding to measles virus culture (6, 16, 18, 30). Eliminating the N-linked glycosylation sites by mutagenesis proved that only the carbohydrate associated with SCR2 is important for virus binding (16, 27, 28). Specific regions of the SCR1 and SCR2 domains have also been implicated in binding by different groups, including ours, using either inhibitory peptides in viral infectivity assays or through use of site-specific mutants in binding assays (6, 16, 29). In a previous publication we demonstrated that a number of hydrophilic residues in CD46 including E36/E37, E45, E58/R59, and R103/D104, appeared to be important for binding to the measles virus H protein (16). Our laboratory recently demonstrated that a mutation at position 481 of the measles virus (Edmonston) hemagglutinin protein, from tyrosine (Y) to asparagine (N), completely abolished binding with CD46 (17). Thus, it appeared that hydrophobic interaction may also be important for binding between measles H protein and

CD46. Therefore, we proposed to systematically replace hydrophobic residues located in SCR1 and SCR2 domains of CD46 with the neutral amino acid, serine, and study the effect of these mutations with respect to measles H protein-CD46 interaction. In this communication, we have identified hydrophobic residues which are important for this interaction, using a binding assay that was previously developed in our laboratory (16). In addition, monoclonal antibodies directed against SCR1 or SCR2 domains of CD46 which inhibited binding to H, mapped near the regions where amino acid substitutions produced the most deleterious effects.

4.2 Materials and Methods

4.2.1 Cell lines and virus

HuTK-143B cells and mouse OST-7 cells, which expressed T7 polymerase, were used in isolating and expressing vaccinia virus recombinants, respectively. They were purchased from ATCC (Rockville, MD). These cells were cultured in Dulbecco's minimal essential medium containing 10% fetal calf serum as previously described (Hsu et al., 1997). Sf9 insect cells were obtained from Invitrogen (San Diego, CA) and cultured in Grace's medium containing 10% fetal calf serum. The Montefiore 89 isolate of measles virus was obtained from M. Sidhu at Wyeth-Lederle Pediatric Vaccine Laboratories and the Edmonston laboratory strain of measles virus, which had been adapted to Vero cells, originally came from E. Norrby (Karolinska Institute, Stockholm, Sweden).

4.2.2 Antibodies

The monoclonal antibody E4.3 (specific for SCR1) was purchased from Bio/Can Scientific (Mississauga, Canada). M75 (specific for SR2) came from Seikugaku (Tokyo, Japan). J4-48 was supplied by Serotec (Kidlington Oxford, UK). 122-2 (specific for SCR2) was purchased from Biodesign International (Kennebunk, ME), and B97 (specific for SCR1) came from the laboratory of J. Schneider-Schaullies and V. ter Meulen (Universitat Würzburg).

4.2.3 Mutagenesis of CD46 coding sequences and DNA sequencing

SCR1 and SCR2 domains were altered with point mutations using gel-purified oligonucleotides consisting of 30 to 40 nucleotides. Mutations were introduced into the CD46 molecule by using the QuickChange site-directed

mutagenesis kit from Stratagene. The CD46-coding region (isotype C2) was cloned into PCR-Script Amp SK(+) plasmid, denatured, and annealed with two complementary oligonucleotides containing the desired mutation, the mutagenized DNA strands were extended with *Pfu* polymerase, the methylated nonmutated parental DNA template was digested with Dpn I, and the mutated plasmid was used to transform XL2-Blue ultracompetent cells. Mutant plasmids were isolated and the CD46 inserts were completely sequenced. The mutagenized CD46 coding regions were excised from the PCR-Script plasmid following digestion with BspHI (5'end) and Bgl II (3'end) and ligated into the vaccinia vector pTM1 (containing the T7 promoter) which had been digested with the compatible restriction enzymes, Nco I and BamH I. Vaccinia virus recombinants were prepared and titered by plaque assays as previously described using HuTK$^-$ 143B cells and bromodeoxyuridine selection (12). CD46 mutants were expressed in mouse OST-7 cells which contained the T7 polymerase and protein synthesis and surface expression was monitored by Western immunoblot and FACS scan analysis.

4.2.4 Binding assays performed between mutant CD46 molecules and insect cells expressing Edmonston measles virus H protein

We previously developed a binding assay which measured the interaction between CD46 molecules expressed in mouse fibroblasts and Sf9 insect cells expressing both the measles virus H protein and ß-galactosidase (16, 17). Mutant CD46 coding sequences were cloned into the pTM1 vaccinia expression vector which uses the T7 promoter to direct transcription of the foreign gene. Recombinant vaccinia virus was prepared as previously described (Earl et al., 1995) and mutant CD46 molecules were expressed in mouse OST7 cells which contain the T7 polymerase. Sf9 insect cells were infected for 48 hr with a recombinant baculovirus (41) which had been generated with the BlueBac2

expression vector (Invitrogen, San Diego, CA) (22, 36) and synthesized both the measles virus H protein and ß-galactosidase. The insect cells were colored blue by adding Bluogal (GIBCO/BRL, Grand Island, NY) at 36 hr infection from a stock solution (50mg/ml in dimethylformamide) to give a 0.05% (w/v) final concentration. Infections with recombinant vaccinia virus were allowed to proceed 12 hrs, Sf9 insect cells expressing the measles H protein and ß-galactosidase were incubated for 1/2 hour with the vaccinia-infected mouse OST7 cells in the presence of PBS containing 5% fetal calf serum. Non-adsorbed insect cells were eluted by washing the mouse cells 2 times with PBS. Binding in 25 cm^2 tissue culture flasks could be quantitated visually under the microscope or quantitatively by the hydrolysis of o-nitrophenylgalactoside (ONPG) using a ß-galactosidase assay kit (Stratagene, La Jolla, CA). Cells were lysed in 0.5 ml of 100mM sodium phosphate buffer (pH 7.5) containing 0.2% Triton-X 100 and 1% NP-40. Aliquots of lysate (50 µl) were added to a 96-well microtitration plate and freshly prepared buffer (100 mM sodium phosphate, 10 mM KCl, 1mM $MgSO_4$, 50mM ß-mercaptoethanol, pH7.5) was mixed with each aliquot to give a final volume of 160 µl. A 50µl volume of ONPG solution (4 mg/ml in 100 mM sodium phosphate, pH 7.5) was added to each well and incubated at 37° until a yellow color was evident after about 30 min. The reaction was terminated by addition of 90µl of 1M Na_2CO_3 and color intensity was measured at 420 nm with a SpectraMax 250 ELISA plate reader purchased from Molecular Devices (Sunnyvale, CA). Results were linear over time for an absorbance range of 0.1-1A420 units.

4.2.5 Fluorescence cytometry of CD46 mutant proteins

Mouse OST7 cells (2 x 10^6 cells) which expressed mutant forms of CD46 were suspended in 1 ml of Cell Dissociation Buffer (Sigma, St. Louis, MO) and washed 2 times by centrifugation with FACS buffer. Cells were resuspended in

100 µl of the same buffer containing 1:100 dilution of either non-immune antibody or a specific monoclonal antibody (E4.3, J4-48, M75, 122-2, or B97) for 1 hour on ice. Following the incubation, cells were washed twice with 3 ml of FACS buffer by centrifugation at 1500g for 5 min. The cells were resuspended in 100 µl of the same buffer containing a 1:100 dilution of fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG(H+L) for 1 hour on ice. After washing twice with the 3 ml FACS buffer, the blood cells were suspended in 0.5 ml of FACS buffer and were subsequently analyzed on a Beckton Dickinson analyzer equipped with 15 mW argon laser at 488 nm. The data were collected and analyzed using CellQuest software. The final pellet was suspended in 100 µl FACS buffer prior to analysis by fluorescence cytometry. The reactivity of the different monoclonal antibodies with the mutagenized portions of CD46 was compared to reactivity with the wild type molecule. Fluorescence from a standard monoclonal (either J4/48 or 122-2) reacting with the non-mutagenized SCR was first normalized to the same level of fluoresence as from wild type CD46. In most cases non-normalized values were already within 10% of each other. The obvious exception was when the mutation effected surface expression of CD46. Following normalization, the signal due to a specific monoclonal antibody reacting with the mutagenized SCR was computed as a geometric mean. The mean fluorescence due to a monoclonal antibody reacting with mutant CD46 was compared to that from the wild type CD46 and expressed as a percentage.

4.2.6 Molecular modeling

Changes in CD46 which reduced binding or recognition by inhibitory monoclonal antibodies were mapped on a structural model of the SCR1 and SCR2 domains which was provided by Drs. R. Cattaneo (University of Zürich) and Dr. W. Braun (University of Texas Medical Branch, Galveston) (6). This model was a

simulation performed with the DIAMOD and FANTOM modeling programs based upon the related structure of Factor H (3, 33). Models were viewed with the RasMol 2.6 Molecular Graphics Visualization Program (Dr. R. Sayle, Glaxo Wellcome, UK). Four possible configurations were reported, but only one model (M1) is consistent with both our laboratories' observations.

4.3 Results

4.3.1 Confirmation of mutant CD46 expression by fluorescence cytometry and immunoblot analysis

Mutation of a single hydrophobic residue (Y481) in the hemagglutinin protein from the Edmonston strain of measles virus appeared to abolish binding to CD46 (17). This observation would suggest that the hydrophobic interactions may play a critical role in the contact between these two proteins. With this hypothesis in mind, we systematically replaced each of the hydrophobic residues in the SCR1 and 2 domains of CD46 with the neutral amino acid serine, and expressed these mutant CD46 molecules in mouse OST-7 cells by using recombinant vaccinia viruses (Figure 4.1A). The surface expression of all the CD46 mutant molecules was quantitated by fluorescence cytometry (Figure 4.1B) and expression of CD46 mutants in mouse OST-7 cells was confirmed by immunoblot analysis (Figure 4.1C). Analysis of mutant CD46 expression and binding assays were performed 10 hr after the OST-7 cells were infected with recombinant vaccinia viruses. This ensured that the CD46 target cells were intact and that receptor surface expression was fairly close to physiological levels. The reactivity of the different monoclonal antibodies with the mutagenized portions of CD46 was compared to reactivity with the wild type molecule. Fluorescence from a standard monoclonal (either J4/48 or 122-2) reacting with the non-mutagenized SCR was first normalized to the same level of fluoresence as from wild type CD46. In most cases non-normalized values were already within 10% of each other. The obvious exception was when the mutation effected surface expression of CD46. Following normalization, the signal due to a specific monoclonal antibody reacting with the mutagenized SCR was compared to that from the wild type SCR and expressed as a percentage. Similar levels of recombinant CD46 were present on the cell surfaces of mouse OST-7 cells

Figure 4.1 Site-specific mutations which were introduced into the SCR1 and SCR2 domains in order to evaluate the importance of hydrophobic interactions during binding of the measles virus H protein (Edmonston strain) to CD46

(A) The amino acid sequence of CD46 is depicted, with SCR1 and SCR2 domains separated, demonstrating the individual hydrophobic residues which were mutated to serine (S). (B) Binding of mutated CD46 molecules to monoclonal antibodies was assessed by fluorescence cytometry in order to demonstrate cell surface expression of the various mutant proteins and to map the reactivity of various monoclonal antibodies to antigenic epitopes. Suspended OST-7 cells expressing mutated CD46 molecules were incubated with the appropriate monoclonal antibody (J4/48, E4.3, B97, M75, or 122-2) and binding was detected with FITC-labeled goat anti-mouse IgG using a Beckton Dickinson analyzer equiped with a 15 mW argon laser at 488 nm. The data were collected and analyzed using CellQuest software. Cell counts are indicated on the y-axis and the logarithm of the fluorescence intensity is represented on the x-axis. These representative plots depict reactivity of SCR1-specific (B97 and J4/48) and SCR2-specific (M75 and 122-2) monoclonal antibodies to wild type CD46 and mutant CD46 molecules (Y70S and L133). The positive and negative controls for antibody binding are CD46 wild type (WT) and non-immune IgG1, respectively. Fluorescence from a standard monoclonal (either J4/48 or 122-2) reacting with the non-mutagenized SCR was first normalized to the same level as that from wild type CD46. For the majority of mutants, the non-normalized fluorescence values were within 10% the levels found for wild type CD46, indicating efficient cell surface expression of the mutant proteins. Following normalization, the signal due to a specific monoclonal antibody reacting with the mutagenized SCR was computed as a geometric mean. The reactivity of the different monoclonal antibodies with the mutagenized portions of CD46 was compared to that with wild type CD46. (C) Western blot analysis with the monoclonal antibodies J4/48 and E4.3 were used to confirm expression of mutant CD46 molecules in mouse OST-7 cells infected with recombinant vaccinia virus. In this example, the effects of 8 individual mutations in SCR1 and SCR2 on recognition by J4/48 and E4.3 are demonstrated. Molecular weight protein standards (Daltons) are located to the left of the blot. Absence of reactivity (L46/47S, Y53S, Y54S, V60S, and Y62S) help define the antigenic epitope recognized by J4/48 and E4.3 antibodies. A non-specific protein band migrating with a molecular mass of 60 kDa is also evident. Expression of all mutant CD46 proteins in OST-7 cells was verified by probing immunoblots with polyclonal CD46 antibodies.

A

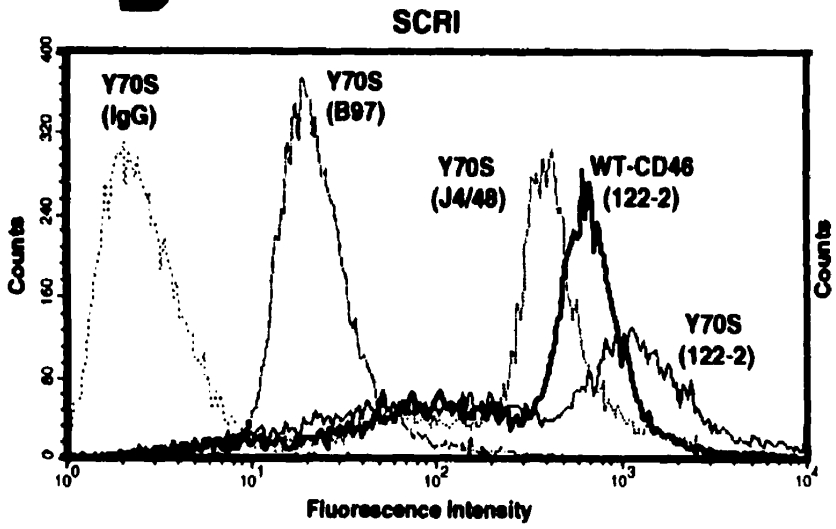
SCRI

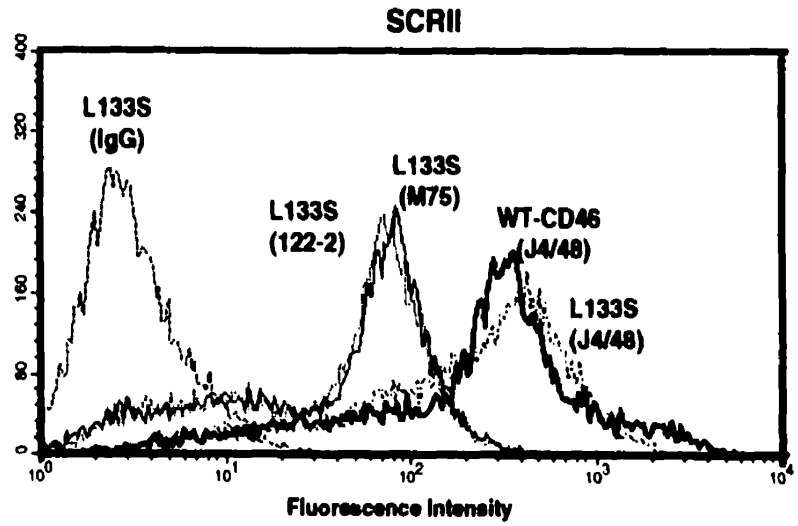
```
35              45              55              65              75              85              95
CEEPPTFEAMELIGKPKPYYEIGERVDYKCKKGYFYIPPLATHTICDRNHTWLPVSDDACYRET
        S   S  SS       SS S    S  S       SSSS  S     S         S S S
```

SCRII

```
100             110             120             130             140             150
CPYIRDPLNGQAVPANGTYEFGYQMHFICNEGYYLIGEEILYCELKGSVAIWSGKPPICE
   SS    S       S       S S S  S  SS     SSSS   SSS  S    S SS       S
```

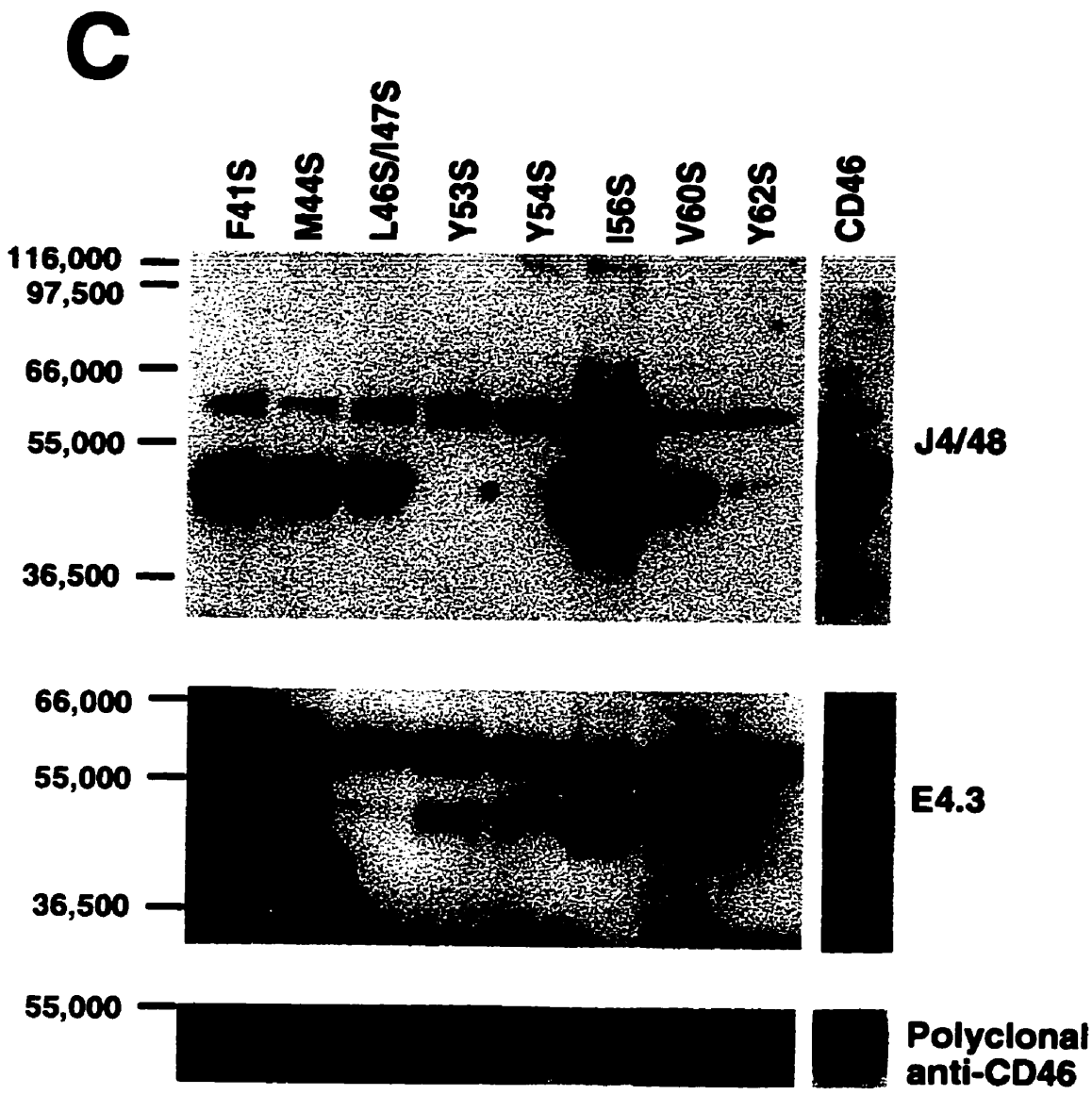
B





	Geo Mean
Y70S (IgG)	2.67
Y70S (J4/48)	222.43
Y70S (122-2)	258.61
Y70S (B97)	62.81
WT-CD46 (122-2)	265.23

	Geo Mean
L133S (IgG)	3.20
L133S (J4/48)	168.08
L133S (122-2)	32.96
L133S (M75)	33.49
WT-CD46 (J4/48)	189.10


C
F41S
M44S
L46S/I47S
Y53S
Y54S
I56S
V60S
Y62S
CD46
116,000
97,500
66,000
55,000
36,500
J4/48
66,000
55,000
36,500
E4.3
55,000
Polyclonal
anti-CD46

expressing most of the CD46 mutants. Mean fluorescence values had a statistical error of ±15%. However, CD46 molecules containing the mutations Y62S and W86S, were not exposed on the cell surface (Tables 4.1 and 4.2) even though immunoblot analysis showed that these proteins were synthesized within the cell. We suspected that these mutations disrupted the folding of CD46 during post-translational processing and prevented the export of this protein to the cell surface.

4.3.2 Binding assays between mutant CD46 molecules and the measles virus H protein

We previously developed a binding assay which measured the interaction between CD46 molecules expressed in mouse fibroblasts and Sf9 insect cells expressing both the measles virus H protein and ß-galactosidase (16, 17). Mutated forms of CD46 were expressed in mouse OST-7 cells using vaccinia virus expression vectors, and Sf9 insect cells were infected for 48 hr with recombinant baculoviruses derived with the vector with the BlueBac II vector, which expressed Edmonston H protein in addition to β-galactosidase. Binding assays between infected insect cells and mouse cells were performed at 37° C for 30 minutes. Nonadherent insect cells were washed away and binding between mutant CD46 molecules and Edmonston H was quantitated with the enzyme substrate o-nitrophenylgalactoside (ONPG) as shown in Figure 4.2 The results with the mutant CD46 molecules were compared to binding obtained with normal CD46. Each experiment was performed 3 separate times in triplicate and values were expressed as standard deviations from the mean. We only considered major decreases in binding to H protein as being relevant.

Mutation of hydrophobic residues at the amino terminus of SCR1 appeared to have very little effect on the binding of CD46 to measles virus H protein. However, substitution of tyrosine at amino acid 54 to serine (Y54S) led to a 30

TABLE 4.1 Site-specific mutagenesis of CD46 SCR1 domain, cell surface expression, and reactivity of CD46 mutant proteins with specific antibodies

Mutation[a]	Surface[b] Expression	Reactivity with monoclonal antibody J4/48[c]	E4.3[c]	B97[d]
CD46 C2	+++	+++	+++	+++
E36A/E37A	+++	-	-	+++
F41S	+++	+++	+++	+++
E42A	+++	+++	+	+++
M44S	+++	+++	+++	+++
E45A	+++	+++	+	+++
L46S/I47S	+++	++	-	+
K49A/K51A	+++	-	-	+++
Y53S	+++	-	+	+++
Y54S	+++	-	+	+++
E55A	+++	+++	+	+++
I56S	+++	+++	+++	+++
E58A/R59A	+++	-	+	-
V60S	+++	+	++	+++
D61A/K63A	+++	+++	+++	-
Y62S	-	+	-	-
K65A/K66A	+++	+++	+++	+++
Y68S	+++	+++	+++	+++
F69S	+++	+++	+++	+++
Y70S	+++	+++	+++	+
I71S	+++	+++	+++	+++
L74S	+++	+++	+++	+++
H77A	+++	+++	+++	+++
I79S	+++	+++	+++	+++
D81A/R82A	+++	+++	+++	+++
N83A	+++	+++	+++	+++
H84A	+++	+++	+++	+++
W86S	-	+	+++	-
L87S	+++	+++	+++	+++
V89S	+++	+++	+++	+++
D91A/D92A	+++	+++	+++	+++

[a]The CD46 molecule (isotype C2) was subjected to site-specific mutagenesis as described previously. The position of the mutation is indicated by the amino acid residue numbered from the amino terminus of the polypeptide, and includes the signal peptide prior to post-translational cleavage. The letter to the left of the number indicates the original amino acid, while the letter to the right indicates the change.

[b]The mean fluorescence of CD46 mutant proteins which were expressed on the surface of mouse OST-7 cells was determined by FACS analysis with CD46 SCR2 specific monoclonal antibody (M75). Analysis was performed twice with an error of ± 15%. The surface expression of each mutant is compared directly with the wild-type CD46. The percentage of standard expression is indicated as follows: - (no surface expression), + (<30%); ++ (31%-65%), and +++ (>66%).

[c]Reactivity of the mutant CD46 molecules with monoclonal antibodies J4/48 and E4.3 was determined by immunoblot analysis of proteins from a mutant CD46 recombinant vaccinia virus-infected cell lysate. -, no band appeared on the blot; +, presence of a faint band; +++, indicates the monoclonal antibody recognized the mutant CD46 protein.

[d]Reactivity of the mutant CD46 molecules with monoclonal antibodies B97 was also determined by FACS analysis. The mean fluorescence values of different antibodies were normalized with wild-type CD46 as described in Materials and Methods. The surface recognition of specific antibody to each mutant was compared to the reactivity with wild-type CD46. The percentage of antibody recognition is indicated as follows: - (no antibody recognition), + (<30% recognition); ++ (31%-65% recognition), and +++ (>66% recognition).

TABLE 4.2 Site-specific mutagenesis of CD46 SCR2 domain, cell surface expression, and reactivity of CD46 mutant proteins with specific antibodies

Mutation[a]	Surface[b] Expression	Reactivity with monoclonal antibody[c] M75	122-2
Y101S	+++	+++	-
I102S	+++	++	+++
R103A/D104A	+++	-	-
L106S	+++	+++	+++
V111S	+++	+++	+++
N114A	+++	-	++
Y117S	+++	+++	+++
E118A	+++	+++	+++
F119S	+++	+++	+++
Y121S	+++	+++	+++
M123S	+++	+	+
H124A	+++	+++	+++
F125S	+++	-	-
I126S	+++	+++	+++
E129A	+++	+++	+++
Y131S	+++	+	+
Y132S	+++	+++	+++
L133S	+++	+	+
I134S	+++	+++	+++
E136A	+++	+++	+++
E137A	+++	+++	+++
I138S	+++	+++	+++
L139S	+++	+	+
V140S	+++	+++	+++
E142A	+++	+++	+++
L143S	+++	+++	+++
E144A	+++	+++	+++
V147S	+++	+++	+++
I149S	+++	+++	+++
W150S	+++	-	-
K153A	+++	+++	+++
I156S	+++	+++	+++

[a]The CD46 molecule (isotype C2) was subjected to site-specific mutagenesis as described previously. The position of the mutation is indicated by the amino acid residue numbered from the amino terminus of the polypeptide, and includes the signal peptide prior to post-translational cleavage. The letter to the left of the number indicates the original amino acid, while the letter to the right indicates the change.

[b]The mean fluorescence of CD46 mutant proteins which were expressed on the surface of mouse OST-7 cells was determined by FACS analysis with CD46 SCR1 specific monoclonal antibody (J4/48). Analysis was performed twice with an error of ± 15%. The surface expression of each mutant is compared directly with the wild-type CD46. The percentage of standard expression is indicated as follows: - (no surface expression), + (<30% expression; ++ (31%-65% expression), and +++ (>66% expression).

[c]Reactivity of the mutant CD46 molecules with monoclonal antibodies M75 and 122-2 was also determined by FACS analysis. The mean fluorescence values of different antibodies were normalized with wild-type CD46 as described in Materials and Methods. Recognition of specific antibody by each mutant was compared to reactivity with wild-type CD46. The percentage of antibody recognition is indicated as follows: - (no antibody recognition), + (<30% recognition); ++ (31%-65% recognition), and +++ (>66% recognition).

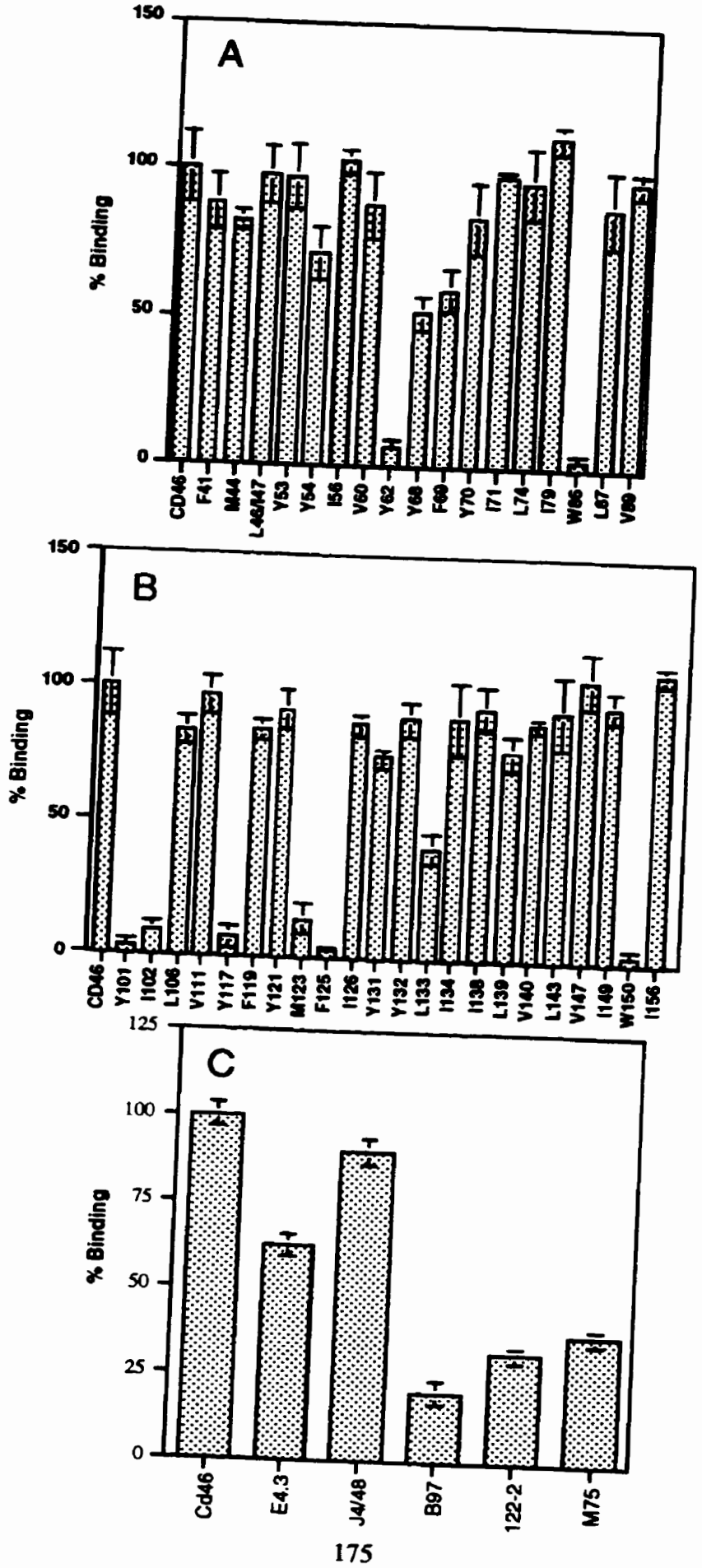
reduction in binding and mutations of tyrosine at amino acid 68 (Y68S) and phenylalanine at amino acid 69 (F69S) led to more substantial inhibition, ranging between 40%-50% (Figure 4.2A). As we expected, mutations of Y62S and W86S abolished expression of CD46 molecule on the cell surface and OST-7 cells containing these mutant proteins did not bind to the H protein (Figure 4.2A). On the other hand, mutation of the hydrophobic residues in the SCR2 domain produced much more dramatic inhibition without affecting expression of mutant CD46 on the cell surface. Substitution of Y101, I102, V117, M123, F125 and W150 to serine completely abolished binding to measles H protein. In addition, mutation of Y131S and L139S produced a moderate 20% reduction in binding while the mutation L133S produced 60% less binding (Figure 4.2B). We and others have previously shown that when R103 and D104 residues of CD46 were mutated to alanine, significant inhibition of binding was also evident (6, 16), suggesting that this region of SCR2 might be crucial for interaction with H protein.

4.3.3 Mapping antigenic epitopes on CD46 which are recognized by monoclonal antibodies that inhibit binding to measles virus H protein

In order to further define specific regions of CD46 which are involved in binding to H, we examined the ability of five different CD46 specific monoclonal antibodies to inhibit interaction between CD46 and measles H protein. Monoclonal antibodies J4/48, E4.3, and B97 were previously shown to interact with the SCR1 domain while M75 was mapped to the SCR2 consensus region (6, 10, 16, 18). In addition, the 122-2 monoclonal antibody was shown to bind SCR2 using CD46 deletion mutants previously generated in our laboratory (data not shown). Human 143B osteosarcoma cells expressing CD46 were incubated with a 1:20 dilution of the monoclonal antibody, Sf9 insect cells with H on their surface were added, and non-adherant cells were washed away. Binding of insect cells expressing measles

Figure 4.2 Interaction between mutant CD46 molecules and measles H (Edmonston strain) protein effects of monoclonal antibodies (E4.4, J4/48, B97, 122-2, M75) on binding

Mutant CD46 molecules were expressed on the surface of mouse OST-7 cells using a recombinant vaccinia virus expression system and measles virus H protein was expressed on the surface of insect Sf9 cells using recombinant baculoviruses which also contained the ß-galactosidase gene. OST-7 and Sf9 cells were incubated for 1/2 hours, non-adherant Sf9 cells were washed away, and binding was quantitated with the ONPG colorimetric assay. Binding relative to that of normal human CD46 is represented on the y-axis of the histogram, and the various CD46 mutations which were assayed are presented on the x-axis. (A) Mutations in the SCR1 domain which were assayed. (B) Mutations in the SCR2 domain which were assayed. (C) Effects of SCR1 specific monoclonal antibodies (E4.3, J4/48, and B97), and SCR2 specific monoclonal antibodies (122-2 and M75) on binding of normal CD46 to measles virus H protein. All results are expressed as means of 3 assays and standard Deviations are indicated.


A
% Binding
150
100
50
0
CD46
F41
M44
L46/47
Y53
Y54
I56
V60
Y62
Y68
F69
Y70
I71
L74
I79
W86
L87
V89
B
% Binding
150
100
50
0
CD46
Y101
I102
L106
V111
Y117
F119
Y121
M123
F125
I126
Y131
Y132
L133
I134
I138
L139
V140
L143
V147
I149
W150
I156
C
% Binding
125
100
75
50
25
0
Cd46
E4.3
J4/48
B97
122-2
M75

virus H protein and β-galactosidase was quantitated by measuring ONPG hydrolysis in a colorimetric assay, and the results are presented in Figure 4.2C. Of the SCR1 specific monoclonal antibodies, E4.3 appeared to inhibit binding by 40%, B97 antibody dramatically reduced binding by 80%, while J4/48 only slightly blocked binding (<12%). On the other hand, both SCR2 specific antibodies, M75 and 122-2, appeared to inhibit binding very efficiently (>70%).

Antigenic epitopes recognized by the 5 different monoclonal antibodies were mapped using either immunoblot or fluorescence cytometry analysis. In the first approach OST-7 cells were infected with the different recombinant vaccinia viruses expressing CD46 mutants, cell lysates were prepared, proteins were subjected to SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The immunoblot was recognized by both J4/48 and E4.3 antibodies, but failed to react with B97, M75, or 122-2 monoclonals. This result indicated that J4/48 and E4.3 recognized either linear or partially renatured epitopes from proteins on the nitrocellulose. The analysis of 8 representative CD46 mutants is shown in Figure 4.1C. Expression of CD46 mutant proteins in all OST-7 cell lysates was verified using a polyclonal CD46 antibody. A nonspecific protein band which migrated with a molecular mass of 60 kDa was recognized by the two monoclonal antibodies. Both E4.3 and J4/48 antibodies appeared to have very similar epitopes involving residues at position 36/37 and between amino acids 46-62. The E4.3 antibody failed to recognize E36A/E37A, L46S/I47S and Y62S mutants, and had reduced recognition for Y53S, Y54S and V60S. Similarly J4/48 antibody failed to recognize E36A/E37A, Y53S and Y54S mutants, and had reduced recognition for L46S/I47S, V60S, Y62S and W86S mutants. Although both of these antibodies have very similar antigenic epitopes, these reagents do not inhibit interaction between CD46 and H equally. This may be due to differences in the binding affinity of these two antibodies with their corresponding epitopes.

Unlike J4/48 and E4.3, monoclonal antibodies B97, M75, and 122-2 did not recognize CD46 on immunoblots and appeared to require native epitopes for interaction. We therefore used a second approach based upon fluorescence cytometry to map the antigenic epitopes for these antibodies. Vaccinia virus recombinants expressing mutated CD46 mutant molecules were used to infect OST-7 cells which were subsequently analyzed for reactivity with B97, M75, and 122-2 antibodies by fluorescence cytometry with M75 and compared to the immune recognition of normal CD46. Fluorescence from a standard monoclonal (either J4/48 or 122-2) reacting with the non-mutagenized SCR was first normalized to the same level of fluoresence as from wild type CD46. In most cases non-normalized values were already within 10% of each other. The obvious exception was when the mutation affected surface expression of CD46. Following normalization, the signal due to a specific monoclonal antibody reacting with the mutagenized SCR was compared to that from the wild type SCR and expressed as a percentage. A representative analysis is shown in Figure 4.1B and the results of all the mutations are summarized in Tables 4.1 and 4.2. B97 antibody reacted weakly with CD46 molecules containing the L46/I47 and Y70S mutations. As expected it did not react with the two mutants (Y62S and W86S) which were not expressed on the cell surface. The antibody 122-2 failed to recognize CD46 molecules containing Y101S, F125S or W150S mutations, and had reduced recognition for proteins with the changes M123S, Y131S, L133S and L139S. The M75 antibody had a similar recognition profile and also did not recognize proteins with F125S and W150S mutations and exhibited reduced binding to molecules containing I102S, M123S, Y131S, L133S, and L139S changes. Our laboratory and another have previously shown that M75 fails to recognize CD46 molecules where polar amino acids between R103 to N114 were replaced with alanine (6, 16). The significance of the epitope mapped by the W150S mutation is not clear, but this mutation also inhibits

the binding of CD46 to H. We believe that the region containing Y101, R103, N114, and F125 must be critical for the interaction between CD46 and H. It seems important to note that the residues implicated as forming antigenic epitopes for both M75 and 122-2 antibodies coincided with regions in SCR2 where mutations produced the most drastic effects.

4.3.4 Molecular model showing amino acid residues on CD46 which seem to be important in binding to measles virus H protein

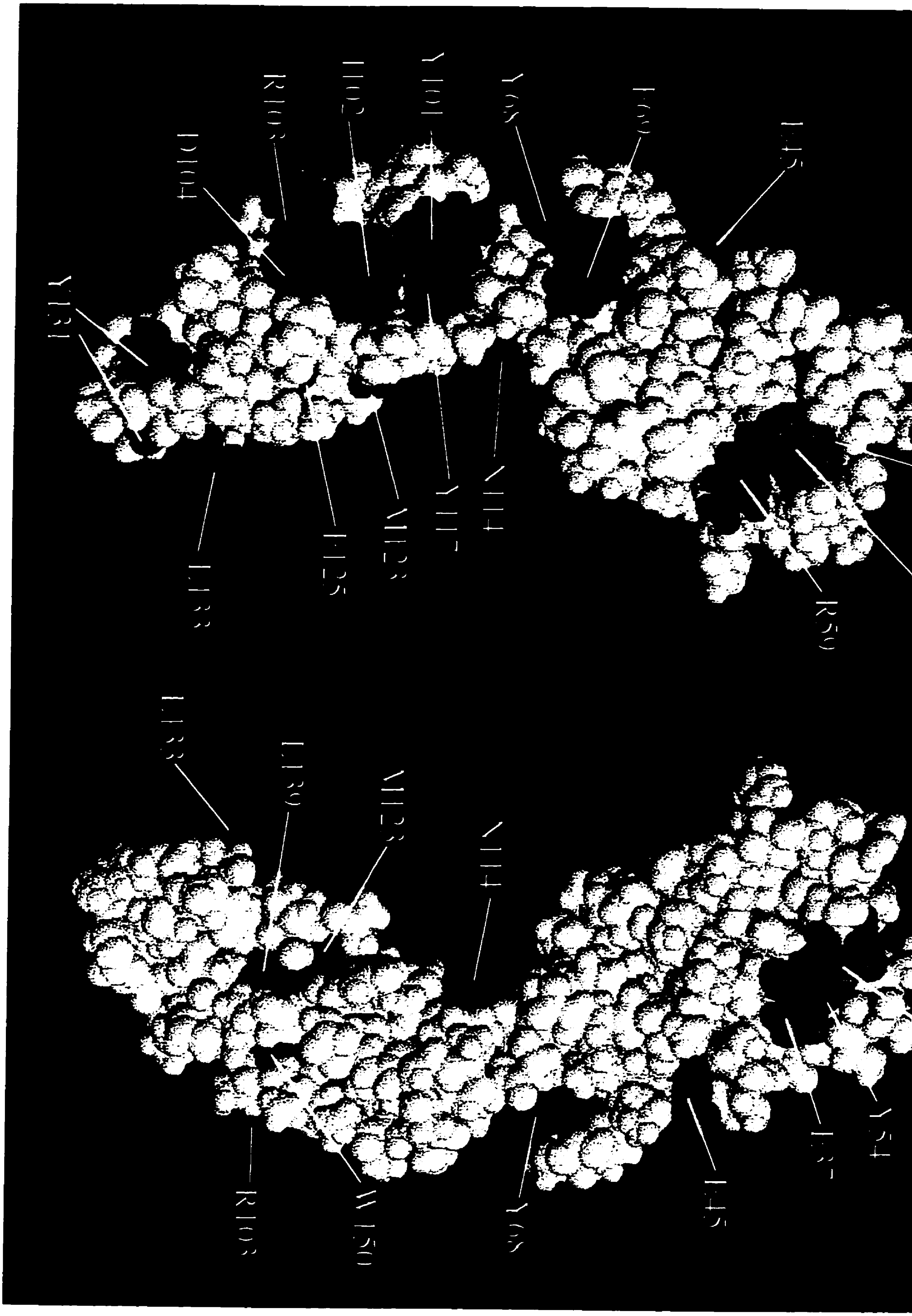
The SCR1 and SCR2 domains of CD46 have previously been modeled based upon the similar SCR15 and SCR16 domains of Factor H, another member of the RCA family (3, 6, 29, 33). Using the RasMol 2.6 Molecular Graphics Visualisation Program and the coordinates for the CD46 SCR1 and 2 domains from the hypothetical model which were provided by Drs. Roberto Catteneo and Werner Braun (33), we were able to visualize the locations of different amino acids contributing to the antigenic epitopes recognized by the inhibitory monoclonal antibodies (Figure 4.3). We also mapped the regions where site-directed mutations yielded reduced binding between CD46 and H (Figure 4.4). For the most part, all mutations which correlate with the reduction or abolition of binding are located on the same face of the model (Figures 4.3A and 4.4A). Hydrophobic amino acids involved in forming the antigenic epitopes for E4.3, and J4/48 antibodies (L46, I47, Y53, Y54, V60) are clustered together in the upper left hand corner of Figure 4.3A. This region lies directly above E45 and adjacent to the 58E/R59, residues which were previously implicated in binding studies following mutagenesis to alanine (16). The antibody E4.3 has also been shown, through site-specific mutagenesis, to interact with E36, E37, E42, E45, K49, K51, E55, E58, and R59 (6, 16), which lie directly behind these hydrophobic residues (Figure 4.3B). The monoclonal antibody J4/48 was determined by our laboratory to have a similar

Figure 4.3 Antigenic epitopes on SCR1 and SCR2 that are recognized by specific monoclonal antibodies which inhibit binding between measles virus H protein and CD46

Assays were performed as described in Tables 4.1 and 4.2. Molecular models of CD46 SCR1 and 2 domains were visualized using the space filled option of the RasMol 2.6 Molecular Graphic Visualization Program. In the SCR1 domain, orange coloured residues represent antigenic sites which react with all three (J4/48, E4.3 and B97) antibodies; green residues represent antigenic sites recognized by both J4/48 and E4.3 antibodies; yellow residues correspond to amino acids interacting with B97. In the SCR2 domain, blue residues are recognized by both 122-2 and M75 antibodies, whereas the red residues reacted only with the 122-2 monoclonal antibody. (A) The face of the model upon which most antigenic residues are located. (B) An opposite rear view of the model (180 degree rotation).

Figure 4.4 Representation of residues implicated in binding of the measles virus H protein to CD46 SCR1 and 2 domains

Molecular models of CD46 SCR1 and 2 domains were visualized using the space filled option of the RasMol 2.6 Molecular Graphic Visualization Program. In the SCR1 domain, orange residues represent hydrophilic amino acids which reduce binding when mutated to alanine, while green residues are the hydrophobic amino acids which affect binding when mutated to serine. In the SCR2 domain, blue residues represent hydrophilic amino acids which reduce binding when mutated to alanine, and red residues are the hydrophobic amino acids that affect binding when mutated to serine. (A) The face of the model upon which residues most sensitive to change are situated. (B) A rear view of the model (180 degree rotation) is shown.



recognition pattern (Table 4.1). Thus, the antigenic recognition site for E4.3 and J4/48 appears to straddle the top portion of SCR1. On the other hand, B97 antibodies appear to interact with another partially overlapping region composed of the hydrophobic amino acids L46, I47, and Y70 and the polar amino acids R59, D61, and K63. This antibody appears to interact with the front face of SCR1 (Figure 4.3A). Taken together, the monoclonal antibody inhibition and site-specific mutagenesis results indicate that R59 is a key residue in SCR1-H protein interaction, since it is common to all epitopes. Residues Y62 and W86 appear to be internal residues which constitute the hydrophobic core of SCR1 and could act to hold the domain together. Therefore, mutation of these two amino acids to serine could result in misfolding and account for the loss in CD46 surface expression (Figure 4.2A and B). Amino acids Y68 and F69 are located below the predicted epitopes for E4.3, J4/48, and B97 antibodies, but they may play some role in H protein interaction, since changes at these positions does yield 50-55% inhibition in binding. However, mutations at Y101, I102, Y117, M123, and F125, and W150 in the SCR2 domain produce dramatic and almost total inhibition of binding. Examination of the model reveals that M123, F125, and W150 are buried residues and they may help retain the active conformation of SCR2. Changes at these residues may result in global distortion of SCR2 and thereby affect both receptor binding and monoclonal antibody recognition. Antigenic epitopes for the M75 and 122-2 monoclonal antibodies appear to contain Y101, I102, R103, D104, and N114 residues. Changes at Y131, L133, and L139 also reduce recognition by M75 and 122-2, but again these residues appear to be buried and mutation of these residues probably affects the antigenic epitope through disruption of the overall SCR2 conformation. SCR2 residues implicated in binding to H all lie on the front side of the model (Figure 4.3A). R103, D104 and N114, are also situated near the Y101 and I102, have also previously been shown to affect binding (16). The

glycosylation site at N114 lies opposite to these residues, and although it was previously shown to be important for binding, mutations of this residue only inhibited interaction between CD46 and H by 45% (16). The carbohydrate associated with this region may serve as a spacer to orient SCR1 and SCR2 relative to each other rather than being actively involved in binding to H protein. (Figure 4.3A). The interpretation of our CD46 mutagenesis results must still be approached with some caution, since the changes may not always produce localized effects. Instead, they may cause alterations in the global structure of the molecule. An exact assignment of residues directly involved in receptor binding awaits the 3-D structural determination of CD46 and the measles virus H protein.

4.4 Discussion

A similar approach involving site-specific mutagenesis and the mapping of inhibitory monoclonal antibody epitopes has previously been used dissect the regions of CD4 (amino acids 39-59) which interact with the gp120 envelope protein of HIV-1 (1, 2, 4, 5, 8, 19, 31, 35, 38, 40). Residues F43 and R59 of CD4 were found to be critical for interaction with D368, E370, and W427 of gp120 through these mutagenesis studies. These results have recently been borne out through crystallographic data obtained from a complex of the two terminal domains of CD4 and gp120 (21). CD4 resides (spanning amino acids 25-64) insert into a depression consisting of 26 amino acids derived from 6 different segments of gp120. Synthetic peptides were also employed by other laboratories in attempts to map binding regions on CD4, but these experiments led to contradictory results (19, 23, 24). Initially we also attempted to perform peptide inhibition studies with measles virus and CD46 by using 25-amino-acid peptides derived from all regions of SCR1 and SCR2, but in our hands peptides at concentrations as high as 200 μM had no effect in Sf9-H binding and measles virus infectivity assays. We presumed that the interaction of measles virus hemagglutinin with CD46 depended more on conformational epitopes rather than linear peptide regions. Our results using mutagenesis and inhibitory monoclonal antibodies implicate E45, 58ER59, Y68, F69, Y101, I102, R103, D104, N114, Y117 in the binding of SCR1/SCR2 domains of CD46 to measles virus hemagglutinin. The carbohydrate attached to N114 probably does not participate directly in binding, but may serve to correctly orient SCR1 with respect to SCR2, and may act to favor binding indirectly. Three dimensional analysis using X-ray crystallography and NMR should eventually complement these studies.

Two other groups have attempted to map the regions of CD46 which bind to measles virus H protein. One laboratory used a series of 26 overlapping peptides spanning SCR1 and SCR2 to inhibit measles virus infections (29). These investigators found that peptides derived from Y70-V89 in SCR1 and E118-E137 in SCR2 could inhibit infections and virus-mediated hemagglutination of monkey red blood cells at concentrations of 100 μM. In addition, these researchers exchanged the peptide regions implicated in binding with analogous stretches of amino acids from CD55, another member of the RCA family. They found that the CD55 polypeptide regions introduced into CD46 blocked viral infections. The opposite experiment whereby putative CD46 binding regions were introduced into CD55 was not reported. However, we previously found that CD46 did not tolerate multiple amino acid changes in almost all regions of SCR1 and SCR2 (16), so these results with chimeric CD55-CD46 should be interpreted with caution. The results of this laboratory imply that measles virus H protein interacts with linear peptide domains on SCR1 and SCR2 and led these investigators to propose a model for CD46 based upon a known 3-D stucture of the SCR's from factor H (3).

Another laboratory has used an approach similar to ours to map the H protein binding domains of CD46 (6). A series of 40 mutations were introduced into the SCR1 and SCR2 domains which changed mainly polar amino acids to alanine. The effect of these CD46 changes on binding to soluble recombinant H protein was measured by FACS analysis. This method proves to be less sensitive than our binding assay which amplifies the H binding signal through ß-galactosidase which is also expressed in the insect cells. In addition, the 40 mutations were used by the other laboratory to map the binding sites of 11 monoclonal antibodies which block the interaction of H protein with CD46. The mutations E45A, K63A, P73A, and D104A reduced binding to H protein. It was surprising that these single amino acid mutations did not have a greater effect on

binding to soluble H, but this could be related to the sensitivity of their assay. In addition, 4 of the monoclonal antibodies tested, E4.3, J4/48, B97, and M75, were the same as those tested in our laboratory. For the most part, these monoclonals mapped to the same regions of CD46 as presented in our paper. As we found, the B97 and M75 antibodies were highly efficient in blocking H protein attachment to CHO-CD46 cells, and E4.3 and J4-48 were less effective in blocking binding. B97 mapped to R59, D61, and K63 in their hands and E58/R59, D61, and K63 in our experiments. M75 mapped to R103 and Q124 in the other group's experiments and I102, 103R/D104, N114, and M123 in our experiments. E4.3 antibody reacted with E36 and E37 in both our hands, but also appears to involve L46/I47 and K49/K51. Slight differences in mapping the epitopes of these monoclonal antibodies may be related to the fact that we utilized immunoblots as well as FACS analysis to monitor the change of reactivity of antibodies towards the mutant proteins. We concluded that J4-48 antibody interacted with SCR1, based upon previous publications (6, 18, 39), and that its epitope consisted of residues K49/K51, Y53, Y54, E58/R59, and V60.

Our results indicate that E45, Y54, E58/R59, Y68, F69, Y101, I102, R103/D104, Y117 seem to be critical residues for the binding of CD46 to measles virus H protein. Hydrophilic interaction with R59 in SCR1, as shown in our previous publication (16), and hydrophobic bonding with residues Y68, F69, Y101, I102, and Y117 seem to be especially important. Other hydrophobic residues such as Y62, W86, M123, F125, L133, and W150 appear to be buried and substitutions of these amino acids probably distort the overall conformation of SCR1 and SCR2 and indirectly inhibit binding of H to CD46. Amino acids implicated in binding seem to lie on one face of SCR1 and SCR2. Our structural interpretations concerning the role of individual amino acids in binding is based upon the most likely model structure derived by superimposing the amino acids of

CD46 SCR1/SCR2 on the related 3-D structure of Factor H domains 15/16 (33). The interpretation of our data is also made with the understanding that individual amino acid changes could distort the global structure of CD46 and indirectly affect its binding to H protein. A similar modeling approach has also been applied to the four SCR domains of the related CD55 molecule (20). While the conformations of SCR1 and SCR2 are generally predictable, uncertainty is derived by possible rotation about the hinge region between the 2 domains. Other viruses such as Epstein-Barr virus (EBV), coxsackie viruses, and echoviruses also bind to members of the RCA complement regulatory family (7, 32). In each of these cases, the virus appears to react with 2 or 3 domains of their receptors. EBV reacts with the 2 terminal domains of CD21 and Coxsackie viruses and echoviruses bind to SCR2-4 of CD55. Confirmation and a more precise assignment of the residues involved in CD46-H binding will depend upon the elucidation of 3-D structures of CD46 and the measles virus H protein through NMR and X-ray crystallography studies, which is currently under way in our laboratory.

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Chapter 5: Conclusion

5.1 Summary and Discussion

The World Health Organization (WHO) has recently targeted measles virus as a candidate for global eradication (7). However, this task may be harder than it seems. Since the introduction of the two-dose measles vaccine in developed countries such as Canada and the US, the rate of measles infection has decreased dramatically (17). Nonetheless in developing countries, measles still remains the number one killer in children, resulting in more than 1.5 million deaths each year. The hallmark of the measles infection is the presence of a rash, which develops 14 days post initial exposure to the virus. In addition, measles virus infected patients are usually immunosuppressed subsequent to the disease (21, 42). In fact, the high mortality rate of measles is a direct result of the secondary infections caused by immunosuppression associated with the virus (20).

Measles virus is a member of the *Paramyxoviridae* virus family. Other viruses in this family are mumps, Sendai, canine distemper virus and respiratory syncytia virus (30). Measles virus is composed of a non-segmented negative strand RNA genome that is associated with the N, P and L proteins. These three proteins, together, mediate viral transcription and replication. The virion also contains the matrix protein (M), which is essential for the packaging of the virus. The membrane of the virus consists of two different glycoproteins, hemagglutinin (H) for attachment and fusion (F) for subsequent viral entry.

Dörig *et al.* and Naniche *et al.* identified CD46 protein as a receptor for the Edmonston laboratory strain of measles virus (16, 37). As one of the complement regulatory proteins, CD46 normally inactivates the complement activation pathway by interacting with the factors, C3b and C4b. CD46 serves to prevent non-specific lysis of the host cell by the complement system of the host cells (32). The structure of CD46 consists of 4 short consensus repeat domains (SCR), a

serine/threonine/proline rich region, a transmembrane region and a cytoplasmic tail. Distinct SCR regions are recognized by complement factors and measles virus. The complement factors bind to SCR3 and 4 and measles virus binds to SCR1 and 2 (22, 25, 34). Detailed mapping of the CD46 regions important for measles virus binding was performed (Chapter 2, 4). Specific monoclonal antibodies were used, as well as site-specific mutagenesis, where charged residues were mutated to alanine and the hydrophobic residues were changed to serine. The effect of these changes on measles virus binding was monitored through a binding assay that measured the interaction between insect cells expressing the measles virus H protein and OST-7 cells expressing mutant CD46 proteins. The amount of interaction was quantified using a colormetric assay for the β-galactosidase activity present in the insect cells. The mutagenesis results indicated that amino acids E45, Y54, E58/R59, Y68 and F69 of the SCR1 domain and Y101, I102, R103, D104, and Y117 of the SCR2 domain are important for interaction with the H protein of measles virus. Furthermore, the antigenic epitopes of five monoclonal antibodies, which inhibited the interaction between CD46 and measles virus H protein, appeared to map to the same amino acids that led to loss of binding following mutation. Finally, the SCR1 and 2 domains of CD46 were modeled through three-dimensional structural data of a functionally analogous protein, SCR 15 and 16 of factor H (5, 12, 33, 35). Amino acids implicated for binding through site-specific mutagenesis and monoclonal antibodies studies appeared to be located on the same planar face of the SCR1 and 2 domains. Recently the crystal structure of SCR1 and 2 domains of CD46 was solved (13). Using the SCR1 and 2 crystal structural coordinates provided by Casasnovas *et al.*, we were able to visualize the effect of our site-specific mutations on this crystal structure with the RasMol 2.6 Molecular Graphics Visualization Program (Figure 5.1). Both the crystal structure and the previously published model structure revealed that each domain folds into

Figure 5.1 Visual representation of residues implicated in binding of the measles H protein to CD46 SCR1 and 2 domains

CD46 SCR1 and 2 domains were visualized using the space filled option of the RasMol 2.6 Molecular Graphic Visualization Program. In the SCR1 domain, orange residues represent hydrophilic amino acids which reduce binding when mutated to alanine, while green residues are the hydrophobic amino acids which affect binding when mutated to serine. In the SCR2 domain, blue residues represent hydrophilic amino acids which reduce binding when mutated to alanine, and red residues are the hydrophobic amino acids that affect binding when mutated to serine. (A) The face of the molecular model we used in chapter 4 upon which residues most sensitive to change are situated. (B) The same view of the CD46 crystal structure published by Casasnovas *et al.* showing the identical residues.

R103
D104
H102
Y101
Y117
R50

β-barrels, which consists of antiparallel β-sheets joined together by 2 disulfide bonds. The hinge region of the SCR1 and 2 domains creates a concave groove and provides limited perpendicular movement between the two domains. Examination of the critical residues implicated by our mutation studies in terms of the 3-D crystal structure supports our previous hypothesis that measles virus H protein binds to same face of the SCR1 and 2 domains of CD46 (13, 23). Nonetheless, the exact interaction between the H protein and CD46 will only be elucidated by future structure studies through X-ray crystallography of an H-CD46 complex. The validity of the above approach used in the interaction studies between CD46 and measles virus H protein was demonstrated by HIV studies (3, 4, 10, 11, 14, 26). More precisely, the recent crystallographic data describing the interaction between CD4 and HIV gp120 protein confirmed that residues which were previously implicated in binding, through mutagenesis and antibody approaches, were actually involved in gp120-CD4 contact. (29).

Our laboratory has demonstrated that South American monkeys have a deletion of their SCR1 domain of the CD46 molecules through a post-transcriptional splicing of CD46 mRNA (Chapter 2). Furthermore, cell lines derived from the South American monkeys were shown to be resistant to the Edmonston laboratory strain of measles virus, which uses CD46 as receptor for viral entry (22). Therefore, we predicted that South American monkeys, such as marmosets, should be resistant to infection by the Edmonston laboratory strain of measles virus. However, researchers in the early 1980s have reported that South American monkeys, including the common marmoset and squirrel monkeys, are susceptible to infection by the wild-type strains of measles virus (1, 2). They exhibit severe gastroenterocolitis and immunosuppression. This revelation, along with reports that wild-type measles virus failed to hemagglutinate African green monkey erythrocytes, which express functional CD46 on their surfaces, led us to

speculate that wild-type strains of measles virus might use another receptor for entry.

We have previously demonstrated that wild-type measles virus can not be propagated in owl monkey or marmoset kidney cell lines, but is able to replicate efficiently in B95-8 marmoset B cell lines without a need for adaptation to cell culture (Chapter 3). This growth phenotype suggestes that the wild-type virus uses another receptor present on B cells. In addition, antibodies directed against CD46 had partially inhibited infection of B95-8 cells by the Edmonston laboratory strain of measles virus, but had no effect on wild-type viral infections of B95-8 cells. This evidence indirectly implies that both the wild-type strains and the Edmonston laboratory strain of measles virus can use the unknown receptor on B95-8 cells for entry (24). Both Murakami *et al.* and Bartz *et al.* have also reported similar findings (6, 36). A direct binding assay, with insect cells expressing the hemagglutinin (H) proteins of either the Edmonston laboratory or wild type (Montefiore 89) strains of measles virus, was used to evaluate the interaction between the H proteins and the receptors. Insect cells expressing the Edmonston laboratory H protein, but not the wild type H protein, bound to CHO cells expressing CD46 on their surface. Conversely, both the Edmonston laboratory and the wild-type H proteins can bind to B95-8 cells. We established that the CD46-binding phenotype is determined by a single amino acid at position 481 of the H protein. Most wild-type H proteins have an asparagine residue at position 481 and can not bind to CD46. Mutating this amino acid to a tyrosine residue converted the wild-type H protein to a CD46 binding molecule. On the other hand, the Edmonston laboratory strain of H protein has a tyrosine at position 481 and can bind to CD46 effectively. Similarly, binding to CD46 by the Edmonston H protein was abolished when the residue at position 481 was mutated to asparagine. Interestingly, both sets of mutants did not affect binding to B95-8 cells. These

results provided evidence, through the use of a novel binding assay developed in our laboratory, that another receptor for measles virus is present on primate B cells (24).

The presence of another receptor may explain the susceptibility of marmoset monkeys to measles virus infection even though their CD46 SCR1 domains are missing. The identity of this unknown measles virus receptor may also elucidate the natural course of measles infection. Since measles is normally spread by aerosol to the nasopharynx, we speculate that the primary cellular target for the virus may be activated lymphocytes in the respiratory tract. In the early days of the viral disease, measles virus may initially use this unknown receptor to infect activated lymphocytes that subsequently facilitate the spread of the virus to the local lymph nodes, where viral replication occurs. As the virus spreads through the body, a block at the level of the receptor prevents the virus from infecting other types of cells and organs. At this point, a N481Y mutation in the H protein favors the use of the CD46 receptor for entry. The mutated virus is now able to infect other organs such as kidneys, liver, lungs and endothelium and epithelium of the skin resulting in the acute measles disease. This hypothesis is supported by the fact that continuous passages of wild-type measles virus in Vero cells yields a similar mutation at position 481 of H protein and results in the use of CD46 as a primary receptor for subsequent infection of the virus.

Changing viral tropism through a minor mutation in viral surface proteins is not without precedent. The epitome for such a shift was recently reported in the human immunodeficiency virus (HIV) which initially infect macrophages through a chemokine receptor, CCR5. The HIV virus subsequently makes minor changes in its V3 loops of the envelope protein and infects T cells through another chemokine receptor, CXCR4 (15, 31). While the transmission of HIV virus from one individual to another is predominantly through the macrophage tropic HIV virus, it

is the T cell tropic virus that leads to AIDS in a single individual (15). There are other examples associated with minor changes in viral attachment proteins that dictate the receptor usage. Coxsackie B viruses can use either CD55 or CAR as receptors depending on the cell lines used to propagate the virus (8, 9). In extreme cases, some viruses can even change animal tropism through small changes in attachment proteins. For example, Ross River virus, a member of the togavirus family, can switch its tropism among mosquitoes and human with single amino acid changes in the E1 and E2 viral membrane proteins (28). Therefore, it is highly plausible that a minor change in the hemagglutinin protein of the measles virus can dictate the specificity for different cellular receptors utilized during the course of measles infection (24).

5.2 Present and Future Experiments

The identity of the measles virus receptor on lymphocytic cells is still unclear. Our laboratory has begun the search for this unknown receptor. Using the binding assay developed in our laboratory, we have been able to establish that the unknown measles virus receptor is present on activated human B cells, myeloma B cells, and some lymphoma B cells. Conversely, the unknown receptor is absent on human T cells such as Jurkat and Molt 4 and resting B cells such as BJAB (Table 5.1). Furthermore, the resting BJAB cell line can be induced to express the unknown measles virus receptor through LPS activation. These results imply that the unknown measles virus receptor is located on the surface of the activated B cell. We have subsequently assessed the ability of known B cell activation markers to function as a wild type measles virus receptor using our binding assay. We have expressed the following markers on the surface of the Chinese hamster ovary cells containing polyoma large T antigen (CHOP): CD9, CD10, CD19, CD20, CD21,

Table 5.1 Binding of wt Measles H to Lymphoid Cell Lines

Cell Line	Type of Cell	Binding Edmonston H	Binding wt H
Raji	activated B	+++	++
Daudi	activated B	+++	++
B95-8	activated B	+++	++
1A2	activated B	+++	+++
H929	myeloma B	+++	+
My5	myeloma B	+++	+
OCI-3	lymphoma B	+++	+
OCI-7	lymphoma B	+++	++
OCI-8	lymphoma B	+++	+
OCI-18	lymphoma B	+++	+
Cassman	lymphoma B	++	-
BJAB	resting B	+++	-
BJAB	LPS act B	+++	++
Jurkat	leukemia T	+++	-
Molt 4	leukemia T	+++	-
OCI-17	leukemia T	+++	-
K562	myel. leukemia	+++	-
U937	premyel. leuk.	+++	++

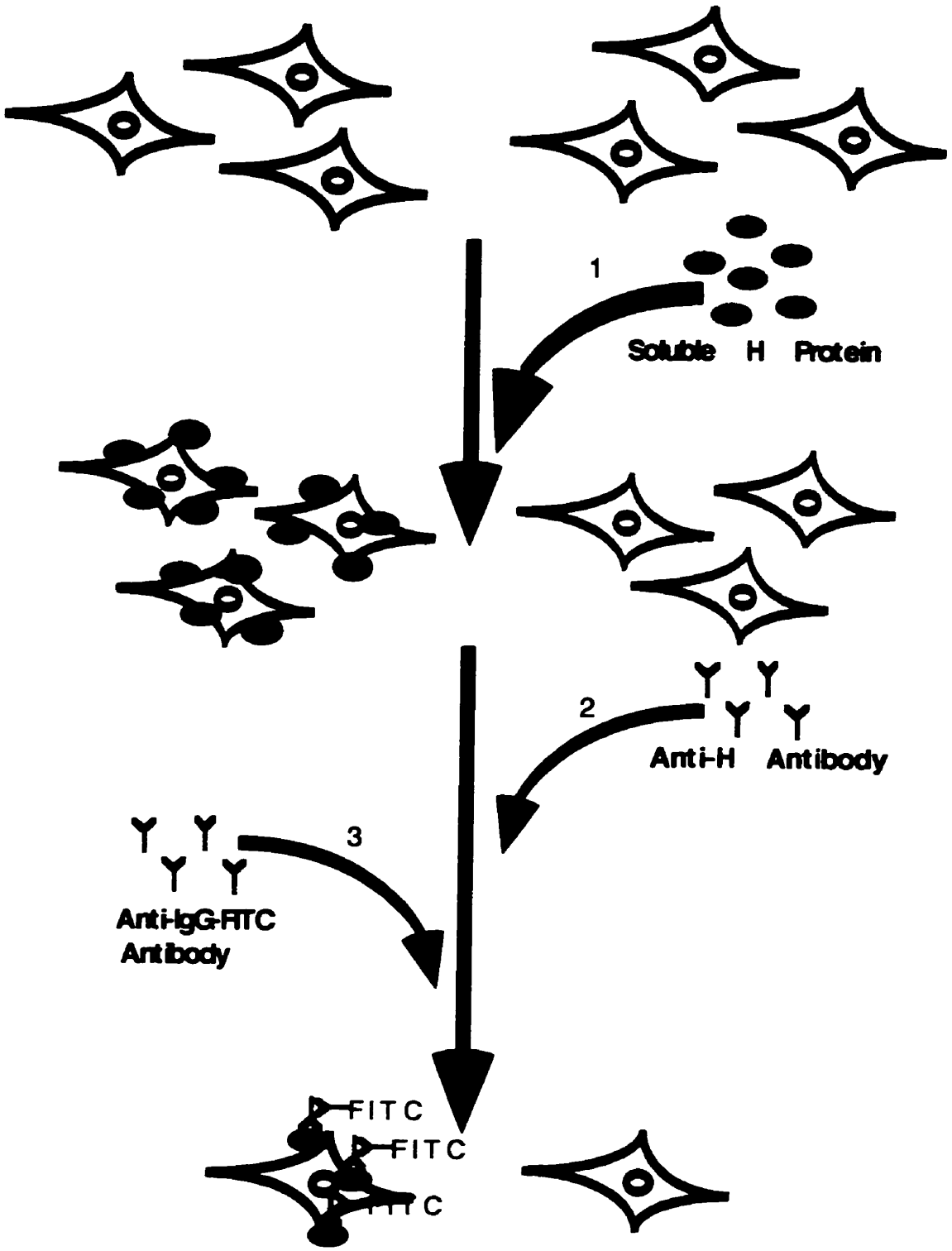
Hematopoietic cancer cells were obtained from Dr. Hans Messner at OCI. Cells were from Bcell lymphoma, T cell leukemia, myeloid leukemia, and premyeloid leukemia patients. Cells were assayed for binding to "blue" insect cells expressing Edmonston H or Montefiore 89 H proteins which also expressed ß-galactosidase. Positive adherant cells bound blue cells while positive suspension cells clumped in the presence of Sf9 cells expressing MV H protein.

CD23, CD24, CD25, CD37, CD39, CD40, CD54, C60, CD79, CD86 and CD119. None of these activated B cell markers, however, are able to bind wild-type H protein in our assay.

Currently, two other approaches are being implemented in our laboratory to identify the unknown measles virus receptor. The first approach utilizes a cDNA expression library prepared from B95-8 cells, and the second approach involves the generation of inhibitory monoclonal antibodies directed against surface proteins of B95-8 cells. Both approaches require a very sensitive and effective screening assay in order to yield reliable results. In the past, the hemagglutination assay and virus infectivity assays have been used to identify CD46 as the measles virus receptor. The hemagglutinin assay involves incubating African monkey erythrocytes, which express CD46, with the Edmonston laboratory strain of measles virus. The virus acted as a "bridge" mediating binding between adjacent erythrocytes. On the other hand, the virus infectivity assay involves inoculating measles virus with cell lines, which express CD46 on their surface. Since measles virus infected cells express viral proteins, H and F, on their cell surface, measles virus infection can be confirmed with a specific antibody against the viral proteins. The presence of measles virus is imperative for the success of both assays. Unfortunately, wild-type measles virus does not hemagglutinate African monkey erythrocytes; therefore, the hemagglutinin assay cannot be used for the screening of the unknown measles receptor. The virus infectivity assay is also not a very sensitive method for screening because measles virus is a very "sticky" virus. Frequently, in culture, the virus can non-specifically attach to and enter cells that do not have the viral receptor on their surface and produce high backgrounds in the assay. To overcome these limitations, we decided to generate soluble wild-type H protein and subsequently use it as the probe for the receptor. The interaction between the soluble H protein and the receptor can be analyzed using a modified binding assay (Figure 5.2).

Figure 5.2 Soluble measles H protein binding assay

Soluble measles H protein will be used in a binding assay. Cells with receptors for the H protein will bind to soluble H protein. This interaction can be quantified in a FACS scan analysis assay using specific monoclonal antibody against the H protein and FITC conjugated anti-mouse IgG antibody. Conversely, cells without the viral receptor will not bind to soluble H protein and cannot be detected with anti-measles H antibody.

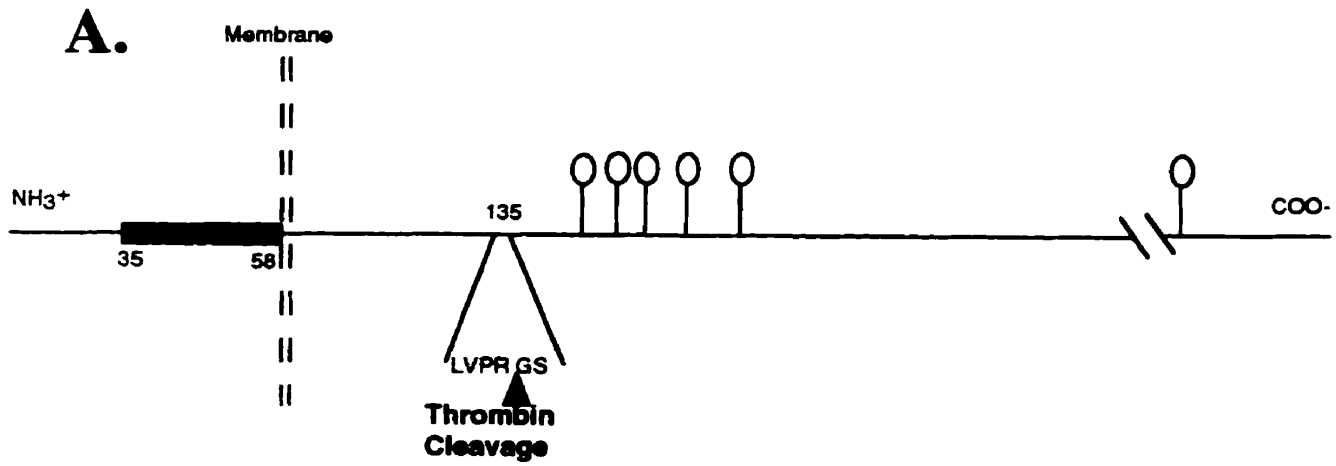

1
Soluble H Protein
2
Anti-H Antibody
3
Anti-IgG-FITC
Antibody
FITC
FITC
FITC

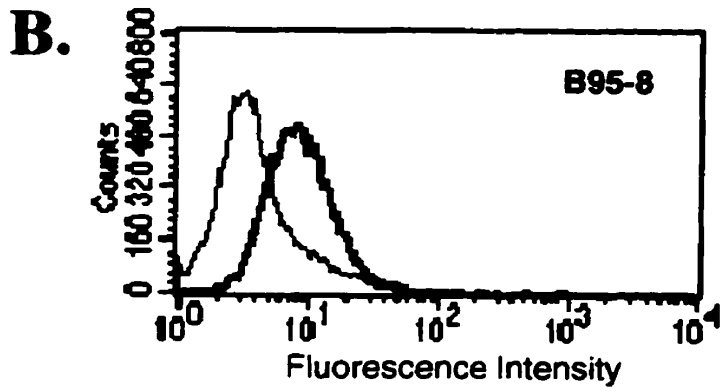
A specific monoclonal antibody against the H protein is used for quantitation of H binding in a FACS scan analysis assay.

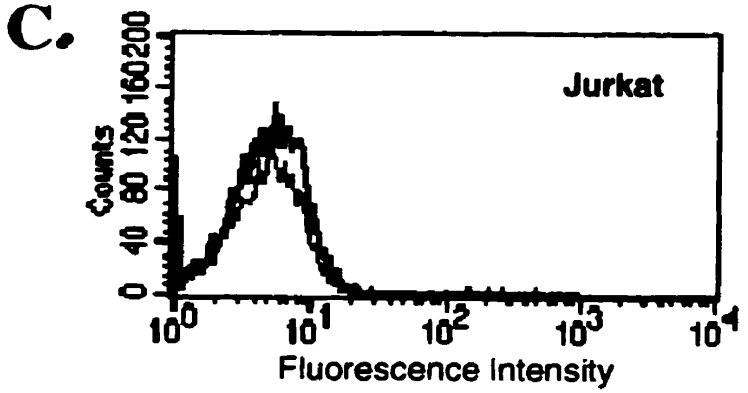
Previous reports have indicated that even minor mutations of the hemagglutinin protein can affect the biological activity of the H protein so it is not inconceivable that a single amino acid change at position 481 could affect the ability of H to bind to CD46 (18). Recently, Sato *et al.* reported the generation of a functionally soluble H protein by digesting the Edmonston laboratory strain of measles virus with Asp-N endoproteinase (40). Using the digestion map of the H protein provided by Sato *et al.* as a blueprint, we inserted a thrombin cleavage sequence at position 135 of the baculovirus recombinant expressing the wild-type measles H protein (Figure 5.3A). No effect on the binding ability of the modified wild-type H protein to B95-8 cells was observed (data not shown). After treating the insect cells expressing H protein with thrombin, the soluble H protein in the media was partially purified by ion exchange chromatography. The purified soluble H protein was subsequently analyzed for biological activity using a modified binding assay (Figure 5.3B). B95-8 cells and Jurkat cells were incubated with either partially purified soluble wild-type H protein or with a different unrelated soluble protein as a negative control. The cells were tested using monoclonal or polyclonal antibodies directed against the H protein and analyzed by FACScan analysis. B95-8 cells exhibited a shift in fluorescence due to the presence of the wild type soluble H protein interacting with the unknown receptor on the cell surface. Conversely, the Jurkat cells did not exhibit a shift in fluorescence because they do not express the wild-type measles receptor (Figure 5.3C). Using soluble wild-type H protein as a probe in the binding assay appears to be very effective in reducing the high backgrounds that was previously observed using measles virus as a probe. Therefore, this modified binding assay will be used to facilitate the cDNA

Figure 5.3 Soluble measles H protein binds to B95-8 cells but not Jurkat cells

A thrombin cleavage sequence was inserted at position 135 of the baculovirus recombinant expressing the wild type measles H protein. The recombinant H proteins was treated with thrombin and was subsequently purified by ion exchange chromatography (A). Following incubation with soluble H protein and mouse anti-measles H antibody. the cells were treated with FITC-labeled goat anti-mouse IgG. The labeled cells was resuspended binding buffer and analyzed using a Beckton Dickinson analyzer equipped with a 15 mW argon laser at 488 nm. The data were collected and analyzed using Cell Quest software. Cell counts are indicated on the y-axis and the logarithm of the fluorescence intensity is represented on the x-axis. (B) FACS analysis of soluble H protein binding to B95-8 cell (red line) vs. B95-8 cells without H protein (green line). (C) Soluble H protein binding to Jurkat cells (red line) vs. Jurkat cells without H protein.


A.
Membrane
NH3+
COO-
35
58
135
LVPR GS
Thrombin
Cleavage


B.
B95-8
Counts
0 160 320 480 640 800
10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity


C.
Jurkat
Counts
0 40 80 120 160 200
10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity

expression cloning screening approach and the monoclonal antibodies inhibition screening approach in our laboratory.

Screening for receptors with a cDNA expression library has been used previously to identify the echovirus receptor, herpes virus receptors and mouse mammary tumor virus receptor (19, 27, 41). We propose to construct a marmoset B cell cDNA expression library by isolating mRNA from the B95-8 cells and cloning it into the expression vector pcDNA1.1. This vector has a strong CMV promoter and a polyoma/SV40 origin of replication which can replicate in the transfected cells and yield high level of protein expression in CHOP or Cos-7 cells which contain the polyoma or SV40 large T antigen. 36 hours post transfection, the cells will be incubated with soluble wild-type H protein and H monoclonal antibody followed by goat anti-mouse antibodies conjugated to magnetic beads. The transfected cells that bind to the soluble H protein will be selected with a magnet and the transfected plasmids will be recovered by the Hirt extraction technique to release episomes that encode the putative receptor. The plasmid encoding the receptor will be amplified by transformation of Top 10 electrocompetent bacterial cells. The identity of the receptor will be determined by DNA sequencing using the T7 and T3 primers which flank the multiple cloning site of the pcDNA1.1 plasmid. The cDNA expression cloning approach will only be successful if the viral receptor consists of a single molecule. If wild-type measles virus utilizes a receptor complex that is composed of several molecules, this approach may fail to yield a result.

The limitation of above approach can be overcome with another more labor intensive method, the monoclonal antibody inhibition of virus binding approach, which has also been used by others to identify molecules present in a receptor complex. For example, monoclonal antibody inhibition was used to identify CD46 as a receptor for the Edmonston laboratory strain of measles virus (37-39). A

similar approach could be used to identify the wild-type measles virus receptor. We propose to generate a battery of monoclonal antibodies directed against the surface proteins of B95-8 cells. The FACS analysis assay that was described previously will be used to screen for specific monoclonal antibodies that can inhibit wild-type soluble H protein attachment to B95-8 cells. Finally, monoclonal antibodies which inhibit binding could be used to immunoprecipitate the viral receptor from a B95-8 cell lysate, and the identity of this immunoprecipitated protein would be determined through N-terminal sequencing.

Finally, we propose to establish that the N481Y mutation of the hemagglutinin protein occurs during the natural course of measles disease (24). Since African monkeys, such as Rhesus macaque, sustain an infection similar to that in humans, we propose to inoculate macaque monkeys with a wild-type strain of measles virus and study the phenotype of the H protein at position 481 during different stages of measles. The mRNA of the measles H from lymphoid tissues (spleen, tonsils, nasopharyngeal exudate and peripheral blood cells) and non lymphoid tissues (liver, kidney, brain and skin) of the infected monkeys will be isolated and sequenced. This experiment will determine whether a switch occurs from the lymphocyte specific receptor to CD46 through a single amino acid mutation (N to Y) at position 481 of the H protein during the course of infection.

5.3 Final Remark

The identity of the wild-type measles receptor will help to further characterize the natural course of measles virus infections *in vivo*. In addition, the normal cellular functions associated with this yet unidentified measles receptor may answer many questions surrounding the disease, such as the well documented phenomenon of measles associated immunosuppression. More importantly, the

technologies developed and the information obtained in our laboratory while studying measles virus can be applied to other less understood pathogens such as hepatitis B virus and hepatitis C virus. Ultimately, we hope that the knowledge contained in this thesis may contribute to the development of anti-viral measles treatments that may complement the already existing viral vaccines and eventually lead to the global eradication of measles.

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