INFLUENCE OF THE MEMBRANE ANCHORING AND CYTOPLASMIC DOMAINS ON THE FUSOGENIC ACTIVITY OF VESICULAR STOMATITIS VIRUS GLYCOPROTEIN G

By

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Abstract

Relatively little is known about the vesicular stomatitis virus (VSV) glycoprotein G fusion mechanism. Vesicular stomatitis virus has a single type 1 integral membrane glycoprotein G embedded in the viral membrane. It is the only viral protein required for VSV induced low pH mediated fusion. Mutations in four regions (H2, A5, A4 and H10) of the VSV G ectodomain have been shown to abolish the fusion activity of the viral glycoprotein (Li et al.,1993). One region H2 (a.a 117-139) has been suggested to be the fusion peptide (Zhang and Ghosh, 1994)(Fredericksen and Whitt, 1995). Amino acids 59-221 of the G protein, an area that encompasses the H2 region, has recently been shown to interact with liposomes through hydrophobic photolabeling experiments (Durrer et al., 1995), suggesting that the H2 region (fusion peptide)is able to interact with hydrophobic target bilayers at low pH. A soluble VSV G protein lacking the transmembrane anchor and cytoplasmic tail of VSV G is not fusogenic, suggesting that G must be anchored to the plasma membrane to promote syncytia (Florkiewicz and Rose, 1984). To better understand the steps involved in the fusion mechanism of VSV G it is important to identify domains within the protein that are involved in the fusion process.

To determine the contributions of the transmembrane anchor and cytoplasmic tail to the VSV fusion mechanism chimeric G proteins were constructed. The transmembrane anchor alone or in conjunction with the cytoplasmic tail of VSV G was replaced with equivalent domains from other viral proteins, HSV-1 glycoproteins gB and gD, adenovirus E3 11.6 K gene, that are not involved in low-pH fusion and the cellular

protein CD4. All chimeras were expressed in COS-1 cells, glycosylated, oligomerized, transported to the cell surface, showed a low-pH induced conformational change and were expressed on the cell surface at levels equivalent to wild-type G. The transmembrane hybrids show extensive syncytia formation at levels similar to wild-type G when induced at pH 5.6. The transmembrane-cytoplasmic tail hybrids showed reduced levels of syncytia as compared to wild-type G at both pH 5.6 and 5.2.

A glycosylphosphatidylinositol lipid-anchored ectodomain of G (GGPI), which lacks both the transmembrane and cytoplasmic tail of G, was expressed in COS-1 cells. The GGPI chimera was glycosylated, expressed on the cell surface,and oligomerized similar to wild-type G. However the chimera was fusion negative, could not promote lipid mixing and had an altered tryptic digestion profile.

A fusion negative chimera GtmΔ12gB was constructed by exchanging the TM of G with the equivalent domain from HSV-1 gB TM plus eight extra amino acids of the gB ectodomain. Deletion of the 11 extra gB amino acids (GgB3G) restored the fusogenic activity of this chimera. Another chimera G10 DAF directly demonstrated that the fusion negative phenotype of GGPI, like chimera GtmΔ12gB, was a result of the 10 extra amino acids at the EC-TM interface. The ectodomain (EC)-transmembrane (TM) interface is highly conserved among 5 vesiculoviruses. Chimeras with a 9 amino acid insertion (G10DAF), deletion (GΔ9) or replacement (GΔ910DAF) were expressed in COS-1 cells. The expressed proteins were glycosylated, underwent a low-pH induced conformational change and were expressed on the cell surface at levels equivalent to wild type, but were

fusion negative. Suggesting that both the sequence and spatial arrangement of amino acids at the EC-TM interface may affect VSV G fusion.

Taken together the data suggests that the specific amino acid sequence of the transmembrane anchor of VSV G is not essential for fusion. Replacement of the TM of VSV G with equivalent domains from other viral and cellular proteins does not affect the fusion activity. The cytoplasmic tail of VSV G may form an entity alone or in conjunction with the transmembrane anchor that can regulate fusion. Another region in the ectodomain of VSV G renders the glycoprotein fusion sensitive in a cell-cell fusion assay and was characterized at the EC-TM interface.

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1. Introduction

1.1 Membrane Fusion

Membrane fusion is a ubiquitous process that occurs when the contents of two separate lipid vesicles or cells mix after the fusion of both lipid bilayers. Some common membrane fusion events are regulated exocytosis, myoblast fusion, sperm-egg fusion and the entry of all enveloped animal viruses into the host cell (White, 1990).

Fusion is an obligate step in the life cycle of all enveloped animal viruses and represents one of the initial stages of virus entry. After receptor binding, viral fusion is important for virus entry into the cell and release of the viral nucleocapsid into the cell cytoplasm (Lenard, 1993). The study of membrane fusion catalyzed by viral fusion proteins is required for a better understanding of the mechanisms viruses use to enter cells. Since most enveloped viruses except herpes virus have only one or two viral glycoproteins directly involved in the membrane fusion process, viral fusion proteins have been studied as a model for protein induced membrane fusion (White 1990) (White, 1992) (Hughson, 1995 and 1995b) (Gaudin et al., 1995) (Hernandez et al., 1996) The information gathered about the viral fusion mechanism may also provide insight into the mechanism of some cellular fusion events, for example a putative fusion protein PH-30 on the cell surface of sperm shares some common characteristics with viral fusion proteins (Blobel et al., 1992)(White, 1992).

In general there are two mechanisms for enveloped viruses to enter cells. Viruses that fuse directly at the plasma membrane in a pH-independent manner. Notable

examples of this class of virus are: human immunodeficiency virus (HIV), Sendai virus and herpes simplex virus. The second class of virus are endocytosed and require the low-pH environment of the endosomal compartment to enter cells in a pH-dependent manner. Notable examples of this class of virus are: influenza, rabies, vesicular stomatitis virus (VSV), tick borne encephalitis virus (TBE) and Semliki Forest virus (SFV). Although there are two different modes of virus entry, all enveloped animal viruses share some common characteristics. All enveloped animal viruses encode genes for integral membrane glycoproteins that induce the fusion reaction. Fusion proteins contain a critical element called the fusion peptide that is required for the fusion process. Some of the salient features of viral fusion proteins will be discussed.

1.2 Viral Fusion Proteins

All of the viral fusion proteins characterized to date have several distinct properties in common. They are all type-1 integral membrane proteins with the amino terminal external to the virus and the carboxyl terminal within the cytoplasm (White, 1990) (White 1992) (Hernandez et al., 1995). The majority of the fusion proteins mass is contained within the ectodomain of the glycoprotein, usually greater than 85% of the proteins mass (White,1990). All viral fusion proteins are glycosylated (White, 1990) (Doms et al., 1993). All viral fusion proteins form higher order oligomers (White, 1990) (White 1992) (Doms et al.,1993) (Hernandez et. al., 1994). They commonly form trimers such as influenza hemagglutinin (HA) protein (Wiley and Skehel, 1987), VSV G glycoprotein (Kreis and Lodish, 1986) (Doms et al., 1988) (Doms et al., 1993), rabies

virus glycoprotein G (Whitt et al., 1991), Semliki Forest virus E protein (Wahlberg et al., 1992), human immunodeficiency virus (HIV) gp41 protein (Lu et al., 1995) and Sendai virus F protein (Hernandez et al., 1996). Viral fusion proteins such as tick-borne encephalitis virus (TBE) glycoprotein E is however a dimer (Heinz and Kunz, 1980). All viral fusion proteins are present on the viral membrane surface at high density, and there is a threshold surface density required to initiate fusion (Ellens et al., 1990) (Puri et al., 1993) (Hernandez et al., 1996). Some viral fusion proteins, such as influenza HA1/HA2, SFV E1/E2, HIV TM gp41/gp120 and Sendai virus F1/F2 are complexes of two glycoproteins subunits that were synthesized as larger precursors and require proteolytic cleavage to activate the fusion activity (Hernandez et al., 1996). Tick-borne encephalitis virus is synthesized as a large precursor and proteolyticaly cleaved, but exists as one subunit on the cell surface (Hernandez et al., 1996). Others, such as vesicular stomatitis virus G and rabies glycoprotein G are not proteolytically cleaved and have only one subunit (Hernandez et al., 1996).

Viral fusion proteins can be divided into the following two main categories: location of the fusion peptide at either the amino terminus or internal and the pH-dependence of the membrane fusion either pH-dependent (acidic pH) or pH-independent (neutral pH) fusion activity. There is no correlation between the position of the fusion peptide in the ectodomain and the pH-dependency for fusion (White, 1990). The fusion peptide is defined as a relatively short hydrophobic stretch of 16 to 24 amino acids that can be modeled as amphipathic α-helices (White, 1990). Fusion peptides are usually

enriched in both glycine and alanine (White, 1990). Some pH-dependent fusion peptides such as influenza HA contain negatively charged amino acids (White, 1990). Internal fusion peptides are unique in that they commonly contain proline residues near the center of the fusion peptide (White, 1992). Under fusogenic conditions, the fusion peptide can interact with hydrophobic target membranes (White,1990) (White,1992) (Hernandez et al., 1996). The fusion peptide for all viral fusion proteins are located in the membrane-anchored subunit of the fusion protein (White, 1992) (Hernandez et al., 1996). Mutational analysis of several cloned viral glycoproteins have been useful in the identification of the fusion peptide location, such as the simian immunodeficiency virus (SIV) (Bosch et al., 1989), HIV-1 (Freed et al., 1990), HIV-2 (Freed et al., 1992), VSV G (Li et al., 1993) (Zhang and Ghosh, 1994) (Fredericksen and Whitt, 1995), influenza (Gething et al., 1986), Semliki Forest virus (Levy-Mintz and Kielian, 1992) and Newcastle disease virus (NDV) (Sergel-Germans et al., 1994) to name but a few.

The hydrophobic fusion peptide of influenza HA2 and the gp41 env protein of human immunodeficiency virus are located at the amino terminus (Freed et al., 1990)(Gething et al., 1986). In contrast, the hydrophobic fusion peptides of tick-borne encephalitis virus E protein and the Semliki Forest virus E1 protein are located internally (Hernandez et al., 1996)(Levy-Mintz et al.,1992). Some viruses enter the cell by fusion within the cellular endosomal compartments. This kind of membrane fusion is induced at acidic pH in the low-pH environment of cellular endosomes (White, 1990). The low-pH induces a conformational change in the fusion protein exposing a hydrophobic fusion

peptide that was previously buried within the protein at neutral pH (White, 1990) (White, 1992) (Hernandez et al., 1996). Viruses such as influenza HA2, tick-borne encephalitis virus E protein, vesicular stomatitis virus glycoprotein G, rabies virus glycoprotein G and the Semliki Forest virus E1 protein infect cells in a pH-dependent manner (Hernandez et al., 1996). The structure of the influenza hemagglutinin is known at the atomic level for both the neutral and low-pH conformations, therefore detailed information is known about its fusion mechanism as compared to any other viral fusion protein (Wilson et al, 1981)(Wiley and Skehel, 1987) (Bullough et al., 1994). Influenza HA is commonly referred to as a model for pH-dependent fusion. Recently, the structure of tick borne encephalitis virus glycoprotein E at neutral pH has been elucidated (Rey et al., 1995). In contrast to influenza, Sendai virus, human immunodeficiency virus and herpes simplex virus-1 enter cells in a pH-independent manner and fuse at the plasma membrane (White,1992). Vesicular stomatitis virus glycoprotein G requires low pH to become fusion competent. Work in our laboratory and by others has recently elucidated a stretch of mainly uncharged amino acids that likely represent the fusion peptide of VSV glycoprotein G (Ohnishi, 1988) (Whitt et al., 1990) (Li et al., 1993) (Zhang and Ghosh, 1994) (Fredericksen and Whitt,1995) (Durrer et al., 1995).

1.3 Influenza HA: The Low-pH Fusion Model

The hemagglutinin protein of influenza is the best studied viral fusion protein to date. The availability of crystal structures for the soluble bromelain treated fragment of hemagglutinin (BHA)(HA2, minus the hydrophobic fusion peptide and transmembrane anchor and cytoplasmic tail) in both the neutral-pH (Wilson et al., 1981) and low-pH conformations (Bullough et al., 1994) have allowed dissection of the initial steps of the fusion process. In contrast to VSV glycoprotein G, a wealth of information is available about influenza hemagglutinin low-pH mediated fusion process making it an ideal model for low-pH mediated fusion. Some of the salient features of the influenza hemagglutinin fusion model will be discussed. For specific information on the influenza HA mediated viral fusion model refer to the following fusion reviews (Gaudin et al., 1995) (Hughson , 1995b) (White, 1995) (Hernandez et al., 1996). Influenza, like VSV enters cells at low-pH. After attachment to its receptor, sialic acid, the influenza virus is endocytosed via clathrin coated pits. In general the low-pH environment of the endosomes causes a conformational change in the hemagglutinin (HA2) protein that exposes a hydrophobic segment (fusion peptide) that interacts with the target membrane. The HA2 molecule promotes lipid mixing between the outer leaflets of the target and viral membrane, followed by opening and then dilation of a fusion pore, resulting in the release of the viral genome into the cell.

The HA protein of influenza HA is sufficient to mediate the fusion reaction. Cells expressing the cloned HA gene, in the absence of any other viral gene, fuse upon

exposure to low-pH (White et al., 1982). Similar to other viral fusion proteins, HA is a type 1 integral protein. The HA protein is synthesized as a large precursor in the ER, denoted as HA_o. HA_o is a homotrimer that oligomerizes in the ER and is transported through the Golgi complex to the cell surface. HA_o is proteolytically cleaved by extracellular protease into two subunit HA1 and HA2 that are linked by a disulphide bridge. HA2 is the membrane anchoring subunit. Proteolytic cleavage of HA_o generates a hydrophobic peptide segment at the amino terminus on the HA_2 subunit called the fusion peptide (Wiley and Skehel, 1987) (White , 1990) (White, 1992) (Hernandez et al., 1996). Proteolytic cleavage is absolutely required for fusion (White et al., 1982). The newly generated amino terminal fusion peptide of HA_2 has the highest sequence conservation in the HA molecule (Wilson et al., 1981). Site directed mutagenesis of the amino acids in the fusion peptide of influenza HA2 abolish or decrease the pH required for fusion (Gething et al., 1986), demonstrating the importance of this element in the fusion mechanism. The HA1 subunit, is a globular protein completely extracellular to the virus. The globular heads contains the binding pocket for receptor recognition of sialic acid (Weiss et al., 1988). In its native conformation at pH 7.0, the HA protein extends 135 Å above the viral membrane from the transmembrane anchor to the top of the globular head of HA1 (Wilson et al, 1981). The fusion peptide is buried within the hydrophobic interior of the HA trimer approximately 100 Å from the top of the HA1 subunit (Wiley and Skehel, 1987). Therefore in the native conformation at pH 7.0 the HA fusion peptide is in close proximity (35 Å) to the viral membrane. Upon acidification

of the endosomal compartment, the low-pH environment induces the HA trimer to undergo a dramatic conformational change (Bullough et al., 1994). The fusion peptide is extruded from inside the HA trimer, and projected towards the target membrane (Carr and Kim, 1993) (Bullough et al., 1994), resulting in hydrophobic binding of HA to the target bilayer (Harter et al., 1989). This results in a transition from a relatively hydrophilic to hydrophobic protein (White,1992) (Hernandez et al., 1996). One of the major structural changes in HA is conversion of a loop region at neutral pH that connects an α-helical stalk of HA2 and the fusion peptide buried with the HA trimer, to an extended α-helix at acidic pH (Bullough et al., 1994). This conversion has been named the "spring-loaded mechanism" by Carr and Kim, since the loop to α-helix transition induced by low-pH propels the fusion peptide from within the HA trimer and into the target membrane (Carr and Kim, 1993). Primary sequence analysis of the loop region shows that it has a heptad repeat, a sequence of amino acids that has a high propensity of forming an α-helix at low-pH (Carr and Kim, 1993). Similar structures have been observed in other viruses such as human parainfluenza virus and HIV, indicating that they might have a similar fusion mechanism to influenza hemagglutinin (Rabenstein and Shin, 1995) (Yao and Compans, 1996). Peptides corresponding to the heptad repeat or mutations in the heptad repeat that block this loop to α-helix transition also block fusion (Rabenstein and Shin, 1995) (Yao and Compans, 1996).

The conformational change that is activated by low-pH is irreversible for influenza, and results in a fusion inactive form of HA (White,1990) (White,1992)

(Hernandez et al., 1996). Exposure of the fusion peptide at acidic pH in the absence of membranes, results in insertion of the fusion peptide into the viral membrane (Weber et al., 1994). Therefore the inactive conformation of HA results in a structure with both the fusion peptide and transmembrane anchor embedded in the viral membrane.

Once the pH is lowered the HA molecule is bound to the target membrane via its fusion peptide (Harter et al., 1989). The fusion peptide of HA inserts into the target membrane before any observable fusion (Stegmann et al., 1991). There is an observed lag phase, presumably to allow a number of HA trimers to cluster at the fusion site(Melikyan et al., 1995). The next observable step is mixing of the lipids of the outer leaflets of the target and viral membrane before any observable contents are mixed. This intermediate is called hemifusion and the experimental data for hemifusion comes from a glycosylphosphatidylinostiol-lipid anchored ectodomain of HA that lacks both the transmembrane anchor and cytoplasmic domain of HA. The HA-GPI chimera is identical to wild-type HA in terms of protein expression, transport, oligomerization and cell surface expression, but is not fusion competent (Kemble et al., 1993) (Kemble et al., 1994) (Melikyan et al., 1995). However, using liposomes labeled with a hydrophobic fluorescent probe, octadecylrhodamine(R18), they were able to show that cells expressing HA-GPI could mediate lipid mixing (Kemble et al., 1994)(Melikyan et al., 1995). This suggests that the HA transmembrane anchor is required both for anchoring the protein to the viral membrane and for the full fusion activity of the HA protein.

The next observable HA fusion stage is the formation of a fusion pore. Using video microscopy and electrical conductance to monitor the fusion of HA with planar membranes the formation of the HA fusion pore can be monitored (Melikyan et al., 1995). The HA fusion pore formation is preceded by a flickering stage where the fusion pore opens and closes rapidly before establishing continuity between aqueous compartments (Melikyan et al., 1995). The HA-GPI chimera showed no observable fusion pore formation, suggesting that the HA-GPI chimera could only mediate lipid mixing (Melikyan et al., 1995).

The hemagglutinin molecule has essentially two critical components; the fusion peptide that is required to establish membrane lipid continuity, while the transmembrane anchor is required for formation of a fusion pore and establishing continuity of the cytosolic compartments (Melikyan et al., 1995). The authors did not examine whether the specific amino acid sequence of the hemagglutinin transmembrane anchor was required for full fusion or not.

1.4 VSV

A good review of rhabdoviridae and their replication can be found in Fields Fundamental Virology (Wagner and Rose, 1996). Vesicular stomatitis virus belong to the family of viruses, rhabdoviridae. The family of rhabdoviruses that infect mammals are divided into two genera, the vesiculovirus genus and Lyssavirus genus, of which vesicular stomatitis virus and rabies are representative of each group respectively

(Wagnaer and Rose, 1996). Rhabdoviridae are bullet shaped with approximate dimensions 180 nm long and 75 nm wide (Wagner and Rose, 1996). The virion is composed of two major structural components, the ribonucleocapsid and the envelope lipid bilayer surrounding the ribonucleocapsid (Wagner and Rose, 1996).

The rhabdoviridae genome contains a single stranded RNA molecule of the negative polarity. The genome encodes 5 genes in the order 3'N-P-M-G-L-5'(Wagner and Rose, 1996). To initiate an infection VSV G binds to its receptor on the surface of its target cell. The VSV receptor is thought to be patches of negatively charged phospholipids. The virus is guided to coated pits by lipid bulk flow, where the virus is endocytosed . The low-pH environment of the endosomal compartment induces a conformational change in the envelope protein G that triggers fusion of the viral and target membranes. Fusion releases the VSV nucleocapsid into the cytoplasm where transcription and replication take place. The VSV genome must be encapsulated with N protein in order to be replicated and transcribed (Wagner and Rose, 1996). The genes are transcribed from left to right forming an RNA gradient, thus N is synthesized in larger quantities than P, P is synthesized in larger quantities than M and so on with L synthesized in the lowest quantities (Wagner and Rose, 1996). The newly synthesized N protein encapsulate the negative sense RNA genome and the virus buds from the viral membrane where the virus obtains its complement of glycoprotein G.

VSV contains the single 67 kDa glycoprotein G with two N-linked glycosylation and fatty acid modifications on the cytoplasmic tail of the protein. The VSV glycoprotein

consists of a large 446 amino acid ectodomain, 20 amino acid transmembrane sequence and a small 29 residue cytoplasmic tail (Lenard,1993).

1.5 Intracellular Localization, Transport and Oligomer Formation of VSV G

VSV G is synthesized in the rough endoplasmic reticulum where folding of the G protein and addition of two N-linked glycosylations occur cotranslationally (Doms et al., 1993). The VSV G protein is transported from the rough ER to the Golgi complex where the N-linked glycosylations are modified and then to the plasma membrane, the site of viral assembly and budding. VSV G glycoprotein exists as a trimer of G molecules in the viral membrane (Dubovi and Wagner, 1990). The majority of the structural information for trimer formation is encoded in the ectodomain of VSV G, since VSV G lacking the transmembrane anchor and cytoplasmic tail can still oligomerize (Crise et al., 1989)(Doms et al., 1993)(Odell et al., 1997). The G protein obtains its correct tertiary and quaternary structure in the rough endoplasmic reticulum (Kreis and Lodish, 1986) (Doms et al., 1988) (Doms et al., 1993). A temperature sensitive mutant of VSV (tsO45), has a single point mutation in the ectodomain of VSV G that is sufficient at the non-permissive temperature (39°C) to cause glycoprotein G to misfold or form aggregates and thus be retained in the rough endoplasmic reticulum (Kreis and Lodish, 1986) (Doms et al., 1987) (Doms et al., 1988) (Doms et al., 1993). At the permissive temperature (32°C), VSV (tsO45) mutant forms trimers in the endoplasmic reticulum (ER) and are transported out of the ER to the Golgi complex (Kreis and Lodish, 1986) (Doms et al., 1987) (Doms et al., 1988) (Doms et al., 1993). VSV G must first oligomerize in the ER, failure of the

glycoprotein to obtain its correct tertiary and quaternary structure inhibits the ability of G to obtain a transport competent state (Kreis and Lodish, 1986) (Doms et al., 1987) (Doms et al., 1988) (Doms et al., 1993).

Pulse-chase experiments have shown that glycoprotein G is synthesized in the ER as a monomer with a $t_{1/2}$ of 3 minutes(Balch et al., 1986)(Kreis and Lodish, 1986)(Doms et al., 1987)(Doms et al., 1993). The G monomers fold cotranslationally followed by oligomerization in the endoplasmic reticulum with a half time $t_{1/2}$ of 6- 8 minutes (Balch et al., 1986)(Kreis and Lodish, 1986)(Doms et al., 1987)(Doms et al., 1993). The G protein is transported out of the ER to the Golgi only after trimerization has occurred with a $t_{1/2}$ of 15-20 minutes (Balch et al., 1986)(Kreis and Lodish, 1986)(Doms et al., 1987)(Doms et al., 1993).

1.6 Heptad Repeats

Heptad repeats have been identified in a number of viral fusion proteins. A heptad repeat contains repeating units of hydrophilic and hydrophobic amino acids in an a-g pattern, and the heptad repeat is commonly found in coil-coiled structures (Carr and Kim, 1993). The residues denoted a and d are typically hydrophobic amino acids that form the hydrophobic interior contacts between helices in the coiled-coil structures (Carr and Kim, 1993). Heptad sequences were identified in viral fusion proteins including influenza HA, with a classical a-g heptad motif that followed the amino terminal fusion peptide (Chambers et al., 1990). It was later shown that the HA heptad repeat has a random coil structure at neutral pH and a coiled coil structure at low-pH (Carr and Kim,

1993). It was suggested that the heptad repeat at low-pH acts like a "spring loaded mechanism"; its structural transition from random coil to a coil-coiled structure propels the fusion peptide toward the target membrane (Carr and Kim, 1993). Similar heptad repeats have also been identified in HIV and the heptad repeat has been identified as critical to HIV fusion activity (Rabenstein and Shin, 1995). Recently paramyxovirus F protein peptides with homology to two HIV peptides corresponding to the HIV heptad repeats were shown to inhibit fusion by paramyxoviruses (respiratory syncytial virus, measles virus and human parainfluenza virus type 3)(Lambert et al., 1996). This suggests the importance of the heptad repeat and its use among viruses in the fusion mechanism (Lambert et al., 1995).

Analysis of the primary sequence of VSV G failed to locate any obvious sequence that corresponds to a heptad repeat (Zhang and Ghosh, 1994). Recently it has been suggested that VSV G has two a-g heptad repeats that are conserved among all rhabdoviridae (Coll, 1995). The VSV G heptad repeats are not the same as other viral fusion peptide heptad repeats and no biological functions have been assigned to the VSV G repeats (Coll 1995).

1.7 Lipid Requirements for Receptor Binding, Fusion and Infectivity

A number of receptors have been identified for enveloped animal virus, most notably sialic acid for influenza HA (Weiss et al., 1988) and the T-cell surface protein CD4 for HIV gp 120 (Weiss, 1992). VSV glycoprotein G mediates both attachment to

the cell surface and low pH induced fusion of the viral and target membrane. In contrast to other viruses, it has been difficult to identify a cellular receptor for vesicular stomatits virus, but the following evidence suggests that the receptor for VSV attachment is a cluster of anionic phospholipids, phosphatidylserine and maybe phosphatidylcholine.

The existence of a receptor for VSV was first identified on Vero cell lines that have a saturable binding site for VSV attachment of approximately 4000 virion per cell (Schlegel et al., 1982). VSV has a wide host range and the receptor that mediates VSV attachment to cells must satisfy the obligate requirement of being expressed on the surface of a wide variety of cell types. Since VSV can compete with rabies virus for binding at the cell surface it suggests that all rhabdoviruses may share a common receptor (Coll, 1995). The VSV G receptor is not a protein since protease treatment of Vero cells or erythrocytes does not reduce, but enhances the binding of VSV(Schloemer and Wagner, 1975)(Schlegel et al., 1982) (Schlegel et al., 1983) (Yamada and Ohnishi, 1986) (Mastromarino et al.,1987).

Two different approaches were used to identify a putative receptor for VSV attachment. The first approach used competition binding experiments using detergent solubilized extracts from Vero cells or goose erythrocytes and identifying the components in the extracts that could effectively inhibit VSV binding attachment (Schlegel et al., 1983) (Mastromarino et al., 1987) (Lenard, 1993). The inhibitory components of VSV attachment were resistant to treatment with protease digestion or heating to 100°C for 10 minutes, but were sensitive to chloroform-methanol treatment and phospholipase C

treatment. This suggested that VSV attachment was inhibited by a phospholipid (Schlegel et al., 1983). Separation of the individual compounds in the extract showed that phosphatidylserine liposomes not only bind specifically to VSV but have the most potent inhibitory activity of all the phospholipids tested against VSV saturable binding and plaque formation with Vero cells (Schlegel et al., 1983) and goose erythrocytes (Mastromarino et al., 1987). In both cases, treatment of Vero cells or erythrocytes with phospholipase C, an enzyme that yields neutral diglycerides, reduced the attachment of VSV to cells (Schlegel et al., 1983) (Mastromarino et al., 1987). This indicates the importance of the phosphatidylserine head group in VSV attachment (Schlegel et al., 1983) (Mastromarino et al., 1987) (Lenard, 1993). The attachment of VSV to Vero or goose erythrocytes could not be inhibited by a soluble phosphoserine head group alone, the phospholipid head group in conjunction with the hydrophobic tail were required for inhibiting VSV attachment (Schlegel et al., 1983) (Mastromarino et al., 1987). It should be noted that these experiments identify compounds that inhibit VSV attachment and therefore represent putative receptors or antagonist of receptor binding. The following lipids, phosphatidylinositiol and GM3 gangliosides, were identified on the basis of inhibiting VSV attachment to goose erythrocytes but not Vero cells (Mastromarino et al., 1987).

Other experiments using phosphatidylserine liposomes confirmed that binding of VSV was dependent on the head group of phosphatidylserine and to a lesser extent on phosphatidylcholine (Schlegel et al., 1983) (Yamada and Ohnishi, 1986). The fatty acid

acyl chain composition of phosphatidylserine and phosphatidylcholine could be varied without affecting receptor binding, indicating the specificity for the phospholipid head group and the lack of specificity in fatty acids composition required for binding (Yamada and Ohnishi, 1986). Taken together, the data suggests that patches of phosphatidylserine and phosphatidylcholine can act as attachment sites for VSV.

The lipid requirements in the target membrane for VSV mediated fusion are different than the lipid requirements previously described for attachment. On the basis of competition binding experiments fusion was suggested to be dependent on a specific phospholipid, as pretreatment of goose erythrocytes with phopholipase C inhibits binding and fusion activity of VSV (Mastromarino et al., 1987). In order to discern between the requirements of phospholipids in fusion, experiments using human erythrocytes as a model system for VSV fusion were studied. Human erythrocytes have a difference in both the lipid content and lipid packing characteristics of the outer and inner leaflet of the plasma membrane (Herrmann, 1990). Using octadecylrhodamine (R18)-labeled human erythrocyte ghosts, it was determined that VSV could fuse with erythrocyte membranes that had been rendered symmetric, but not with the naturally occurring asymmetric erythrocyte membranes (Herrmann et al., 1990). In contrast to this study, neither incorporation of phosphatidylserine, normally located in the inner leaflet of asymmetric ghosts, into the outer leaflet of asymmetric ghosts increase fusion nor did treatment of lipid symmetric ghosts with phosphatidylserine decarboxylase decrease fusion (Herrmann et al., 1990). This confirmed results of Yamada and Ohnishi, that there is no specific

phospholipid requirement for fusion. (Yamada and Ohnishi, 1986) (Hermmann et al., 1990). The fusion dependency on lipid symmetric erythrocyte ghosts is a result of lipid packing, and redistributing unsaturated fatty acid that are concentrated in the inner leaflet into both leaflets (Herrmann et al., 1990). This confirms previous experiments that first described the requirements of cis-unsaturated acyl chains in biological membranes or liposomes to render them excellent target membranes for VSV fusion (Yamada and Ohnishi, 1986) (Herrmann,1990) (Lenard,1993). The biological requirement for cis-unsaturated acyl chains in the target membrane may be due to the tendency of these lipids to form non-bilayer intermediates (Lenard,1993).

Alphaviruses such as Semliki Forest Virus require cholesterol and sphingomyelin in the target membrane to mediate fusion (White and Helenius, 1980) (Phalen and Kielian, 1991) (Wahlberg et al., 1992) (Nieva et al., 1994). In contrast to Semliki Forest virus, VSV does not have a specific lipid requirement for fusion. VSV can infect cholesterol depleted mosquito cells or G protein reconstituted into phospholipid vesicles will fuse with liposomes in the absence of cholesterol (Eidelman et al.,1984) (Phalen and Kielian, 1991). This demonstrates that cholesterol is not required for VSV fusion (Eidelman et al.,1984) (Phalen and Kielian, 1991), although its presence in the target membrane enhances the fusion activity of glycoprotein G (Yamada and Ohnishi, 1986) (Herrmann et al., 1990) (Lenard, 1993) (Hug and Sleight, 1994). Specifically it seems that incorporation of cholesterol into the target membrane can enhance the extent of

fusion but does not affect the rate of fusion (Eidelman et al., 1984) (Hug and Sleight, 1994).

The cytoplasmic tails of both viral and cellular integral membrane glycoproteins are commonly modified with fatty acids. In viruses such as influenza HA A/Japan/305/57 virus, alphaviruses Sindbis and Semliki Forest virus removal of the fatty acid acylation abolishes the fusogenic activity or retards the release and alters the morphology of virions (Gaedigk-Nitschko et al., 1990) (Gaedigk-Nitschko and Schlesinger 1991) (Naeve and Williams, 1990). The Indiana serotype of VSV G is modified at amino acid Cys_{489} in the cytoplasmic tail with a palmitate fatty acid residue (Rose et al., 1984) (Whitt and Rose,1991). Mutation of Cys_{489} amino acid to Ser blocked fatty acid acylation of the VSV G, but did not alter the transport, anchoring to the plasma membrane, fusogenic activity or the incorporation of the mutant glycoprotein G into a mutant temperature sensitive strain of VSV (ts045) (Rose et al., 1984) (Whitt and Rose,1991). It appears that the fatty acid modification of the glycoprotein G cytoplasmic tail has no apparent biological role. It is also notable that G proteins of other serotypes of VSV, such as, New Jersey and cocal, have no fatty acid modifications, supporting the conclusion that fatty acid acylation is not essential for VSV replication (Kotwal and Ghosh, 1984) (Whitt and Rose, 1991).

1.8 Factors that Influence Fusion

The cell surface density of VSV G is essential for both the extent and rate of the fusion activity (Puri et al., 1993). As the cell surface density of G is increased past a threshold level, both the rate and extent of fusion increase with increasing cell surface expression of the protein (Puri et al., 1993). Purified G protein was partitioned into preformed liposomes, or virosomes, and the fusion activity was monitored by a resonance energy transfer assay. Increasing the G protein concentration in the virosome membrane also increased the extent of fusion (Hug and Sleight, 1994). The optimal concentration of G protein in the virosome membrane was 0.1 mol % (Hug and Sleight, 1994). This value correlates approximately to 1000 VSV G molecules per liposome, a value that is close to the approximated 1200 molecules per virion (Hug and Sleight, 1994)(Thomas et al., 1985). Increasing the G protein concentration above this optimal molar concentration of G and the extent of fusion increased slowly, decreasing the G protein concentration below 0.1% and the extent of fusion decreased rapidly (Hug and Sleight,1994).

Only VSV glycoprotein G is required to mediate fusion, as cell lines expressing stable or transiently VSV glycoprotein G on the cell surface form polykaryons when exposed to low-pH (Florkiewicz and Rose, 1984) (Reidel et al., 1984), showing that glycoprotein G in the absence of other viral genes is sufficient to support low-pH mediated fusion (Florkiewicz and Rose, 1984) (Reidel et al., 1984). The other viral proteins, N,P L and M do not influence the fusion activity of glycoprotein G, as cell lines infected with either a recombinant adenovirus expressing the VSV glycoprotein G or

wild-type VSV, each expressing similar cell surface densities of VSV glycoprotein G on the cell surface fuse to the same extent as measured by R18-labeled plasma membrane vesicles, suggesting that the matrix protein or other viral proteins of VSV do not influence the extent of fusion (Schneider et al., 1989) (Puri et al., 1993). The extent and rate of VSV G fusion are dependent on pH, temperature and cholesterol concentration (Puri et al., 1993) (Hug and Sleight,1994).

1.9 Kinetics and Conformational change of VSV G

Considerably more information is known about the conformational change of rabies virus G protein as compared to VSV G. Since both rabies and VSV are rhabdoviridae, with approximately 20% sequence homology (Rose et al., 1982), it is useful to examine the conformational change of rabies to better understand and postulate changes in vesicular stomatitis virus glycoprotein G. Rabies is known to have at least three conformations, native state, active state, and inactive state, that can be distinguished on the basis, of length by electron microscopy, bromelain sensitivity and by conformational specific monoclonal antibodies (Gaudin et al., 1991)(Gaudin et al, 1993) (Gaudin et al., 1995). The rabies viral G protein exists in the viral membrane as a trimer in the native state (Gaudin et al., 1995). The active state is more hydrophobic than the native state and occurs at pH 6.4 above the threshold that fusion is detected (Gaudin et al., 1991). It is the active state of the rabies protein that interacts with target membranes (Gaudin et al., 1991)(Gaudin et al, 1993) (Gaudin et al., 1995). Two protonation steps have been suggested for rabies, one at pH 6.4 that is required for hydrophobic binding to

membranes, and one below pH 6.0 that is required for fusion activation (Gaudin et al., 1991). The inactive conformation of rabies G is detected at 0.5 pH units above the threshold that fusion can be detected (Gaudin et al., 1993). The inactive and active forms of the protein exist in an equilibrium together, yet the transition to the active state is kinetically faster than the transition to the inactive state (Gaudin et al., 1993). The conformational change of the protein is reversible. The inactive state of the rabies G is also associated with the transport state of the protein from the ER to the plasma membrane (Gaudin et al., 1995). The inactive conformation prevents the newly synthesized G protein from fusing in the acidic Golgi compartments (Gaudin et al., 1995). This may suggest that the transition of rabies G to the inactive state is irrelevant to the fusion process (Gaudin et al., 1995). All three conformations have been suggested for VSV G on the basis of kinetic data (Clague et al., 1989)(Puri et al., 1992) and recently has been shown with biochemical techniques (Pak et al., 1997).

Using an electron microscope, studies with intact VSV virions have shown that the envelope glycoprotein G of VSV shows a pH-dependent accumulation of its glycoproteins at the ends of the bullet shaped virions (Brown et al., 1988). The G protein can move laterally in the viral membrane, and accumulates at the ends of virions in a pH dependent fashion (Brown et al., 1988). Although the biological significance of this phenomenon is not clear, both the accumulation of G at the ends of virions and low-pH-dependent activation of the G protein have the following similar properties: both are

induced by low pH, both are reversible and both are not dependent on temperature (Brown et al., 1988).

The fusion peptides of pH-dependent viral fusion proteins contain acidic residues in or bordering their fusion peptides (White, 1990)(White, 1992). In VSV G protein, Asp137 and Glu139 are conserved in all 5 vesiculoviruses including Indiana, Cocal, New Jersey and Chandipura with exception of Glu139 in Cocal which is substituted with a Val. Mutations at D137→L and E139→L or the double mutant D137/E139→S affect the pH threshold of fusion and the pH induced conformational change of the G protein above pH 5.7, however all three mutants showed wild-type fusion below pH 5.7 (Fredericksen and Whitt, 1996).

Experiments by Durrer et al. were the first to identify subtle differences in the way VSV G and influenza HA interact with target membranes under pre- and post-fusion conditions. (Durrer et al., 1995). Under pre-fusion conditions (pH 6.4, 0^0C) both glycoproteins are labeled in the ectodomain, presumably at the fusion peptide (Durrer et al., 1995). In transition from the pre-fusion state (pH 6.4, 0°C) to the post-fusion state (pH 5.8, 23°C) there was a 3 fold increase in the labeling of VSV G protein, a result of labeling the transmembrane anchor (Durrer et al., 1995). Influenza HA was labeled 200 fold greater in the post-fusion state as compared to the pre-fusion state (Durrer et al., 1995). The difference in labeling of the viral glycoproteins under pre- and post-fusion conditions may suggest that the transition between pre- and post fusion states are mechanisticaly different for influenza HA and VSV G(Durrer et al., 1995).

1.10 Characterization of the VSV G (H2 region) Fusion Peptide

Fusion peptides are located in a the ectodomain of type 1 integral membrane proteins either at the amino terminus or internal. In general, fusion peptides are hydrophobic in nature and approximately 16 -20 amino acids in length. Under low pH conditions the fusion proteins are induced to change conformation extruding a previously buried fusion peptide, at neutral pH, and inserting the fusion peptide in the target bilayer (White, 1990)(White, 1992)(Hernandez et al., 1996). Therefore at low pH the fusion peptide can interact with and presumably destabilize the target lipid bilayers. Primary sequence analysis of VSV G have not revealed any obvious hydrophobic stretch of amino acids that could act as a putative fusion peptide other than the signal sequence and transmembrane anchor (Li et al., 1993). The amino terminal region of VSV G, after cleavage of the signal sequence was not particularly hydrophobic, making this an unlikely candidate for the fusion peptide. Although, earlier studies have shown that a synthetic peptide corresponding to the amino terminal 25 amino acids of VSV G has a pH-dependent hemolytic activity (Schlegel and Wade, 1984)(Schlegel and Wade, 1985). The hemolytic activity of the 25 amino acid synthetic peptide appeared to be sequence specific, as a single amino acid mutation of lysine to glutamic acid at the amino terminus of the peptide abolished its pH-dependent hemolytic activity(Schlegel and Wade, 1984)(Schlegel and Wade, 1985) . Although the corresponding mutation in wild-type glycoprotein G resulted in a level of fusion activity indistinguishable from wild-type as measured by a cell-cell fusion assay (Woodget and Rose, 1986). This suggests that the

hemolytic activity of the synthetic G peptide is different than the activity that causes low-pH induced G fusion (Woodget and Rose, 1986).

Based solely on sequence homology between Indiana and New Jersey serotypes of vesicular stomatitis virus glycoprotein G it was predicted that an internal stretch of uncharged amino acids from 102-131 was a strong candidate for the internal fusion peptide (Ohnishi, 1988). Another region of VSV G at amino acids 175-199 was also proposed to have hydrophobic properties at acidic pH (Ohnishi, 1988).

A mutant protein (QN-1) of vesicular stomatitis virus glycoprotein G was constructed that contained a third N-linked glycosylation site at amino acid 117(Machamer and Rose,1988). The mutant protein QN-1 was found to be expressed, transported to the cell surface (Machamer and Rose, 1988) and formed trimers efficiently at low pH (Doms et al., 1988). The mutant QN-1 was however fusion defective when HeLa cells expressing G protein on the cell surface were exposed briefly to acidic media (Whitt et al., 1990). The QN-1 mutant could also incorporate into a temperature-sensitive VSV G mutant (ts045) at the non-permissive temperature (39°C), but could not rescue infectivity of the ts045 virus (Whitt et al., 1990). The QN-1 mutant was defective at the level of fusion. The presence of an extra glycosylation site in close proximity to the fusion peptide would likely disrupt the interaction of the fusion peptide with the target bilayer (Whitt et al., 1990).

Support for involvement of this region in the fusion process was elucidated by our laboratory using linker insertion mutagenesis. Linker insertion mutagenesis was used to

identify three regions in the VSV G ectodoman where inserting tripeptides or dipeptide LEN, SRD and SRD/SS at amino acid positions 123 (H2 region), 194 (H5 region) and 410/415 (H10/A4 region) respectively, abolished the fusion activity of G protein (Li et al., 1993). Mutants H2, H5 and H10/A4 were expressed in COS-1 cells with cell surface localization, transport and trimerization properties similar to wild-type, but all three mutants were defective at syncytia formation (Li et al., 1993). The H2 insertion (a.a 123) mutant was in close proximity to the QN-1 mutation at amino acid 117 that also confers a fusion negative phenotype. Sequence analysis of this region (amino acids 123-137) revealed a stretch of uncharged amino acids that were conserved among all 5 VSV serotypes including VSV Indiana, New Jersey, Cocal, Chandipura, and Piry (Li et al., 1993). The H2 region encompasses an area that was also previously predicted by Ohnishi to be a candidate for the fusion peptide. The H2 region of the protein (a.a. 123-137) also contains three proline residues at P^{123}, P^{126} and P^{127}, a requirement that has been previously suggested for internal fusion peptides (White., 1992). Taken together, this suggests an important biological role for this region of the VSV G glycoprotein (Li et al., 1993).

^{123}Pro Gly Phe Pro Pro Gln Ser Cys Gly Tyr Ala Thr Val Thr Asp Ala Glu^{139}

Figure 1: The amino acid sequence of the putative fusion peptide of VSV G Indiana. The numbering of the amino acid sequence were taken from VSV G Indiana sequence with amino acid number 1 indicated before cleavage of the signal sequence. The underlined amino acids represent amino acids that are conserved in all 5 VSV serotypes

Site directed mutagenesis of the H2 insertion region was used to characterize the biological role of amino acids 123 -137 in the fusion process (Zhang and Ghosh, 1994). The mutations performed by Zhang and Ghosh can be divided into the following three categories based on the levels of fusion activity: wild-type fusion, fusion negative and reduced levels of fusion. The following two mutants, Gly124→Ala, Pro127→Leu, were completely fusion defective when assayed for syncytia formation in COS-1 cells. Three mutants Phe125→Tyr, Pro127→Gly and Asp137→Asn showed fusion 30 %, 20 % and 50% of wild-type G respectively and the pH optima required for fusion were shifted to more acidic values (Zhang and Ghosh, 1994). Mutation of Pro123→Leu resulted in a mutant that had wild-type fusion activity. The results of Zhang and Ghosh were confirmed by another lab, with two mutations Gly124→Glu and Pro127→Asp that also abolish fusion activity at pH 5.7, but show some syncytia formation at pH 5.2 (Fredericksen and Whitt, 1995). A mutation at Ala133→Lys completely abolished all fusion activity at all pH tested(Fredericksen and Whitt, 1995). They were also able to incorporate Gly 124→Glu and Pro127→Asp into VSV tsO45 virions and show that both mutations could not rescue virus infectivity of BHK-21 cells (Fredericksen and Whitt, 1995).

The H2 region primary sequence although not strictly hydrophobic shares some similarities with other internal fusion peptides. By definition fusion peptides are approximately 16 to 26 amino acids in length and bordered by positively and negatively

charged amino acids (White,1990). Internal fusion peptides are also known to contain proline residues near the center, VSV G has three proline residues at amino acids 123,126 and 127 (White, 1992)(Zhang and Ghosh, 1994). The H2 domain also shares some sequence homology to the putative internal fusion peptide of the alphavirus family Semliki Forest virus E1 fusion protein. Both the SFV E1 glycoprotein and VSV G are internal fusion peptide that have pH-dependent fusogenic activities (Kielian, 1993). They share a common motif with similar sequences and spatial arrangements of proline, glycine and aspartic acid shown in Figure 2 (Zhang and Ghosh, 1994).

Fusion Protein	Sequence of Fusion Peptide
VSV G	^{123}Pro Gly Phe Pro Pro Gln Ser Cys Gly Tyr Ala Thr Val Thr Asp^{137}
SFV E1	^{82}Thr Gly Val Tyr Pro Phe Met Trp Gly Gly Ala Tyr Cys Phe Cys Asp^{97}
Shared Motif	__ Gly __ __ Pro __ __ __ Gly __ __ __ __ __ __ Asp

Figure 2: Comparison of the sequences of the internal fusion peptides of vesicular stomatitis virus glycoprotein G and Semliki Forest virus E1 protein and the shared motif of both fusion peptides. Conserved amino acid residues are underlined. The VSV underlined amino acid are conserved among among all 5 VSV serotypes. Underlined SFV E1 amino acids are conserved in all alphaviruses and rotavirus VP4.

Mutations in the internal fusion peptide of Semliki Forest virus corresponding to this shared motif of Gly83→Ala, and Gly91→Ala decrease the pH to induce fusion to more acidic values, while Gly91→Asp completely abolishes the fusion activity (Levy-Mintz and Kielian, 1991). Mutations that affect SFV fusion activity are in conserved amino acids in all alphaviruses (Levy-mintz and Kielian,1991), and correspond to glycine

and proline residues that are shared in the suggested SFV-VSV fusion peptide motif (Zhang and Ghosh, 1994).

Dr. J. Brunner's laboratory has used photoactivatable lipids to label areas of a protein capable of interacting with the lipid membranes, a technique that has proved useful in proving that the amino terminus fusion peptide of influenza HA can insert into target membranes under pre-fusion conditions (pH 6.4,0^0C) (Tsurudome et al., 1992). Using this same technique with rabies and VSV, they were able to identify a region in the ectodomain of the corresponding viral glycoprotein that interacts with target membranes under pre-fusion conditions (pH 6.4, 0^0C) (Durrer et al., 1995). Vesicular stomatitis virus ectodomain was labeled from amino acid 59 - 221 of VSV G. This region of VSV G encompasses the H2 region (a.a 123-137) that was identified as a putative fusion peptide by mutagenesis. The H2 region of the VSV consists of mainly uncharged amino acids and is capable of interacting with target bilayers at acidic pH. Further experiments will be needed to define the precise region of the VSV G glycoprotein that is labeled under pre-fusion conditions before a precise region of VSV G can be designated as a fusion peptide with any certainty. The results also confirm that the activated state of VSV G interacts with target membranes (Durrer et al., 1995), (Puri et al., 1988).

Work in our lab has also identified insertion mutations at two different regions of the VSV G ectodomain H5 and H10/A4 that abolish the fusion activity of VSV G. The H5 linker insertion region was located at amino acid 206 (Li et al.,1993). This region has low sequence conservation, with only amino acid Cys193 and Gly 215 common between

all 5 vesiculoviruses. It is noteworthy, that the H5 insertion region is contained within the photolabeled ectodomain fragment previously described by Durrer et al., 1995. Point mutations in this region, Leu192→Lys/Asp, Leu197→Lys Ser212→Glu were all found to be as fusogenic as wild-type G, while two other mutants Thr203→Asp and Gly209→Glu were retained in the endoplasmic reticulum (Fredericksen and Whitt, 1995). This region is also in close proximity to a glycosylation residue that may increase the hydrophilic character of this region while imposing steric constraints that would limit the hydrophobic interactions of this region with the target membrane (Fredericksen and Whitt, 1995).

Linker insertions at amino acid 410 and 415 in regions H10 and A4 respectively, abolished the fusion activity of VSV G (Li et al., 1993). The H10/A4 insertions borders a region between amino acids 383 and 409 where 63 percent of the amino acids are completely conserved between all 5 vesiculoviruses Indiana, Cocal, New Jersey and Chandipura. This region could have a direct role in the VSV G low pH induced fusion mechanism, or alternatively these mutations might disrupt fusion indirectly by misfolding of G. The role of H10/A4 as the fusion peptide that directly interacts with the target membrane tends to be discounted since this region is not contained within Durrer's photolabeled fragment (Durrer et al., 1995). This implies that H10/A4 has an indirect role on the fusion process, possibly in the conformational change of the glycoprotein.

The H10 region involvement in the conformational change of glycoprotien G was suggested from a recent study with rabies virus. Rabies virus mutants (RAIN mutants,

resistant to acid-induced neutralization) were selected that could escape low-pH neutralization with monoclonal antibodies specific for the low-pH inactive conformation of rabies glycoprotein G (Gaudin et al., 1996). The RAIN mutants typically contained mutations in glycoprotein G at either amino acid V392→G or M396→T and the mutations did not affect either the rate or extent of fusion of the rabies virus RAIN mutants (Gaudin et al., 1996). However, both RAIN mutants were slowed in the kinetics of the low-pH induced conformational change of glycoprotein G from the native state to the inactive-state (Gaudin et al., 1996). The RAIN mutations are contained in a region of the rabies glycoprotein G that has been predicted to contain two amphipathic α-helices separated by a small 5 amino acid hinge (Gaudin et al., 1996). The hing also contains a histidine and a proline motif conserved among all Rhabdoviridae (Gaudin et al., 1996). Sequence alignments of both the rabies and VSV show that both RAIN mutations of rabies and the H10 mutations of VSV occur in similar regions of the G protein (Gaudin et al., 1996). Based on this evidence, it could be suggested that the mutations in the H10 region of VSV G may abolish the fusion activity by stabilizing the inactive conformation of the VSV glycoprotein G (Gaudin et al., 1996). Recent work in our lab has shown that mutations in the region of amino acids 395-418 can decrease or abolish the fusion activitiy of VSV G protein(Shokrolla et al., 1998). Five of these mutations, G404→K/A, D409→N/A and D411→N also have altered tryptic digestion profiles in response to low-pH. This suggests that the H10 region may have a role in the the low-pH conformational change of the G protein (Shokrolla et al., 1998). It is also interesting to note that the H10

region falls close to one of two predicted heptad repeats at amino acid position 130-160 and 330-370 (Coll, 1995).

1.11 Role of Transmembrane Anchor and Cytoplasmic Tail

The fusion peptide is located on the protein subunit that is anchored to the membrane through a peptide segment. Soluble ectodomains of three different low-pH induced viral fusion proteins, influenza HA2 (White et al., 1982) (Wiley and Skehel, 1987), SFV E1 (Klimjack et al., 1994) and VSV G (Florkiewicz and Rose, 1984), lacking both the transmembrane and cytoplasmic domains, were not fusogenic. The absence of fusogenic activity demonstrates the importance of anchoring the ectodomain to the plasma membrane for its activity.

The role of the transmembrane anchor and cytoplasmic tail in the fusion mechanism has been demonstrated for different viruses. A chimeric rabies glycoprotein G with the transmembrane anchor in conjunction with the cytoplasmic tail exchanged with equivalent domains from VSV glycoprotein G was not fusogenic in a cell-cell fusion assay (Whitt et al., 1991). The transmembrane anchor of the pH-independent viral fusion proteins gp41 of HIV and the TM protein of Moloney murine leukemia virus were both suggested to have a role in the fusion process. Both a GPI anchored TM protein and deletion mutants in the transmembrane anchor of the TM protein of Moloney Murine Leukemia virus can block the fusion activity of this protein (Ragheb and Anderson, 1994). Mutations within the transmembrane anchor of the HIV envelope protein can abolish its fusion activity (Helseth et al., 1990) (Owens et al., 1994). It was suggested

that basic amino acid residues 696 and 707 of the HIV transmembrane anchor were required for the fusion activity of this protein (Helseth et al., 1990) (Owens et al., 1994). A chimeric HIV env protein lacking both the transmembrane anchor and cytoplasmic domains were exchanged with a signal for GPI-lipid anchor addition from DAF and Thy 1.1; both chimeras were shown to be fusion negative (Salzwedel et al., 1993)(Weiss and White, 1993). Taken together, the data suggests an important link between the transmembrane anchor and the fusion activity of the HIV gp41 protein (Owens et al., 1994).

Influenza is the best characterized viral fusion protein. It has an amino terminal hydrophobic fusion peptide and requires low pH to induce fusion. The hydrophobic transmembrane anchor of influenza HA mediates two functions: it anchors the ectodomain in to the viral membrane and it is required for the full fusion activity of influenza HA. A bromelain cleaved HA fragment lacking the cytoplasmic and transmembrane domains binds to liposomes but can not induce fusion (White et al., 1982) (Wiley and Skehel, 1987). When the HA ectodomain was anchored to the plasma membrane with a lipid anchor (glycosylphosphatidylinositol-lipid tail), it was not fusogenic (Kemble et al., 1994). Although, the GPI-linked HA chimera is sufficient to support lipid mixing (Kemble et al., 1994)(Melikyan et al., 1995). While a lipid anchor can satisfy the initial step of fusion lipid mixing, fusion pore formation and dilation will not occur without the transmembrane anchor (Kemble et al., 1994) (Melikyan et al., 1995). Recently it has been shown that chimeric HA proteins in which the cytoplasmic

tail and/or transmembrane domain were replaced with equivalent domains of Sendai virus F protein were all fusogenic as tested in a fusion assay (Schroth-Diez et al., 1998). This suggests that the specific amino acid sequence of the transmembrane anchor and cytoplasmic tail are not essential for HA's fusion activity (Schroth-Diez et al., 1998)

The cytoplasmic tail can also influence the fusion activity of viral fusion proteins in both a positive and negative manner. A deletion of the cytoplasmic tail of the paramyxovirus SV5 F fusion protein has recently been shown to promote hemifusion but not full fusion (Bagai and Lamb, 1996). This suggests an important role of the F protein cytoplasmic tail in the fusion process. The regulatory role of the cytoplasmic tail in fusion of both HIV and SIV has been demonstrated, where deletion of an R peptide of the cytoplasmic tail can enhance viral fusion (Ritter et al., 1993) (Mulligan et al., 1992). Mutations in the cytoplasmic tail of Newcastle disease virus have identified a region (amino acid 540-550) that is required for the fusion activity of the fusion protein (Sergel and Morrison, 1995). The importance of the cytoplasmic tail and transmembrane anchor on the fusion process has become apparent, both domains, the transmembrane anchor and cytoplasmic tail can affect the fusogenic activity of viral fusion proteins.

A common theme is emerging among viral fusion proteins that have been characterized to date. It has become apparent that the fusion peptide is a critical element of all viral fusion proteins. Mutations in the fusion peptides of different viruses can abolish the proteins fusion activity (Bosch et al., 1989)(Feed et al., 1990)(Feed et al., 1992)(Gething et al., 1986)(Levy-Mintz and Kielian, 1992)(Sergel-Germans et al.,

1994)(Zhang and Ghosh, 1994)(Fredericksen and Whitt, 1995). To better understand the contributions of other domains of VSV G glycoprotein in the fusion mechanism, specifically the transmembrane anchor and cytoplasmic tail, we have constructed a number of chimeric proteins in which the transmembrane anchor alone or the transmembrane anchor in conjunction with the cytoplasmic tail of VSV G were exchanged with equivalent domains from other viral glycoproteins not involved in low-pH induced fusion or the T-cell surface protein CD4. The results suggest that the specific amino acid sequence of VSV G transmembrane or cytoplasmic tail are not essential for fusion. Although, VSV G chimeras with the transmembrane anchor in conjunction with the cytoplasmic tail replaced show reduced amounts of fusion as compared to wild-type. To investigate the requirement of a GPI-lipid anchor on VSV G fusion, a chimera GGPI was constructed. In contrast to an influenza GPI-linked hemagglutinin, the ectodomain of VSV G was not able to mediate hemifusion. The lack of fusogenicity of the GGPI construct is a result of the 9 amino acid signal sequence of DAF retained in the GGPI construct after GPI-lipid addition. This has led to the identification of another region in the ectodomain of VSV G at the EC-TM interface, where insertion (G10DAF), deletion (GΔ9) or replacement (GΔ910DAF) of the EC-TM amino acids of VSV G with 9 amino acids of DAF abolishes the fusion activity of the G protein.

2. Materials and Methods

2.1 Chemicals and Reagents

CHEMICAL	SUPPLIER
Acrylamide	Research Organics Inc.
Adenosine 5'- triphosphate	Pharmacia
Agar	Difco
Agarose	Gibco/BRL
Ampicillin	Sigma
Aprotinin	Miles Canada Inc.
Bactotryptone	Difco
Bromophenol Blue	Sigma
Deoxynucleotides (dNTP's)	Pharmacia
Dithiotreitol	Calbiochem
Dimethyldioctadecylammonium bromide	Sigma
Ethylenediaminetetraacetic acid disodium salt	BDH
Ethylene glycol-bis(ß-aminoehtyl ether) N,N,N',N'tetraacetic acid	Sigma
Ethidium Bromide	Sigma
Fetal Bovine Serum (FBS)	Gibco/BRL
L-Methionine	General Biochemicals
N-2-hydroxyethylpiperazine-N-2-ethanesulfoic acid (HEPES)	Boehinger Mannheim
2-[N-morpholino] ethanesulfonic acid (MES)	Sigma
3-[N-morpholino] propanesulfonic acid (MOPS)	Sigma
N,N-methylenebisacrylamide	Gibco/BRL
N,N,N,N,-tetramethylethylene-diamine (TEMED)	Gibco/BRL
Non-essential amino acids (100X)	Gibco/BRL
Nonidet P40	BDH
Octadecyl rhodamine (R18)	Molecular Probes
Paraformaldehyde	Sigma
Penicillin (5000 U/ml),Strepto-mycin (5 mg/ml)	Gibco/BRL
Phenylmethylsulfonide fluoride (PMSF)	Sigma

CHEMICAL	SUPPLIER
Phenol (liquefied)	Fisher Scientific
Phosphatidylethanolamine	Sigma
Protein A-Sepharose	Pharmacia
Rubidium Chloride	Sigma
Salicylic acid	Sigma
Sodium azide	Sigma
Sodium deoxycholate	Calbiochem
Sodium dodecyl sulfate (SDS)	BDH
Sucrose	Gibco/BRL
Tris(hydroxymethyl)aminomethane (Tris)	Boehringer Mannheim
Triton X-100	Bio-Rad
Urea	Research Organics Inc.
Vitamin solution (100X)	Gibco/BRL
Xylene cyanol FF	Baker Chemical
Yeast Extract	DIFCO

2.2 Radiochemicals

[^{35}S]-dATP 8 μCi/μl	Dupont-New England Nuclear
[^{35}S]-methionine (1175 Ci/mmol)	Dupont-New England Nuclear
Na ^{125}I, carrier free (50 μCi/μl)	Dupont-New England Nuclear

2.3 Proteins and Enzymes

Enzymes	Supplier
Calf Intestinal Alkaline Phosphatase	Pharmacia
Restriction enzymes	BRL/Gibco, Bohringer Mannheim, Pharmacia, New England Biolabs
Ribonuclease A	Sigma
T4 DNA Ligase	BRL/Gibco
Taq DNA Polymerase	Perkin Elmer
T4 Polynucleotide Kinase	New England Biolabs
Trypsin/EDTA	Gibco/BRL
Trypsin TPCK	Bohringer Mannheim

2.4 Antibodies

Rabbit polyclonal anti-VSV glycoprotein G (Indiana) antibody was made by Essam Wanas (McMaster University) by injecting rabbits with a recombinant adenovirus vector expressing the VSV G protein, AdG12. The adenovirus vector expressing VSV G Indiana was a gift from Dr. L. Prevec (McMaster University).Goat anti-rabbit IgG conjugated to fluorescin was purchased from Cappel Laboratories.

2.5 Molecular Biology Kits

Geneclean II® Kit	Bio 101 Inc.
Muta-Gene® M13 In Vitro Mutagenesis Kit Version 2	Bio-Rad
Sequenase® Version 2.0, DNA Sequencing Kit	United States Biochemical

2.6 Oligonucleotides

Oligonucleotides were synthesized at the Institute for Molecular Biology and Biotechnology at McMaster University. The following oligonucleotides were used for sequencing DNA, creating deletions and PCR.

Primer	Oligonucleotides (Sequences with homology)	Description
AB5405	5' C AGT AGT TGG AAA TTT GGG GCG CT 3' (VSV G 1403-1415, HSV-1 gB 2870-2881)	Deletion of 10 extra amino acids of the gB ectodomain of GtmΔ 12gB to construct GgB3G
AB6331	5' CTC GAG CTC GAG TCC AAA TAA AGG AAG TGG A 3 (human DAF 1098-1114)	PCR amplification of the C-terminal 37 amino acids of human decay acceleration factor, to construct a GPI lipid anchored VSV G ectodomain (G-GPI, GΔ GPI)
AB6332	5' GAA TTC GAA TTC TTT CTC CTT GCT CTG TTG A 3' (human DAF 1216-1099)	PCR amplification of the C-terminal 37 amino acids of human decay acceleration factor, to construct a GPI lipid anchored VSV G ectodomain (G-GPI, GΔ 9GPI)
AB7846	5' CTC GAG CTC GAG C GCC CTG ATT GTG CTG GGG 3' (human CD4 1267-1284)	PCR amplification of the transmembrane domain or transmembrane domain and cytoplasmic tail of CD4
AB7848	5' T TAC GCG CGC GAA GAA GAT GCC TAG CCC AA 3' (human CD4 1329-1310)	PCR amplification of the transmembrane domain of CD4
AB7849	5' GCG CGC GCG CGC GGG AGG CTG CAA GTG CGA 3' (human CD4 1490-1471)	PCR amplification of the transmembrane domain and cytoplasmic tail of CD4
AB9189	5' TCG AGC CCA AAT AAA GGA AGT GGA 3' (anneals to AB 9190)	A linker with Xho1 sticky ends used to construct G10DAF and G Δ910DAF
AB9190	5' CG GGT TTA TTT CCT TCA CCT AGC T 3' (anneals to AB 9189)	A linker with Xho1 sticky ends used to construct G10DAF and G Δ910DAF
AB1885	5' GAT ACT GGG CTA TCC AAA 3' (VSV G 1356-1373)	Sequencing primer for VSV G transmembrane domain and cytoplasmic tail
AB 6809	5' ATGGATACCAACTCGGAGAACCAAGAATA G -3' (VSV G) (VSVG 1490-1461)	Sequencing primer for GΔ 910DAF
AB 8844	5' CGTAACTCGAGCTTTTCTCGATTGGATTTT TGGATAG-3' (VSV G1401-1365)	PCR amplification of VSV G sequences between Kpn1 and Xho1 to construct GΔ9
AB1891	5' AA TGG GTC ACT ACT TGT G 3' (VSV G 265-282)	PCR amplification of a VSV G sequence between Kpn1 and Xho1 to construct GΔ9

2.7 Media and Buffers

The following list describes the composition of common buffers and media.

Media/ Buffer	Components
LB Media	1% Bactotryptone, 0.5% yeast extract, 171 mM NaCl
2xYT	1.6% Bactotryptone, 1% yeast extract, 86 mM NaCl
H Medium	1% Bactotryptone, 86 mM NaCl
Glucose Minimal Media	42 mM Na_2PO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 1 mM $MgSO_4$, 0.001% thiamine Hcl, 0.02% glucose
PBS	137 mM NaCl, 2.7 mM KCl, 8 mM Na_2PO_4, 1.5 mM KH_2PO_4, pH 7.4
TE	10 mM Tris-HCl, 1 mM EDTA, pH 8.0
TBE	90 mM Tirs-borate, 2 mM EDTA
Lysis Buffer	1% (v/v) NP40, 0.4% (w/v) Sodium deoxycholate, 66 mM EDTA, 10 mM Tris pH 7.4, 0.3 % (v/v) SDS
4xMNT	80 mM MES, 120 mM Tris, 400 mM NaCl, 4 mM EDTA, 4 mM EGTA
2xMNT	40 mM MES, 60 mM Tris, 400 mM NaCl, 4 mM EDTA, 4mM EGTA
Detergent Lysis Buffer	1% Triton X-100, 10 mM Tris pH 7.4, 66 mM EDTA and 0.02% sodium azide
RIPA	1% (v/v) NP40, 0.4% (w/v) sodium deoxycholate, 12.5 mM EDTA, 50 mM Tris pH 8.0, 0.3% (v/v) SDS

2.8 Molecular Weight Markers

Radioactive [^{35}S]methionine VSV (Indiana) marker was prepared by Essam Wanas (McMaster University). This marker displays 5 predominant bands on a 10% polyacrylamide gel. The apparent molecular weight of the bands correspond to L (190 kDa), G (69 kDa), N and P (50 and 49 kDa) and M (29 kDa) proteins. The DNA marker, 1 KB ladder was purchased from Gibco/BRL and displayed the following banding pattern

on an agarose gel 12216,11198, 10180, 9160, 8144, 7126, 6180, 5090,4072, 3054, 2036, 1636, 1018, 506, 517, 396, 344, 298 bp.

2.9 Plasmids and Bacterial Strains

The eukaryotic expression vector pXM was provided by Dr. G.G. Wong (Genetics Institute Inc., Cambridge, MA). The plasmid pCDNA1CD4 encoding the gene for human CD4, the HIV receptor on the surface of T-cells was a generous gift from Dr. A. Jabbar (The Cleveland Clinic Foundation, Cleveland, Ohio). The plasmid pBS-SK-N-Tϕ encoding the gene for VSV N (nucleocapsid protein Indiana) was a generous gift from Dr. J. K. Rose (Yale University School of Medicine, New Haven Connecticut). The plasmid pDAF12, which contains the sequence for human decay-accelerating factor cDNA was a generous gift from Dr. Judy White (University of California, San Francisco, California).

E.coli DH5α (supE44, ΔlacU169, (Φ80lacZΔM15), hsdR17, recA1, end A1 gyrA96, thi-1, relA1) was purchased from Gibco/BRL and was used for propagation and preparation of all plasmid DNA.

E.coli CJ236 (dut-1, ung-1, thi-1, rel A-1; pCJ105(Cm^r)) from BIO-RAD and was used for substituting uracil for thymine in all plasmid DNA template preparation for M13 mutagenesis.

E.coli MV1190 (Δ(lac-pro AB), thi, sup E, Δ(srl-rec A) 306::Tn10(tet^r)[F':traD36,pro AB, $lacI^qZ\Delta M15$]) from BIO-RAD was used for selection of mutants from parental wild-type templates.

2.10 Restriction Endonuclease

Restriction digests of plasmid DNA was performed as specified by the manufacture of the restriction enzyme. Double restriction enzyme digests were performed in Pharmacia One Phor ALL Buffer Plus. When an enzyme required unique restriction digest conditions, the first restriction enzyme was heat inactivated, extracted with phenol:chloroform and ethanol precipitated. The DNA pellet was suspended in a small volume of TE buffer, pH 8.0 and digested with the second restriction enzyme. All digestions were stopped by the addition 6X loading buffer and the DNA fragments were separated by 0.8 to1.5% (w/v) agarose gel electrophoresis.

2.11 Dephosphorylation of DNA

Plasmid DNA that was digested with a single restriction endonuclease was dephosphorylated with calf intestinal alkaline phosphatase prior to use in ligations. The dephosphorylation of plasmid DNA digested with a single enzyme reduced the transformation background resulting from ligation of compatible sticky ends during ligations. The protocol for dephosphorylation was followed as described by the manufacturer Pharmacia. Approximately 1-10 μg of plasmid DNA was digested with a single restriction endonuclease in Pharmacia One Phor ALL Plus Buffer system. The restriction enzyme was heat inactivated for 15 minutes and then cooled on ice. One unit of calf intestinal alkaline phosphatase (CIAP) was used to dephosphorylate 1 to 10 μg of plasmid DNA at 37°C for 30 minutes. The CIAP was heat inactivated at 85°C for 15 minutes, phenol:chloroform extracted and precipitated with 2 volumes of absolute

ethanol. The dephosphorylated plasmid DNA was suspended in a small volume of sterile ddH_2O. The dephosphorylated plasmid transforms bacteria at approximately 1% of the efficiency of plasmid DNA that has not been dephosporylated.

2.12 Production of Blunt Ends from 3'→5' Overhangs

Native T7 DNA polymerase has a high 3'→5' exonuclease activity and was used to degrade 3' overhangs by the methods described in Current Protocols in Molecular Biology. The plasmid DNA was digested with the appropriate restriction endonuclease in a 20 µl reaction volume. The DNA was separated by agarose gel electrophoresis to assure that the plasmid was completely cut. The DNA was extracted with phenol:chloroform, precipitated with absolute ethanol and suspended in 10 µl 1x synthesis buffer [5mM each deoxynucleotide triphosphate, 10 mM ATP, 100 mM Tris pH 7.4, 50 mM $MgCl_2$, 20 mM ditthiothreitol] and 0.5 U native T7 polymerase, incubate at 37°C for 30 minutes. The reaction was terminated by heating at 75°C for 10 minutes.

2.13 PCR Amplification with Sticky Foot Mutagenesis

Two primers were designed to amplify a specific DNA segment by PCR. Each primer has twelve nucleotides at the 5' side that do not anneal to template but instead contain a duplicated novel restriction enzyme site. The procedure for PCR amplification of DNA was followed as described in Current Protocols in Molecular Biology. In a 500 µl microcentrifuge tube the following components were added to a final concentration of 1x PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 $MgCl_2$, 0.01% (w/v) gelatin autoclaved), 0.5 µM Primer 1, 0.5 µM Primer 2, 0.1 ng of CsCl purified plasmid DNA

template, 0.2mM 4dNTP mix and sterile water to make the final volume up to 100 µl. The reactions were held on ice prior to the addition of 2.5 U of Taq polymerase (Perkin Elmer Cetus). The microcentrifuge tubes were loaded in a Techne PHC-3 Thermal Cycler, that was programmed with the following parameters: Hot start for 5 minutes at 94°C for 1 cycle, 30 complete cycles of (denaturation step for 1 min at 94°C, annealing step for 1 minute at 55°C if the GC content is less than 50% or at 60°C if the GC content is greater than 50%, and an extention step at 72°C for 1 min if the product is less than 500 bp in length or 3 minutes for products grater than 500 bp in length). The last step was 1 cycle at 72°C for 10 minutes to assure that all PCR products were completely extended. Ten percent of the PCR reaction was run on a 0.7- 1.5 % agarose gel to determine the PCR product size and purity. The PCR products were extracted with phenol:chloroform, precipitated with absolute ethanol, suspended in a small volume of TE buffer and stored at -20°C until use.

2.14 Agarose Gel Electrophoresis

Agarose gels (0.7%-1.5% (w/v)) were prepared by dissolving agarose in 1X TBE (0.09M Tris-borate, 0.002M EDTA) and 0.5 µg/ml ethidium bromide. To each DNA sample 1/6th volume of 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30%glycerol) was added before loading. Samples were separated by agarose gel electrophoresis at 100V for 1 hour. The DNA was visualized by short wavelength ultra-violet light and photographed. DNA fragments that were recovered from the agarose gel for cloning were visualized by long wave ultra-violet light, excised from the

gel with a sterile razor blade and the fragments recovered by using Gene.clean glass milk procedure.

2.15 Neutralization of Phenol

The phenol was equilibrated before use to remove oxidation products that may result in the crosslinking of DNA (Sambrook et al. (1989)). The method for phenol neutralization was followed from the methods described by Sambrook et al. (1989). Liquefied phenol, was removed from the -20°C freezer and allowed to thaw at room temperature and 8-hydroxyquinoline was added to a final concentration of 0.1%. The phenol was equilibrated with an equal volume of 1M Tris, pH 8.0 in a sepratory funnel. The Tris phase was removed, and the phenol was extracted several times with 0.1 M Tris, pH 8.0. The extractions were complete when the pH of the Tris phase was greater than pH 7.8. The neutralized phenol was stored in a dark container at 4°C with 0.1 volume of 0.1 M Tris, pH 8.0 containing 0.2% β-mercaptoethanol final concentration.

2.16 Extraction of DNA

Proteins were removed from DNA preparations with phenol/chloroform extractions as described (Sambrook et al. (1989)). The DNA preparations were extracted with an equal volume of phenol:chloroform. The phenol chloroform was prepared in the following ratios (phenol:chloroform:isoamyl alcohol 25:24:1). The contents were mixed by vortexing for 30 seconds, then centrifuged at 12,000g for 15 seconds at room temperature in a microcentrifuge. The aqueous upper phase was saved and the extraction

was repeated until the cloudy protein phase disappeared at the aqueous-organic interface. The aqueous phase was then removed and the DNA recovered by precipitation with absolute ethanol.

2.17 Ethanol Precipitation

Plasmid DNA was concentrated by precipitation with absolute ethanol. The method of ethanol precipitation was followed as described (Sambrook et al. (1989)). DNA solutions that were suspended in distilled water or TE were precipitated with the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes 95 % ice cold ethanol. The solution was vortexed briefly then stored at -20°C or -70°C for 15 minutes to allow the DNA to precipitate. The solution was centrifuged at 14,000g in a microcentrifuge at 4°C for 15 minutes. The supernatant was removed and the DNA pellet was washed with cold 70% ethanol. The DNA pellet was inverted, allowed to dry and suspended in a small volume of TE buffer, pH 8.0.

2.18 Isolation of DNA from Agarose Gels.

Plasmid DNA digested with a restriction endonuclease and separated by agarose gel electrophoresis was purified from the agarose gel using the Gene Clean II kit. The bands are visualized with Model UVL-56 Black Ray long wavelength UV lamp. The appropriate size DNA band was isolated from the gel with a fresh sterile razor blade, and the gel slice placed in a 1.5 ml microcentrifuge tube. The mass of the gel slice in mg was converted to µl (1 gm equals approximately 1 ml) and 4.5 volumes of NaI and 0.5

volumes of TBE modifier were added to the microcentrifuge tube. The solution was mixed and the agarose dissolved at 45°C - 55°C. The microcentrifuge tube was placed on ice and the DNA was incubated with 5 µl of glassmilk® (1 µl per µg DNA) with occasional mixing by using a vortex mixer. After 5 minutes the glass milk was washed three times with New Wash Buffer, with a brief 5 sec centrifuge between washes. The DNA was eluted with 10 µl of ddH_2O at 55°C for 10 minutes. The glassmilk was centrifuged for 5 minutes and the supernatant removed. A small aliquot of supernatant was analyzed by agarose gel electrophoresis to quantify the recovery of DNA from the agarose gel.

2.19 Ligation

The protocol for ligation of plasmid DNA was followed as described (Sambrook et al. (1989)). A ligation was setup with a 3:1 insert to vector ratio diluted up to 10 µl in 1x Ligase buffer [50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000]. The ligation reaction was incubated with 1 (Weiss) unit of T4 ligase overnight at 16 °C. The ligation reaction was stored at -20°C until transformation.

2.20 Preparation of Competent E.coli Cells by Rubidium Chloride Method

Competent cells were prepared as described by the protocol of Hanahan (1985). Fresh LB streak plates of E.coli DH5α were made from frozen stocks. A single colony was inoculated into 2.5 ml of LB and grown overnight at 37°C. The bacteria were subcultured 1:100 into 250 ml of LB containing 20 mM $MgSO_4$. The culture was grown

at 37°C for approximately 2-3 hours until the $O.D._{590}$ reached 0.4-0.6. The bacterial culture was centrifuged at 5000 RPM for 5 minutes at 4°C in a Sorval GS3 rotor. The supernatant was removed and the bacterial pellet was gently suspended 0.4 original volume ice cold TFB1 [30 mM potassium acetate, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$ and 15% glycerol, adjust solution to pH 5.8 with acetic acid]. The bacteria were combined in one 250 ml centrifuge tube and incubated on ice for 5minutes. The bacteria were centrifuged at 5000 rpm for 5 minutes at 4°C using a Sorval GS3 rotor. The supernatant was removed and the bacterial pellet was gently suspended on ice with 1/25 original volume cold TFB2 [10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15 % glycerol, adjust pH to 6.5 with KOH]. The bacteria were incubated on ice for 15-60 minutes and aliquots of 100 µl of competent cells were placed in microcentrifuge tubes, dropped in liquid nitrogen and stored at -70°C. The transformation efficiency of the E.coli DH5α competent cells was approximately 10^6-10^7 cfu/µg of closed circular plasmid DNA.

2.21 Transformation of Bacteria

The transformation of E.coli DH5α was followed from the procedures described by Sambrook et al., 1989. The frozen competent cell stocks were placed on ice for approximately 20 minutes to thaw. Approximately 1/10 to 1/2 of the ligation reaction mixture was added to the competent cells with gentle mixing, followed by a 30 minute incubation on ice. The bacteria were heat shocked at 42°C for 90 seconds, incubated on ice for 2 minutes and diluted 15 fold with LB. The bacteria were placed at 37°C for 20 minutes with shaking to allow phenotypic expression of the antibiotic resistance gene.

The bacteria were plated on LB agar plates containing 50 μg/ml - 100 μg/ml ampicillin. The plates were inverted and incubated for 16 hours at 37°C. Plates were analyzed for colony formation the next day, wrapped with parafilm and stored at 4°C.

2.22 Small Scale Preparation of Plasmid DNA

2.22.1 Lysis by Alkali

Plasmid DNA was grown up in E.coli DH5α and isolated from bacteria by the alkali lysis method. The protocol is described in Sambrook et al. (1989), and was modified from the original method described by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). A single colony of transformed E.coli was used to inoculate 4 ml of LB containing 50 μg/ml ampicillin in a sterile 15 ml capped falcon tube. The cultures were incubated at 37°C with shaking overnight. The overnight cultures were stored at 4°C until use, and were stable for up to 1 week at 4°C. An aliquot of 1.5 ml of the overnight culture was centrifuged at 14,000g at 4°C for 5 minutes to pellet the bacteria. The supernatant was aspirated and the bacterial pellet was suspended in 100 μl of ice cold solution #1 (50 mM glucose, 25 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0). Next, 200 μl of freshly prepared solution #2 (0.2 N NaOH (freshly diluted from a 10N stock), 1% SDS) was added to the suspended bacteria, inverted several times to mix the contents and placed on ice for 5 minutes. To the lysate 150 μl of ice cold solution 3 was added (60% 5 M potassium acetate, 11.5% glacial acetic acid), the contents were vortexed briefly and stored on ice for 5 minutes. The solution was clarified by centrifugation at 14,000g for 5 minutes at 4°C. The supernatant was transferred to a fresh

tube and extracted once with an equal volume of phenol:chloroform. The extracted aqueous phase was then precipitated with 2.5 volumes of 95% ethanol at room temperature for 2 minutes. The solution was clarified by centrifugation, the supernatant was decanted and the pellet was washed with 70% ethanol. The pellet was dried at 37^0C for 5 minutes and suspended in 50 µl of sterile ddH_2O containing DNase free pancreatic RNase (20 µg/ml). A small aliquot of miniprep DNA (1-2 µl) was used for restriction endonuclease digested and analyzed by agarose gel electrophoresis to verify the successful recovery of recombinant plasmid DNA.

2.23 Large Scale Preparation of Plasmid DNA

2.23.1 Lysis By Alkali

This protocol was used for the preparation of milligram quantities of plasmid DNA. The following protocol was taken form Sambrook et al. (1989), and is a modification of the procedures of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). Recombinant plasmids were produced by inoculating 500 ml of LB containing the appropriate antibiotic (50 µg/ml Amp). The bacteria were grown at 37°C with shaking overnight. The bacterial inoculation can be either 1 ml of an overnight culture that is not more than 7 days old or a scraping form a 15% glycerol frozen bacterial stock. The following day, the overnight culture was divided evenly into two 500 ml sterile centrifuge tubes. The bacteria was pelleted by centrifugation at 4000 rpm for 15 minutes at 4°C in a Sorval GS3 rotor. The pellet was suspended by vortexing in 18 ml of solution #1(50 mM glucose, 25 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0). At room

temperature, 20 ml of freshly prepared solution #2 was added (0.2 N NaOH (freshly prepared from a 10N stock), 1% SDS) with gentle mixing by inverting the flask 5 - 10 times. The lysate was incubated at room temperature for 10 minutes. To the lysate, 15 ml of solution #3 was added(60% potassium acetate, 11.5 % glacial acetic acid), mixed by vigorous shaking, and incubated on ice for 10 minutes. The bacterial lysate was clarified by centrifugation at 4000 rpm at 4°C for 15 minutes in a Sorval GS3 rotor. The supernatant was filtered through four layers of cheese cloth into a sterile 250 ml centrifugation tube. The plasmid DNA was precipitated with 0.6 volumes of isopropanol at room temperature for 10 minutes. The DNA was centrifuged at 5000 rpm for 15 minutes at room temperature in a Sorval GS3 rotor. The pellet was washed with 70% ethanol and then evaporated to dryness. The DNA pellet was suspended in 5 ml of TE buffer (pH 8.0). The DNA was purified by equilibrium centrifugation in CsCl gradients containg ethidium bromide.

2.23.2 CsCl Gradients

The maxiprep DNA was purified by equilibrium centrifugation in CsCl gradients in the presence of ethidium bromide. The DNA pellet was suspended in 5ml of TE (pH 8.0), transferred to a 15 ml capped Falcon tube and diluted up to 8 ml with TE, pH 8.0. Exactly 8.8 g of CsCl (1.1 g/ml of DNA solution) was weighed out and fully dissolved in the DNA solution. For every 10 ml of DNA/CsCl solution 0.8 ml of ethidium bromide solution was added (10 mg/ml), and mixed by vortexing. The DNA-CsCl-ethidium bromide solution was loaded with a 5cc syringe with an 18 gauge needle into two

Beckman polyallomer Quick Seal centrifuge tubes (13 x 51 mm). Each Beckman tube has a maximum capacity of 5 ml. Once the Beckman tubes were loaded and balanced, the tops were sealed with a Beckman Quick Seal Tube Topper. The tubes were loaded in a Beckman Vti65 rotor and centrifuged at 45,000 rpm for 16 hours at 20 °C in a Beckman L8-70M Ultracentrifuge. The closed circular plasmid DNA was removed from the density gradient with a 5 ml syringe with an 18 gauge needle. The ethidium bromide was removed from the DNA by 5-6 extractions with equal volumes of 1-butanol saturated with water. The extractions were complete when all traces of ethidium bromide were removed from both the organic and aqueous phases. The DNA was diluted with 3 volumes of ddH_2O and precipitated with 2 volumes of absolute ethanol at 4°C overnight. The DNA was centrifuged at 10,000g at 4 °C for 15 minutes in a Sorval SS34 rotor. The supernatant was removed and the pellet dried at 37°C for 15 minutes. The DNA pellet was suspended in 1 ml of sterile TE (pH 8.0). The OD_{260} and OD_{280} of the final DNA solution was determined to calculate the concentration and purity of the recovered DNA.

2.24 Site Directed Mutagenesis

The following protocols for site-directed mutagenesis are described in the Muat-Gene® M13 In vitro Mutagenesis KIT form BIO-RAD.

2.24.1 Growth of Bacterial Strains

Growth of Bacterial strains was followed as described in BioRad protocols. E.coli strains MV1190 and CJ236 contain pili that encode for the F' plasmid and is expressed only above 35°C and therefore the bacteria were grown routinely at 37°C. Bacterial

stocks of E.coli MV1190 and CJ236 were grown on the appropriate agar plates at 4°C. To maintain the selection for the F' plasmid at 4°C E.coli MV1190 and CJ236 are grown on glucose minimal media and LB containing chloramphenicol, respectively. E.coli MV1190 has lost the chromosomal gene encoding for synthesis of proline. The F' plasmid contains the gene for proline synthesis and therefore it maintains selection pressure for proline synthesis on glucose minimal plates the F' plasmid will always be retained. The F' plasmid in E.coli CJ236 has the chloramphenicol resistance gene and therefore this bacteria is maintained on LB or H agar plates containing 30 μg/ml chloramphenicol

2.24.2 Titering Phage

Phage stocks containing uracil were titered on CJ236 and non-uracil containing phage stocks were titered on either CJ236 or MV1190 bacteria. A 20 ml MV1190 culture was grown in LB or a 20 ml CJ236 culture was grown in LB containing 30 μg/ml of chloramphenicol overnight with shaking at 37°C. Four 5 ml sterile Falcon tubes were prepared with 2.5 ml molten LB top agar that was cooled in a 50°C - 55°C waterbath until use. Four 100 fold serial dilutions (10^2-fold, 10^4-fold, 10^6-fold, 10^8-fold for MV1190) or (10^6-fold 10^8-fold, 10^{10}-fold, 10^{12}-fold, for CJ236) in LB were prepared of the appropriate phage stock in separate 5 ml sterile Falcon tubes. In four separate sterile 5 ml Falcon tubes 0.2 ml of MV1190 or CJ236 overnight bacterial culture were aliquoted with 100 μl of the first three phage dilutions with the last tube serving as a

control. The cultures and phage were incubated at room temperature for 5 minutes then mixed with one tube of LB top agar, vortexed, and poured on LB agar plate. The top agar was allowed to harden for 15 minutes at room temperature, the plates were inverted and incubated at 37°C overnight. The titer was calculated according to the following equation:

titer = (# of plaques x 10 x dilution factor) pfu/ml

2.24.3 Transformation

Transformation of competent E.coli MV1190, competent E.coli stocks were thawed on ice for 20 minutes. Approximately 1 µl - 5 µl of ligation mix or diluted synthesis reaction were added to 0.3 ml E.coli MV1190 competent cells with gentle mixing. The reaction was incubated for 30 minutes on ice, heat shocked at 42°C for 3 minutes and returned to ice. The transformants were placed in 10 µl, 50 µl and 100 µl aliquots and added to 0.3 ml of an E.coli MV1190 overnight culture and 2.5 ml molten agar previously cooled to 50°C. The top agar-transformants were mixed by vortexing and poured on H agar plates. The plates were inverted and incubated at 37°C overnight.

2.24.4 Growth of Uracil-Containing Phage

Frozen stocks of E.coli strain CJ236 were streaked out on LB plates containing 30 µg/ml chloramphenicol. Streak plates were grown at 37°C until individual colonies appeared. An isolated colony was placed in 20 ml of LB containing 30 µg/ml chloramphenicol and incubated overnight with shaking at 37°C. One milliliter of the

CJ236 overnight culture was used to inoculate 50 ml of 2xYT in a sterile 250 ml flask. The culture was incubated at 37°C with shaking until an OD_{600} of 0.3 was obtained, that corresponds to 1 x 10^8 cfu/ml. Uridine was added to the culture at a final concentration of 0.5 μg/ml and the culture was infected with a recombinant phage at an MOI of 0.2 or less. The culture was incubated with shaking at 37°C for 4-6 hours. Thirty milliliters were transferred to a 50 ml centrifuge tube and centrifuged at 12,000 rpm in a Sorval SS34 rotor for 15 minutes at 4°C. The supernatant was transferred to a fresh centrifuge tube and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was transferred to a fresh polyallomer 50 ml centrifuge tube. The RNA was removed with 150 μg of DNase-free RNase A digestion for 30 minutes at room temperature. The phage particles were precipitated on ice for 30 minutes with ¼ volume of a solution containing 3.5 M ammonium acetate and 20% PEG 8000. Phage particles were recovered by centrifugation at 12,000 rpm for 15 minutes at 4°C. The supernatant was discarded and excess liquid was wiped off with a Kim-wipe. The phage pellet was suspended in 200 μl of high salt buffer (300 mM NaCl, 100 mM Tris, pH 8.0, 1 mM EDTA), and held on ice for 30 minutes. Insoluble material was removed by centrifugation for 2 minutes at 14,000 rpm in an Eppendorf microcentrifuge. The supernatant was transferred to a fresh microcentrifuge tube and the unextracted phage was stored at 4°C for no longer than 1 week. The uracil containing phage stock was then titered on CJ236 and MV1190. The phage DNA if prepared correctly should contain sufficient uracil to be inactivated on

E.coli MV1190, the efficiency of the titer on E.coli MV1190 will be 10^4 fold lower than on E.coli CJ236.

2.24.5 Titering Uracil Containing Phage

Phage with uracil containg DNA will be inactivated in E.coli MV1190 strains, but not in E.coli CJ236 strains. To determine the uracil content, the phage stock was titered on CJ236 and MV1190 E.coli strains. A preparation of uracil containing phage will yield a titer of about 5 x 10^{11} pfu/ml on CJ236 and the efficiency on MV1190 will be at least 10^4 fold lower. A 20 ml MV1190 culture was grown in LB and a 20 ml CJ236 culture was grown in LB containing 30 µg/ml of chloramphenicol overnight with shaking at 37°C. Eight 5 ml sterile Falcon tubes were prepared with 2.5 ml molten LB top agar that was cooled in a 50°C - 55°C waterbath until use. Six 100 fold serial dilutions (10^2-fold, 10^4-fold, 10^6-fold, 10^8-fold, 10^{10}-fold, 10^{12}-fold) in LB were prepared of uracil containing phage stock in separate 5 ml sterile Falcon tubes. In four separate sterile 5 ml Falcon tubes 0.2 ml of MV1190 overnight culture was aliquoted with 100 µl each of the first three phage dilutions into one of these with the last tube serving as a control. In four separate 5 ml Falcon tubes 0.2 ml of CJ236 overnight culture was aliquoted with 100 µl each of the three last phage dilutions into on of these with the last tube serving as a control. The cultures and phage were incubated at room temperature for 5 minutes. The cultures were then mixed with one tube of LB top agar, vortexed, and poured on an LB agar plate. The top agar was allowed to harden for 15 minutes at room temperature. The

plates were inverted and incubated at 37°C overnight. The titer was calculated according to the following equation:

titer = (# of plaques x 10 x dilution factor) pfu/ml

2.24.6 Extraction of Single-Stranded DNA

The uracil DNA stock must be purified before its use as a template for the mutagenesis reaction. The entire 200 µl phage stock was extracted with 200 µl of neutralized phenol, 1x with phenol/chloroform (1:1:1/48 phenol:chloroform: isoamyl alcohol) and 4 to 5 times with chloroform/isoamyl alcohol. Extraction of the phage DNA was complete when the organic-aqueous interface was clear of any visible proteins (cloudy interface). Each extraction was vortexed for at least 30 seconds then centrifuged for 2 minutes. The organic phases were back extracted with 200 µl of TE (10 mM Tris pH 8.0, 1 mM EDTA) to increase the final recovery of phage DNA. The aqueous fractions were pooled and precipitated at -70°C for 30 minutes with 0.1 volume 7.8 M ammonium acetate and 2.5 volume absolute ethanol. The DNA was centrifuged for 15 minutes at 4°C then the supernatant was removed and the pellet was washed with 90% ethanol. Residual ethanol was allowed to evaporate from the side of the microcentrifuge tube and the pellet was suspended in 20 µl TE. The suspended DNA was transferred to a fresh microcentrifuge tube. Approximately 1/20 th of the DNA was analyzed on a 1% agarose gel with a known amount of single stranded DNA to quantify the concentration of uracil template.

2.24.7 Synthesis of the Mutagenic Strand

2.24.7.1 Phosphorylation of the Oligonucleotides

Lyophilized oligonucleotides were suspended in distilled water at 20 pmol/µl. In a 500 µl microcentrifuge tube the following components were mixed: 200 pmol oligonucleotides, 100 mM Tris, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 0.4 mM ATP (neutralized) and distilled water to a final total volume of 30 µl. The components were briefly mixed and 4.5 units of T4 polynucleotide kinase (BioRad) was added. The reaction was mixed and incubated at 37°C for 45 minutes. The reaction was stopped by heating at 65°C for 10 minutes. The oligonucleotide was diluted to a final concentration of 6 pmol/µl with TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and stored frozen until use.

2.24.7.2 Annealing the primer to the template

The following reaction was prepared in a 500 µl microcentrifuge tube: 0.1 pmol (200 ng) uracil-containing DNA, 2-3 pmol phosphorylated mutagenic oligonucleotide, 1 µl 10X annealing buffer (20 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 50 mM NaCl) and distilled water to a final total volume of 10 µl . A second control reaction was prepared with all the above components except the primer to test for non-endogenous priming. The reactions were placed in a 70°C water bath and were allowed to cool at an approximate rate of 1° C/min to 30°C over a 40 minute period. The reactions were then placed on ice and used for synthesis of the complementary DNA strand.

2.24.7.3 Synthesis of the Complementary DNA Strand

Once the primer has been annealed to the template, the following components were added to the primer annealed template and the reactions were held on ice: 1µl of 10x synthesis buffer (final concentration, 0.4 mM each dNTP, 0.75 mM ATP, 17.5 mM Tris-HCl, pH 7.4, 3.75 mM $MgCl_2$, 21.5 mM DTT), and 2 - 5 units T4 DNA ligase (BioRad) and 0.5 units T7 DNA polymerase (prediluted 1:2, T7 DNA polymerase: 20 mM potassium phosphate, pH 7.5, 1 mM DTT, 0.1 mM EDTA and 50 % glycerol). The reaction was incubated on ice for 5 minutes to initiate DNA synthesis and stabilize the primer. The reaction was then incubated at 25°C for 5 minutes and finally 37°C for 30 minutes. The reaction was terminated by the addition of 90 µl of stop buffer (10 mM Tris, pH 8.0, 10 mM EDTA) and freezing at -20°C (stable for 1 month).

2.25 Transformation of E.coli MV1190

The following protocol is used to transform competent MV1190 bacteria with synthesized of the mutagenic strand of DNA. For every synthesis reaction, 0.3 ml competent E.coli MV1190 cells were thawed on ice. Approximately 10 µl of synthesis reaction after dilution with stop buffer was added to the E.coli MV1190 competent cells, mixed gently, and incubated on ice for 60 minutes. The bacteria were heat shocked at 42°C for 3 minutes and then returned to ice. Aliquots of 0.3 ml E.coli MV1190 overnight culture were placed in to three 13 x 100 mm test tubes. Ten, 50 and 100 µl of the transformed cells were added separately to the three test tubes. The tubes were mixed

gently, and 2.5 ml of molten H top agar cooled to 50°C was added, and poured on the H agar plate. After allowing the top agar to solidify the plates were inverted and incubated at 37°C overnight. Immediately the next morning plaques were picked with sterile Pasteur pipettes and placed into 1 ml of sterile TE. The phage, approximately 10^7 per plaque were stable at 4 ° C for months.

2.26 Sequencing Plasmid DNA

The protocols for sequencing single and double stranded plasmid DNA are described in the Sequenase® Version II Kit from United States Biochemical.

2.26.1 Double Stranded Miniprep Plasmid DNA Preparation for Sequencing

Miniprep plasmid DNA was purified for sequencing with RNase A for 1 hour at 37°C, followed by 2 phenol:chloroform extractions and 1 chloroform extraction. The plasmid DNA was ethanol precipitated as described previously. Approximately 2 -5 µg CsCl purified DNA or miniprep DNA was dissolved in 100 µl ddH_2O and denatured by the alkaline denaturation method at 37°C for 30 minutes with 0.1 volume 2M NaOH, 2 mM EDTA. The mixture was neutralized with 0.1 volumes of 3M sodium acetate pH 4.5-55 and precipitated with 4 volumes of absolute ethanol at -70°C for 15 minutes. The DNA was pelleted at 14,000 rpm for 10 minutes at 4°C. The pellet was washed with 70% ethanol at 4°C and centrifuged. The ethanol was decanted, the pellet was air dried and suspended in 7 µl of ddH_2O.

2.26.2 Single Stranded Plasmid DNA Preparation for Sequencing

Single stranded DNA for sequencing was isolated from M13 phage. Large scale preparation of mutant phage were prepared to isolate enough mutant single stranded DNA to sequence the desired mutation. One milliliter of a MV1190 overnight culture was added to 30 ml of LB media and 100 µl of mutant phage. The cultures were grown with shaking for 5 hours at 37°C. Cultures were centrifuged at 12,000 rpm in a Sorval SS34 rotor for 15 minutes at 4°C. The supernatant that contains the phage particles was transferred to a fresh tube and the single stranded DNA isolated as descibed previously. The single stranded DNA was used for sequencing to verify the presence of the desired mutation. The pellet contains the double stranded replicative form and was saved at -20°C, once the appropriate mutation was identified by its sequence

2.26.3 Annealing primer and the Sequencing Reaction

The purified single stranded DNA or double stranded plasmid DNA was suspended in 7 µl of ddH_2O, 2µl 5x Sequenase buffer[final concentration, 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 25 mM NaCl] and 1 µl sequencing primer. For sequencing typically 1 µg of M13 template and 2-5 µg of plasmid DNA were used, and the primer to template molar ratio was approximately 1:1. The annealing reaction for both single stranded templates and double stranded plasmid DNA were heated to 65°C for 2 minutes, then allowed to cool to 30°C over a 30 minute period. The reactions were immediately placed on ice. To the annealed template 1 µl of 0.1 M DTT, 2 µl of 5x dilute labeling

mix [1.5 μM of each, dGTP, dCTP and dTTP], 0.5 μl [^{35}S]dATP (5 μCi), 2 μl of 8 times diluted Sequenase T7 DNA polymerase). In order to read sequences close to the primer within the first 150-200 base pairs, 1 μl of Mn buffer [0.15 M sodium isocitrate, 0.1 M $MnCl_2$] was added to the labeling mix prior to the polymerase addition. The reaction was mixed gently, and incubated at room-temperature for 5 minutes. To terminate the reactions, 3.5 μl aliquots of reaction mix was placed into four capped tubes that contained 2.5 μl each of termination mix [tubes labeled G,C,A and T, with 80 μM of each dNTP, 8 μM of one of the four ddNTPs and 50 mM NaCl]. The termination reactions were incubated at 37°C for 5 minutes. The reactions were terminated by the addition of 4 μl stop solution [95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF. The sequencing reactions were stored at -20°C until use and were stable for up to 1 week.

2.26.4 Separation of the Products of the Sequencing Reaction by Gel Electrophoresis

Sequencing reactions were analyzed on 6% acrylamide gels made by the following recipe: 5.7 % (w/v) acrylamide, 0.3 % (w/v) bis-acrylamide, 7 M urea, dissolved in TBE buffer with stirring and the final volume adjusted to 100 ml with TBE. When ready to pour the gel, 1ml of 10% ammonium sulphate prepared fresh and 25 μl TEMED were added initiate polymerization of the gel. Approximately, 2-2.5 μl of the sequencing reaction was loaded on the gel. Samples were separated from 2- 5 hours at 60 watts, 1500 volts and 30 mA with the Model S2 electrophoresis apparatus from BRL.

2.27 Virus, Cell Culture and Transfections

COS-1 and Vero cells were grown in high glucose Dulbecco's media supplemented with 7% calf serum, 2% sodium bicarbonate, 2% glutamine, 1% penicillin / streptomycin and 1% Hepes. BHK (Baby Hamster Kidney) cells were grown in DMEM supplemented with 7% fetal bovine serum, 2% sodium bicarbonate, 2% glutamine, 1% penicillin/streptomycin and 1% Hepes. VTF7-3 is a recombinant vaccinia virus that expresses T7 RNA Polymerase.

2.28 Mammalian Cell Culture Transfections

2.28.1 Transfections by Calcium Phosphate

COS-1 cell transfections were performed using the calcium phosphate technique as previously described (Graham and van der Eb,1973). COS-1 cells were transfected at approximately 80% confluence with 5 μg wild-type pXM-G or 20 μg of plasmids encoding the genes for chimeric G proteins. Constructs pXM-GgDgD and G-GPI were transfected using 30 μg of plasmid DNA. The genes encoding for wild-type and chimeric G proteins were cloned into the unique EcoR1 site of the high efficiency eukaryotic expression vector pXM (Yang et al.,1986).

2.28.2 Transfection by Cationic Liposomes

BHK cells were transfected with cationic liposomes, prepared as described by Rose et al., (1991). Phosphatidylethanolamine (5mg in 500 μl chloroform) Sigma, cat# P0510)(100 μl) dissolved in chloroform at a stock concentration of 10 mg/ml was mixed

with 4 μl of dimethyldioctadecylammonium bromide (Sigma, cat#D2779)(stock 0.1 mg/ml). A thin film of lipid was made on the sides of a glass test tube by evaporating the lipid mixture with Argon gas. Residual chloroform was removed from the test tube in a liquid nitrogen cooled evaporator from Buchler instruments for 2 hours. The lipids were suspended in 1 ml distilled water and sonicated with a Fisher Probe microprobe sonicator at maximum power, on ice for 10 minutes. The cationic liposomes were stored at 4°C and remained stable for several months. The efficiency of each liposome batch was standardized by transfection of COS-1 cells with 5, 10, 15, 20, 25 and 30 μg of pBS*G or pBS*GGPI plasmid DNA and the cells were processed for indirect-immunofluorescence. The DNA concentrations that showed the greatest transfection efficiency were used for subsequent transfections. Liposome mediated transfections were performed as described (Rose et al., 1991). COS-1 cells were grown to 60% confluence. The COS-1 cells were washed with DMEM (minus serum) and infected with vTF7-3 (recombinant vaccinia virus expressing T7 polymerase) at an moi of 2-4 for 30 minutes at 37°C in 0.5 ml DMEM (minus serum). Thirty (30) μl of liposome were added per 5 μg of plasmid DNA to 1 ml DMEM (minus serum). The solution was briefly mixed with the plasmid DNA with a vortex mixer, followed by incubation at room temperature for 5-10 minutes. The vTF7-3 was removed from the COS-1 cells and replaced with the liposome mixture and incubated at 37°C for 3 hours. The liposome mixture was replaced with 10 ml DMEM containing 10% calf serum. The next day the cells were processed for indirect immunofluorescence or R18- fluorescence assay.

2.29 Metabolic Labeling of Cells

COS-1 cells expressing the genes of wild-type G or chimeric G proteins or pXM (vector alone) at 24 hour post transfection were washed twice with PBS and incubated at 37°C with 1 ml Met-free DMEM (-Met) medium for 1 hour. Proteins were radiolabeled with the addition of 50 uCi/ml of [^{35}S] Met and incubated at 37°C for 2 hours. The media was removed from the plates, washed twice with PBS and the cells were lysed with the addition of 1 ml ice cold Lysis Buffer [1% NP40, 0.4% NaDeoxycholate, 66 mM EDTA, 10 mM Tris pH 7.4, 1 mM PMSF and 100 U/ml Trasylol]. The lysis buffer was removed from the plates, centrifuged at 4°C for 5 minutes to remove nuclei and cell debris.

2.30 Immunoprecipitation

Cell lysates were incubated with 1ul crude rabbit anti-G antibody at 4°C for 2 hours in a Labquake shaker. Samples were then incubated for 1 hour at 4°C upon the addition of 50 uL of 10%(v/v) Protein A Sepharose beads. Beads were collected, washed 4 times with lysis buffer containing 0.3% SDS. The lysis buffer containing 0.3% SDS was removed by aspiration and the Protein A sepharose beads were boiled in 20 µl of 2X SDS loading buffer for 2 minutes at 100°C. The samples were analyzed on a 10% SDS-PAGE gel, and fluorographed gels were exposed to Kodak XPR-1 X-ray film overnight at -70°C.

2.31 Cell Surface Expression

2.31.1 Indirect Immunofluorescence

Surface expression of wild-type G and G hybrids was determined by two methods, indirect immunofluoresence and quantitated by cell surface iodination as described previously (Zhang and Ghosh,1994). Coverslips with transfected COS-1 cells were washed with PBS twice and fixed with 2% paraformaldehyde 24 hours post-transfection. Cells were washed with PBS 3 times followed by 1% BSA in PBS. Coverslips were incubated at room temperature for 1 hour with rabbit anti-G antibody (1:50 dilution of ammonium sulphate purified rabbit anti G antibody in PBS containing 1% BSA). The cells were washed with PBS and the reincubated for 1 hour at room temperature with affinity purified Fluorescein-conjugated goat antibody to rabbit IgG (1:100 dilution of antibody in PBS containing 1% BSA). The coverslips were washed three times with PBS, mounted on slides with a 15 µl 80% glycerol/ 20% PBS solution and examined for cell surface immunofluoresence by epifluorescent microscopy.

2.31.2 Lactoperoxidase catalyzed iodination of cell surface proteins

To quantify the G protein expression on the cell surface a lactoperoxidase catalyzed iodination was performed essentially as described earlier (Guan et al., 1985) (Li et. al., 1993). Lactoperoxidase (E.C. 1.11.17) was suspended in 3.2 M $(NH_4)_2SO_4$ pH 7.0, 20 mM CH_3COONa to a final concentration of 5 µg/µl and stored at 4°C until use. Glucose oxidase, was suspended in 0.1 M CH_3COONa pH 4.0 to a final concentration of 2 units /ml and stored at 4°C until use. Twenty four hours post-transfection, COS-1cells

were washed two times with PB-NaCl [10 mM Na_2HPO_4 pH7.4, 150 mM NaCl], suspended in 3 ml PB-NaCl containing 135 µL of a 2% glucose stock solution, 12.5 µL of (60µg) lactoperoxidase (Sigma) and 2.5 µL $Na^{125}I$ (250 µCi). The reaction was initiated by the addition of 10 µL (0.5 U) of glucose-oxidase (Boehinger Mannheim). The reaction was incubated for 40 minutes at room temperature, washed 2 times PB-NaI [10 mM Na_4HPO_4 pH 7.4, 150 mM NaI], washed 4 times with PB-NaCl. The cells were lysed and immunoprecipitated as described previously.

2.32 Syncytia Assay

Cell-cell fusion assay was performed essentially as described previously (Florkiewicz, 1984). The assay was conducted at 24 hour post-transfection when the cells are approximately 90% confluent. The DMEM media was removed and replaced with 1mL of fusion media [1.85 mM $NaH_2PO_4 \cdot H_2O$, 8.39 mM $NaHPO_4 \cdot 7H_2O$, 2.5 mM NaCl, 10 mM Hepes and 10 mM MES (2-(N-mopholino)ethanesulfonic acid)] at 37°C for 1minute. The media was removed and replaced with DMEM containing 10% serum and incubate at 37°C for 2.5 hours. The cells were briefly examined for polykaryon formation and then the media was removed from the cells and replaced with a second fusion media shock for 1 minute at 37°C. The fusion media was removed, replaced with DMEM containing 10% serum and incubated at 37°C for 2 hours. Polykaryon formation was visible after 1 hour of the first fusion shock, but a second fusion shock was used to ensure maximal fusion was obtained for each construct tested. After the 1.5 hour

incubation of the second fusion shock the media was removed and the cells were fixed with Carnoy's solution, and stained with 0.1% Crystal violet.

2.33 Oligomerization Assay

Oligomerization assay was performed as described by Doms et al. 1987. Twenty-four hour post-transfection, COS-1 cells were starved with 1mL Met-Free DMEM (-Met) media for 1 hour, labeled with 100 μCi/ml [^{35}S]-methionine for 30 minutes and chased with 1mL DMEM containing 2.5 mM filtered Met for 1.5 hours. Cells were washed twice with PBS, and lysed at 4°C for 1 hour with 300 μL of 4X MNT [80 mM MES (Morpholino-ethane-sulfonic acid), 120 mM Tris, 400 mM NaCl, 4 mM EDTA, and 4 mM EGTA containing1% Triton X-100 at pH to 5.5 or 7.4]. Cell lysates were centrifuged at 10,000 rpm for 5 minutes at 4°C. The lysates (250 μL) were transferred to a 5 - 20 % continuous sucrose gradient made in 2X MNT [40mM MES, 60 mM Tris, 200 mM NaCl, 2 mM EDTA, 2 mM EGTA containing 0.1 % Triton X-100 at pH 5.5 or 7.4]. One sucrose gradient contained only markers 100 μg BSA (4S) and 100 μg Aldolase (8S) to mark the position of the G monomer and G trimer respectively. The gradients were centrifuged in a SW41 swinging bucket rotor at 40,000 RPM at 4°C for 20 hours in a Beckman Ultracentrifuge. The gradients were fractionated from bottom to top, approximately 24 fractions in total. The position of the BSA and Aldolase marker were estimated using the BCA Protein Assay Kit. To each fraction 1 volume of Ripa buffer (-SDS) [1% NP40, 0.4% Na-deoxycholate, 12.5 mM SDS, 50 mM Tris (pH 8.0)] was

added. Alternate fractions from 5 to 21 were immunoprecipitated as previously described and analyzed by SDS-PAGE.

2.34 Endoglycosidase H Resistance

COS-1 cells were transfected with the appropriate plasmids, and the proteins were metabolic labeled as described previously except with 75 μCi/ml of [^{35}S]-Met for 15 minutes. The media were removed and washed twice with PBS and chased with DMEM containing 2.5 mM Met for either 0 or 60 minutes. Cells were lysed and immmunoprecipitated as described previously. Immunoprecipitates were suspended in 20 μL of 1% SDS and 50 mM Tris pH 6.8. The samples were boiled for 2 minutes, centrifuged briefly and split into two equal 10 μl fractions, and 1 mUof endo-H (Genzyme) was added to one fraction only. Both fractions were then incubated at 37°C for 16 hours. Samples were analyzed by SDS-PAGE.

2.35 R18 Lipid mixing Assay

2.35.1 Preparation of Plasma Membrane Vesicles

Plasma membrane vesicles were prepared from Vero cells. Vero cells represent a good target membrane for VSV G fusion (Puri et al., 1993). Plasma-membrane vesicles (PMV's) were prepared as described by Puri et al., 1992. Vero cells were grown to 95% confluence on ten 150 mm Falcon tissue culture plates. The cells were washed with PBS twice and trypsinized to remove the Vero cells from the plates. Cells were suspended in 50 ml DMEM with 7% calf serum to inhibit the trypsin . Cells were centrifuged at

500xg, washed 3 times with PBS and suspended in homogenization buffer [10 mM Na_2HPO_4, 1 mM $MgCl_2$, 30 mMNaCl, 1 mM DTT, 0.02 % NaN_3, 0.005 mM PMSF at a concentration of $1x10^8$ cells/ ml. Homogenization was performed at 4°C by means of a Thomas Teflon coated Homogenizer (approximately 6-8 strokes). The nuclei were removed by centrifugation at 500xg at 4°C for 5 minutes. The supernatant was layered on a 2 ml 41% sucrose cushion and centrifuged at 100,000 x g for 60 minutes at 4°C in a Beckman SW55Ti swinging bucket rotor. The plasma membrane fraction was dialyzed against 2L of PBS overnight to remove the sucrose. Recovery of plasma-membrane vesicles were analazyed by determining the protein concentration as assayed by Pierce BCA protein assay kit. The PMV's were frozen in liquid nitrogen and stored in aliquots at -70°C.

2.35.2 R18 Labeling of Plasma Membrane Vesicles

Labeling of PMV's with R18 was performed as described by Puri et. al.,1993. Two hundred micrograms of PMV's were labeled with10 µl from a R18 stock (10µg of R18 in 10 µL absolute ethanol) with mixing in a vortex mixer at room temperature under low intensity lighting. The R18 was added one drop at a time with vortexing using a 10 µl Hamilton syringe. The vesicles and R18 were incubated at room temperature for 30 minutes in the dark. Labeled PMV's were layered on 10%/41% sucrose step gradient and centrifuged at 4°C for 60 minutes in a Beckman SW55Ti swinging bucket rotor. The R18 labeled PMV fraction was collected and dialyzed against 2 liters of PBS at 4°C

overnight. A Sephadex column was not used in this protocol as the recovery of R18-labeled PMV's were to low for subsequent use in the R18 fluorescence assay. Aliquots of R18-PMV's were stored at -70°C.

2.35.3 R18 Lipid mixing assay

R18 labeled PMV's were bound to BHK cells expresing wild-type G or G-GPI proteins on the surface of transfected BHK cells or VSV infected VERO cells. R18 labeled-PMV's were bound to target cells at 4°C in D-PBS [PBS containing 2 mM Ca^{2+} and 2 mM Mg^{2+}] for 30 minutes in the dark. Monolayers were washed with D-PBS, and scraped with a sterile rubber policeman into 0.5 ml D-PBS, placed in a microcentrifuge tube and stored on ice in the dark. Fluorescence dequenching was measured using an Aminco-Bowman Series 2 Luminescence Spectrometer with 1s time resolution at 560 nm and 585 nm excitation and emission wavelengths, respectively. To monitor the fusion reaction (fluorescence) a 100μL aliquot of R18-PMV-cell suspensions in 2 ml HEPES-NaCl [10 mM HEPES, 145 mM NaCl, pH7.4] in a cuvette were pre-equilibrated at 37°C. The fluorescence was measured for 30 seconds to 1 minute to establish a background fluorescence level. Fusion of G protein expressing BHK cells and R18-labeled PMV's were induced by lowering the pH to 5.5 with 100 μL 0.5 M MES at 100 seconds and the fluorescence dequenching was monitored for several minutes. Full dequenching was determined by the addition of 250 μL 1% Triton X-100. Percent dequenching was determined by the following equation: $100(F-F_0)/(F_t-F_0)$, where F, F_0 and F_t are fluorescence values at any given time, time zero and after total dequenching with 1%

Triton X-100 respectively. At the end of each assay the pH in the cuvette was checked. The R18 assay was standardized with VSV infected Vero cells expressing the G protein on the cell surface. Vero cells grown to confluence on a 60 mm Falcon plate were infected at an moi of 6 for 4 hours in 0.5 ml DMEM serum free for 30 minutes at 37°C.

2.36 Trypsin Sensitivity

In order to detect the low-pH induced conformational change of either wild-type or chimeric G proteins, the increased resistance of G protein to tryptic digestion was monitored as a function of decreasing pH. COS-1 cells expressing wild-type G or chimeric G proteins were analyzed for differences in the low-pH induced conformational change. The protocol for trypsin digestion was followed from the methods of Fredericksen and Whitt (1996). Four 60mm plates of COS-1 cells were transfected with the appropriate plasmid construct. Twenty four hours post-transfection, COS-1 cells were washed two times with PBS and starved in methionine free DMEM media for 60 minutes at 37°C. Cells were labeled with 50 μCi [^{35}S]-methionine per milliliter for 30 minutes and chased for 60 minutes with DMEM supplemented with 2.5 mM methionine at 37°C. Cells were washed two times with PBS and each plate was lysed with 1 ml of 2xMNT containing 1 % Triton X-100 (40 mM MES, 60 mM Tris, 200 mM NaCl, 2.5 mM EDTA) at pH's 7.4, 6.5, 6.1 or 5.6. The lysates were clarified at 14,000g for 10 minutes at 4°C. Aliquots of 200 μl of clarified supernatants were incubated with or without 10 μg TPCK-Trypsin at 37°C for 30 minutes. TPCK-Trypsin was suspended in 100 mM KCl pH 7.1 at a final concentration of 1 mg/ml and stored in 100 μl aliquots at -20°C until use.

To show that the trypsin is active at pH 5.6, one extra sample is included with 200 µl pH 5.6 supernatant adjusted to 0.3% SDS final concentration and 10 µg TPCK-Trypsin. The reactions were stopped by the addition of Aprotinin (0.1 trypsin inhibitor unit). The samples were centrifuged at 14,000g for 10 minutes at 4°C to remove any precipitate. An aliquot of 170 µl of the clarified supernatant was transferred to a fresh microcentrifuge tube. The pH of each sample was neutralized before immunoprecipitations by the addition of 4 volumes of detergent lysis buffer (10 mM Tris, pH 7.4, 66 mM EDTA 1% Triton X-100, and 0.02% sodium azide). G proteins were immunoprecipitated with the addition of polyclonal G antibody and analyzed by SDS-PAGE electrophoresis through 10% polyacrylamide gels and visualized by fluorography.

3. Results

VSV glycoprotein G is a type 1 integral protein embedded in the viral membrane. Previously, specific regions in the ectodomain of VSV G were identified by linker-insertion and site directed mutagenesis that abolish or shift the pH threshold required for fusion (Li et al., 1993) (Zhang and Ghosh, 1994) (Fredericksen and Whitt, 1995). The H2 region of VSV G (amino acid 117 to 137) has been characterized by mutagenesis studies and suggested by our lab and others as the putative fusion peptide of VSV G (Li et al., 1993) (Zhang and Ghosh, 1994) (Fredericksen and Whitt, 1995). It has also been shown that a peptide segment from amino acid residues 59--221 can interact with a target bilayer at low-pH (Durrer et al., 1995). This labeled peptide segment also contains the proposed H2 fusion peptide of VSV G Indiana.

The fusion peptide has been identified as a critical element of the viral fusion protein (White, 1990) (White,1992)(Hernandez et al., 1996). It has previously been shown to be required for fusion with other virus systems as well as VSV G (Gething et al., 1986) (Bosch et al, 1989) (Freed et al., 1990) (Freed et al., 1992) (Levy-Mintz and Keilian, 1992) (Li et al., 1993) (Zhang and Ghosh, 1994) (Sergel-Germans et al., 1994) (Fredericksen and Whitt, 1995). We were interested in identifying other domains within glycoprotein G that may also be important to the fusion mechanism. To study the role of transmembrane domain and cytoplasmic tail, VSV G chimeras were constructed that have the transmembrane alone or in conjunction with the cytoplasmic tail exchanged with the equivalent domain of other integral proteins.

3.1 Description of Chimeric G Proteins

Four kinds of chimeras were constructed to study the role of the transmembrane anchor and cytoplasmic tail on the fusion process. A schematic representation of the following 4 classes of chimeras are shown Fig.3. The first class of hybrid was constructed to investigate the requirements for a specific amino acid sequence of the VSV G transmembrane anchor on the fusion process. This class of chimera had the transmembrane anchor of VSV G exchanged with the equivalent domain from other viral glycoproteins HSV-1 gB and gD (Ward and Roizman, 1994), adenovirus type 5 E3-11.6 K protein (Scaria et al., 1992) and the T-cell integral membrane protein CD4 (Weiss, 1992). Similar to VSV, HSV-1 is an enveloped animal virus. However, HSV-1 contains twelve glycoproteins embedded in the viral membrane including gB, gD, gC, gH and gK (Spear, 1993) (Ward and Roizman, 1994) (Hernandez et al., 1996). Mutations in glycoproteins gD and gB along with gH and gK abolish the fusion activity, suggesting that the four glycoproteins are required for fusion (Spear, 1993) (Ward and Roizman, 1994) (Hernandez et al., 1996). HSV-1 gB is a dimer (Highlander et al., 1991) and gD is a monomer (Spear, 1993) while VSV G is a trimer (Kreis and Lodish, 1986)(Doms et al., 1988). HSV-1 gB and gD localize to the nuclear envelope and plasma membrane while VSV G localizes exclusively at the plasma membrane (Spear, 1993) (Ward and Roizman, 1994) (Lenard, 1993). Adenovirus type 5 is not an enveloped animal virus and therefore does not encode for any fusion proteins.

Fig. 3. Schematic diagram of wild-type and chimeric G proteins. Open boxes represent VSV G amino acid sequences, closed boxes represent amino acid sequences from foreign proteins HSV-1 gB, gD, adenovirus type 5 E3-11.6 K (Ad), human CD4 (CD4) or decay accelerating factor (DAF). Double black line represents the addition of a glycosylphosphatidylinositol (GPI) lipid anchor. The grey square represents the 9 conserved amino acids of the VSV G extracellular juxtamembrane amino acids. The broken line represents a deletion of the 9 conserved extracellular juxtamembrane amino acids of VSV G. Amino acid sequences and construction of chimeric G constructs are described in the methods and materials and in Table I and Table II. EC, ectodomain; TM, transmembrane anchor; CT, cytoplasmic tail.

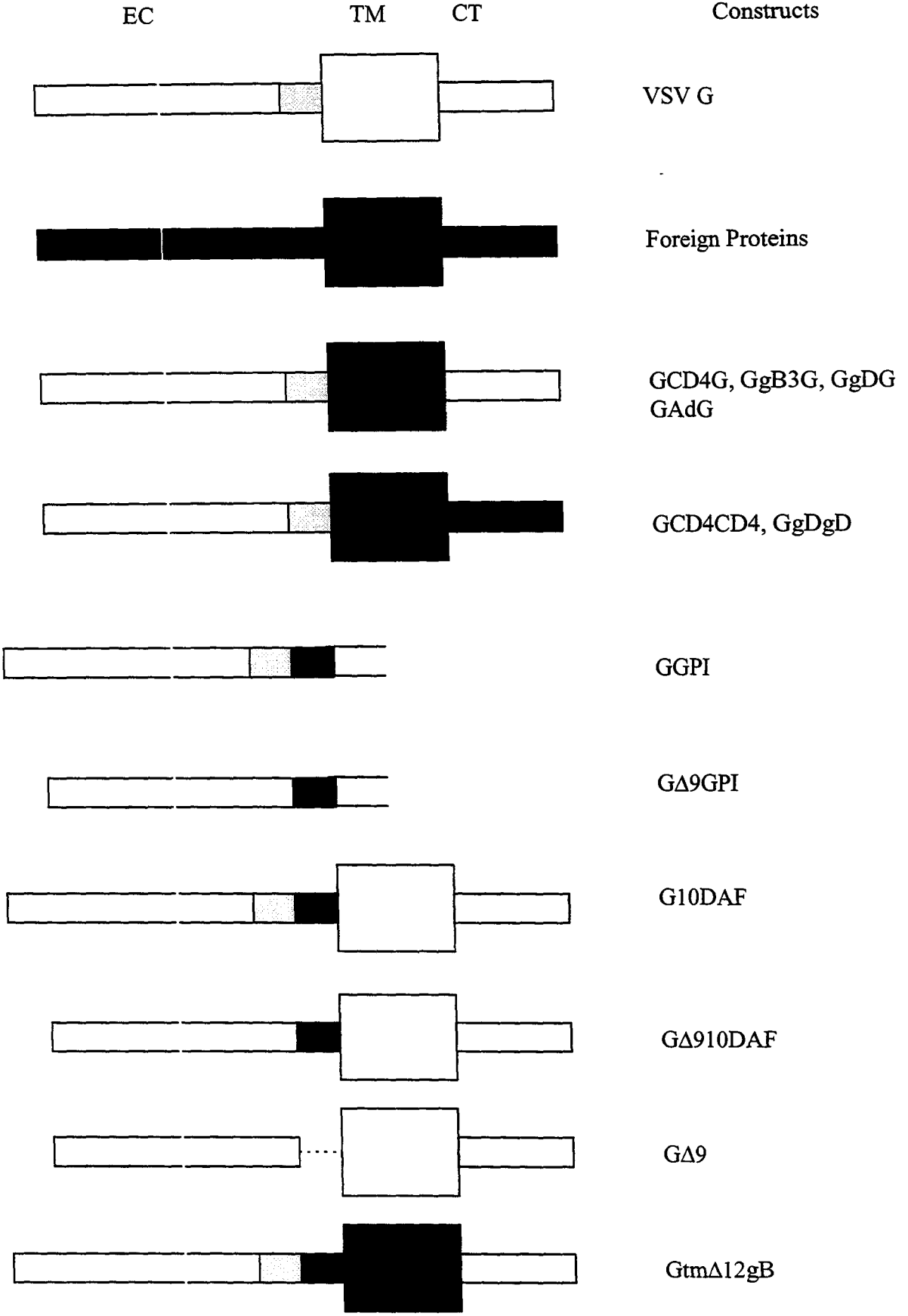

EC
TM
CT
Constructs
VSV G
Foreign Proteins
GCD4G, GgB3G, GgDG
GAdG
GCD4CD4, GgDgD
GGPI
GΔ9GPI
G10DAF
GΔ910DAF
GΔ9
GtmΔ12gB

Table I

Amino acid sequences of the membrane spanning and the cytoplasmic domains of VSV G protein and the chimeras constructed.

	Ectodomain	Transmembrane Anchor	Cytoplasmic Tail
G-wt	FSSWK	SSIASFFFIIGLIIGLFLVL	RVGIHLCIKLKHTKKRQIYTDIEMNRLGK
G(AXB)	FSSWK	SSSSIASFFFIIGLIIGLFLVA	RVGIHLCIKLKHTKKRQIYTDIEMNRLGK
GgB3G	FSSWK	FGALAVGLLVLAGLAAAFFAF	RVGIHLCIKLKHTKKRQIYTDIEMNRLGK
GCD4G	FSSWK	SSSALIVLGGVAGLLLFIGLGIFFA	RVGIHLCIKLKHTKKRQIYTDIEMNRLGK
GAdG	FSSWK	SSLGMWWFSIALMFVCLIIMWLICCL	RVGIHLCIKLKHTKKRQIYTDIEMNRLGK
GgDG	FSSWK	NMGLIAGAVGGSLLAALVICGIVYWM	RRVGIHLCIKLKHTKKRQIYTDIEMNRLGK
GgDgD	FSSWK	NMGLIAGAVGGSLLAALVICGIVYWM	RRRTRKAPKRIRLPHIREDDQPSSHQPLFY
GCD4CD4	FSSWK	SSSALIVLGGVAGLLLFIGLGIFFCV	RCRHRRRQAERMSQIKRLLSEKKTCQCPHRFQKTCSPI

The construct G(AXB) contained an ApaI restriction enzyme site at nucleotide 299 (Zhang and Ghosh, 1994)); a XhoI site at nucleotide 1416 (Li et al., 1993) and a BssHII site at nucleotide 1473, created by site-directed mutagenesis using a primer 5'ATGGATACCAACGCGCGCAACCAA-GAATAG-3'. The construct G(AXB) contained two additional serine inserted between amino acids 463 and 464 and the leucine at residue 482 was replaced by alanine. The G protein coded by G(AXB) gene showed wild-type properties with respect to transport to cell surface, oligomerization at low pH and low-pH induced polykaryon formation and was, therefore, used as a control. The construct GgB3G contained amino acids 775-795 of HSV-1 gB between residues 462 and 482 of VSV G protein. Construct GCD4G was made by inserting amino acids 375-395 of CD4 between residues 465 and 484 of G(AXB) while GCD4CD4 was made by joining residues 375-435 of CD4 after amino acid 465 of G(AXB). GAdG were made by insertion of residues 32-55 of adenovirus type 5 E3-11.6 K protein between amino acids 464 and 483 of VSV Gm6 (Xho1 site only). GgDG was made by inserting residue 339-365 of HSV-1 gD between amino acids 462 and 483 of VSV G while GgDgD was made by joining residues 339-394 of HSV-1 gD to amino acid 465 of VSV Gm6 (Xho1 site only)

Table II

Amino acid sequences of the a portion of the ectodomain and the transmembrane anchor of VSVG and the chimeras constucted

	Ectodomain	Transmembrane Anchor
G-wt	KNPIELVEGWFSSWK	SSIASFFFIIGLIIGLFLVL
G(AXB)	KNPIELVEGWFSSWK	SSSSIASFFFIIGLIIGLFLVA
GΔ9	KNPIE----------K	SSSSIASFFFIIGLIIGLFLVA
GGPI	KNPIELVEGWFSSWK	SSSPNKGSGTTS*
GΔ9GPI	KNPIE----------K	SSSPNKGSGTTS*
G10DAF	KNPIELVEGWFSSWKSSSPNKGSG	SSSIASFFFIIGLIIGLFLVA
GΔ910DAF	KNPIE----------KSSSPNKGSG	SSSIASFFFIIGLIIGLFLVA
GtmΔ12gB	KNPIELVEGWFSSWKSSSTVIHADNP	FGALAVGLLVLAGLAAAFFAF

The construct G(AXB) contained an ApaI restriction enzyme site at nucleotide 299 (Zhang and Ghosh, 1994); a XhoI site at nucleotide 1416 (Li et al., 1993) and a BssHII site at nucleotide 1473, created by site-directed mutagenesis using a primer 5'ATGGATACCAACGCGCGCAACCAA-GAATAG-3'. The construct G(AXB) contained two additional serine inserted between amino acids 463 and 464 and the leucine at residue 482 was replaced by alanine. The G protein coded by G(AXB) gene showed wild-type properties with respect to transport to cell surface, oligomerization at low pH and low-pH induced polykaryon formation and was, therefore, used as a control. Construct GΔ9 was made by deleting amino acids 453-461 of the VSV G (AXB) ectodomain. Construct GGPI and GΔ9GPI was made by joining amino acids 311-347 of decay accelerating factor (DAF) after amino acid 464 of VSV G (AXB) and GΔ9 respectively. Construct G10DAF and GΔ910DAF were made by inserting 9 amino acids of DAF (311-319) between amino acids 464 and 465 of VSV G (AXB) and GΔ9 respectively. Chimera GtmΔ12gB contained amino acids 1-464 and 483-511 of VSV G and 721-726 plus 773-795 of the HSV-1 gB ectodomain of HSV-1 gB third transmembrane anchor. This chimera contains an extra serine residue to maintian reading frame.

* predicted point of attachment of GPI lipid anchor depicts a deletion

Underlined sequences represent 9 amino acids from DAF or the 8 amino acids from the ectodomain of HSV-1 gB (721-726 and 773-774).

The Adenovirus genome is spatially organized into different regions, where the genes in each region encode for proteins with similar biological functions (Wold and Gooding, 1989). The genes of the adenovirus E3 region encodes for integral membrane proteins that are involved in countering the hosts immune system (Scaria et al., 1992). One gene from the E3 region, the 11.6 K gene, is a type 1 integral membrane protein of unknown function (Scaria et al., 1992). The 11.6 K gene is a small 101 amino acid protein with a single transmembrane anchor (Scaria et al., 1992). The 11.6 K gene localizes exclusively to the nuclear envelope (Scaria et al., 1992). CD4 is expressed on the surface of T-cells, and is the primary receptor for HIV attachment and fusion to human T-cells (Weiss, 1992). CD4 is involved in T-cell activation (Weiss,1992). The CD4 protein is a monomer (Weiss, 1992).

The second class of chimeras was constructed to investigate the effect of the transmembrane anchor in conjunction with the cytoplasmic tail on fusion. This class of chimera had the transmembrane anchor in conjunction with the cytoplasmic tail exchanged with the equivalent domains from HSV-1 gD and CD4.

The third class of chimera was constructed to investigate the requirements of a peptide anchoring domain versus a lipid anchoring domain on the fusion process. This class of chimera exchanged the transmembrane anchor and cytoplasmic tail of VSV G with the carboxyl terminal 37 amino acid of decay accelerating factor (DAF). The C-terminal 37 amino acids of DAF encodes a signal for a glycosylphosphatidylinositol-lipid anchor attachment (Caras et al., 1987). GPI attachment signals from decay-accelerating

factor or Thy1.1 have been used to anchor viral glycoproteins to the plasma membrane(Kreis et al., 1986) (Kemble et al., 1993) (Salzwedel et al., 1993) (Weiss and White, 1993) (Gilbert et al., 1993) (Kemble et al., 1994) (Melikyan et al., 1995). All the GPI-linked viral glycoproteins were expressed in cell culture, transported to the cell surface and oligomerized correctly. GPI-linked chimeras retain 9 amino acids (PNKGSGTTS) of DAF after cleavage and GPI attachment (Kemble et al., 1994). This produces chimeras that have a 9 amino acid insert between the ectodomain of VSV G and the lipid anchor. Previously, an HA-GPI linked construct of influenza was made by deleting 9 juxtamembrane exoplasmic amino acids of the HA (Kemble et al., 1994). This resulted in a HA-GPI linked chimera with a spatial arrangement similar to wild-type HA. When the 9 juxtamembrane exoplasmic amino acids of HA were replaced with the 9 amino acids form the DAF attachment signal as a control, it did not affect the fusion activity of the HA protein. This suggests that the 9 juxtamembrane amino acids of hemagglutinin are not essential for the fusion mechanism of HA and can be replaced (Kemble at al., 1994).

The amino acids 442-462 of the VSV G ectodomain that border the transmembrane anchor are highly conserved among 5 VSV serotypes. To investigate the role of the 9 juxtamembrane exoplasmic amino acids of VSV G on the fusion mechanism and to distinguish between the effect of the 9 amino acid of DAF and the GPI-lipid anchor on fusion a fourth class of chimera was constructed. The fourth class of chimera are controls for the VSV G-GPI linked chimeras in which the 9 juxtamembrane

exoplasmic amino acids of VSV G are either deleted , replaced or 9 amino acids inserted with the corresponding 9 amino acids of DAF. Also one hybrid, GtmΔ12gB, retains 8 extra amino acids from gB ectodomain plus three serine residue with the HSV-1 gB TM. The 11 amino acids were deleted to construct GgB3G.

3.1.1 Transmembrane Anchor Hybrids

To study the affect of the transmembrane anchor on the fusion activity of VSV G, chimeric G proteins were constructed in which the transmembrane anchor exclusively was exchanged with the transmembrane anchor of other glycoproteins to construct GtmΔ12gB, GgB3G, GgDG, GAdG and GCD4G. The specific amino acid sequence of each chimera is shown in Table 1. All transmembrane hybrids contained the amino acid sequence 1-462 of VSV G and 483-511. Chimera GtmΔ12gB contained amino acids 1-464 and 483-511 of VSV G and 721-726 plus 773-795 of the HSV-1 gB ectodomain of HSV-1 gB third transmembrane anchor. This chimera contains an extra serine residue to maintain reading frame. This chimera was not fusogenic. To determine if the loss of fusogenic activity was due to the extra amino acids of the HSV-1 gB ectodomain, eleven amino acids were deleted from GtmΔ12gB, including three serine residues from VSV G to construct GgB3G. This chimera has the EC of VSV G and the third transmembrane anchor of HSV-1. The resulting chimera GgB3G consisted of amino acids 1-462 and 483-511 of VSV G and amino acids 775-795 of HSV-1 gB. Chimera GgDG also had amino acids 463-482 of VSV G replaced with 339-365 of HSV-1 gD. Both chimeras, GAdG and GCD4G, have amino acids 465-481 replaced with amino acids 32-55 of

adenovirus type 5 E3-11.6 K gene and 375-395 of CD4 respectively. Construct GCD4G had one additional serine residue at the hybrid EC-TM interface to maintain reading frame.

3.1.2 Transmembrane and Cytoplasmic Domains Hybrids

To study the role of the cytoplasmic tail on fusion the transmembrane anchor and cytoplasmic tail were exchanged with equivalent domains from HSV-1 gD and CD4 to construct GgDgD and GCD4CD4 respectively. The specific amino acid sequence of each chimera is shown in Table 1. Construct GCD4CD4 has one additional serine residue at the hybrid EC-TM interface to maintain reading frame.

3.1.3 GPI linked Hybrids

To study the requirements for a peptide anchor versus a lipid anchor on fusion, the ectodomain of VSV G was linked to the C-terminal 37 amino acids of DAF that encodes a signal for GPI-lipid anchor addition (Caras et al., 1987). After cleavage of the DAF signal sequence and attachment of the GPI-lipid anchor 9 amino acids from DAF remain in the final G chimera between the VSV G EC and the GPI anchor. The specific amino acid sequence of each chimera is shown in Table 2. Both constructs, GGPI and GΔ9GPI, were constructed by attaching amino acids 311-347 of DAF after amino acids 464 of VSV G(AXB) and GΔ9 respectively. Chimera GΔ9GPI has 9 amino acids deleted from the VSV G ectodomain at the EC-TM interface. GGPI and GΔ9GPI have one additional serine residue at the EC-TM hybrid interface to maintain reading frame.

3.1.4 Ectodomain-Transmembrane Interface Chimeras

The GPI anchored constructs contain 9 amino acids of DAF retained in the final GGPI and GΔ9GPI construct. The GPI signal introduces a 9 amino acid space between the ectodomain of VSV G and the GPI lipid anchoring to the plasma membrane. To investigate the effect of this 9 amino acid spacing on fusion, hybrids were constructed at the TM-EC interface of VSV G. Nine amino acids of the VSV G ectodomain were either deleted alone, replaced with 9 amino acids from DAF plus one serine or 9 amino acids of DAF plus one serine were inserted at the EC-TM interface called GΔ9, GΔ910DAF and G10DAF respectively. Amino acids of 311-319 of DAF were inserted between amino acids 464 and 465 of G(AXB) and GΔ9 to construct G10DAF and GΔ910DAF respectively. Also 9 amino acids were deleted between 452 and 462 of VSV G (AXB) to construct GΔ9. All three hybrids have serine inserted at the hybrid interface to maintain reading frame. The specific amino acid sequence of each chimera is shown in Table 2.

3.2 Plasmid Construction and Mutagenesis

The VSV G chimeras were constructed as follows: the gene encoding the wild type G protein of VSV Indiana serotype was cloned in the unique EcoR1 site of the eukaryotic expression vector pXM (Yang et al., 1986) to produce the plasmid pXM-G as described earlier (Li et al., 1993).

3.2.1 Construction of pXM-GtmΔ12gB and pXM-GgB3G

Plasmid pXM-GgB3G was constructed from a plasmid pXM-GtmgBΔ12(or pXM-GtmΔ12gB) (Gilbert et al., 1994). A 632 bp Eco R1-Kpn1 fragment was subcloned

from pXM-GtmgBΔ12 into Eco R1- Kpn 1 site of M13mp18. Using oligonucleotide directed mutagenesis by the procedure of Kunkel et al (1987), 11 amino acids of the gB ectodomain from pXM-GtmgBΔ12 were deleted with the following primer '5CAGTAGTTGGAAATTTGGGGCGCT3'. The 1038 bp Kpn1-Eco R1 fragment of wild type pXM-G and the 600 bp Kpn1-Eco R1 mutagenized fragment were subcloned into the EcoR1 site of the pXM eukaryotic expression vector (5.13 kbp) to create pXM-GgB3G. The deletion was verified by sequencing DNA with Sequenase 2.0 (United States Biochemical Corp.).

3.2.2 Construction of pXM-Gm6

Chimera Gm6 was obtained from Yun Li. It was constructed by introducing a Xho 1 site into wild type pXM-G at nucleotide position 1416 as described previously (Li et al., 1993).

3.2.3 Construction of pXM-GgDG and pXM-GgDgD

The plasmids pXM-GgDG and pXM-GgDgD were constructed in our lab by a fourth year project student Jieshi Yan. Plasmid pXM-GgDgD was constructed by isolating a 1.75 kbp cDNA fragment of HSV-1 gD from the plasmid p9gD (Butcher et al., 1990). The 1.75 kbp gD fragment was digested by Ava1 and Hinf 1 and a 360 bp fragment was agarose gel purified and digested with Nla IV to isolate a 300 bp gel purified fragment of gD containing the transmembrane anchor and cytoplasmic tail sequences. The 300 bp Nla IV fragment of gD was blunt ended into the Xho1 site to create pXM-Gm6 to construct the intermediate pXM-GgDgDG. The intermediate was

digested with Eco R1 and the 1.97 kbp GgDgDG fragment was subcloned into the EcoR1 site of M13mp18 vector to construct M13mp18-GgDgD. The ectodomain-transmembrane interface was mutagenized with the following primer 5'CAGTAGTTGGAAAAACATGGGCCTGAT3'. The 1.95 kbp EcoR1 mutagenized fragment was sequenced and subcloned into the EcoR1 site of the pXM vector to produce pXM-GgDgD. Plasmid pXM-GgDG was constructed from the intermediate M13mp18-GgDgD and mutagenized with the following primer 5'GTGTACTGGATGCGCC-GAGTTGGTATCCAT3'. The 1.7 kbp EcoR1 GgDG fragment was sequenced and subcloned into pXM to create pXM-GgDG. The chimeras were verified by sequencing with Sequenase 2.0 (United States Biochemical Corp.).

3.2.4 Construction of pXM-GAdG

The plasmid pXM-GAdG was constructed in our lab by Essam Wanas. Creation of pXM-GAdG from the Adenovirus 5 E3 region 10 .5 k gene. The transmembrane anchor of the 10.5 K gene was amplified by PCR primers 5'CTCGAGCTCGAGTAGGCAGCAGATGAGCCACAT3' and 5'CTCGAGCTCGAGTTGGGCATGTGGTGGTTCTCCATAGCG3' with Xho1 sites engineered on both the 5' and 3' sides of the PCR product. The 84 bp PCR product was digested with Xho1 and cloned into the Xho1 site of pXM-Gm6. The clone was sequenced for orientation and the 700 bp Kpn1-EcoR1 fragment was cloned into M13mp19 to create the intermediate M13mp19-GtmAd. The following primers, 5'GATGGATACCAACTCGTAGGCAGCAGATGA3' and

5'CCACATGCCCAACGAGCTTTTCCA3' were designed to delete the transmembrane anchor of G and two extra bases of the Xho1 site at the 3' end of the ectodomain of G to preserve reading frame respectively. The clones were sequenced and the 646 bp Kpn1-EcoR1 fragment of M13mp19-GtmAd and a 1038 bp fragment of wild type pXM-G were subcloned into the pXM vector to create pXM-GAdG.

3.2.5 Construction of G(AXB)

The plasmid pXM-G(AXB) was constructed in our lab by Essam Wanas. A G cassette was created that has an Apa1, Xho1 and BssHII site at nucleotide position 299, 1416 and 1473 respectively. The 629 bp Kpn1-Eco1R1 fragment of pXM-Gm6 was subcloned into Kpn1-EcoR1 site of the M13mp19 vector. A BssHII site was introduced at nucleotide position 1473 changing CTC CGA to GCG CGC with the following primer 5'ATGGATACCAACGCGCGCAACCAAGAATAG3' to create M13mp19-G(XB). The mutation was verified by sequencing analysis and the 1038 Kpn1-EcoR1 fragment of pXM-G (+A) described earlier (Zhang and Ghosh, 1994) and the 629 bp Kpn1-EcoR1 fragment of M13mp19-G(XB) into the EcoR1 site of pGEM4z and pXM to create G cassettes pGEM4z-G(AXB) and pXM-G(AXB) respectively.

3.2.6 Construction of pXM-GCD4G and pXM-GCD4CD4

The HIV receptor CD4 on human T cells was cloned into the pCDNA1 vector to produce pCDNA1-CD4 as described earlier (Raja et al., 1993) Using primers

5'TTACGCGCGCGAAGAAGATGCCTAGCCCAA3',

5'CTCGAGCTCGAGCGCCCTGATTGTGCTGGGG3',

5'GCGCGCGCGCGCGGGAGGCTGCAAGTGGGA3', the transmembrane anchor and the transmembrane anchor and cytoplasmic tail sequences of CD4 were amplified by PCR with flanking Xho1 site at the 5' end and BssHII site flanking the 3' end. The transmembrane domain of CD4 and the transmembrane domain and cytoplasmic tail of CD4 , an 85 bp and 258 bp fragment respectively, were cloned into the Xho1-BssHII site of pGEM4z-G(AXB) to create pGEM4z-GCD4G and pGEM4z-GCD4CD4 respectively. The 1.7 kbp and 1.9 kbp EcoR1 fragments containing GCD4G and GCD4CD4 respectively were subcloned into the unique EcoR1 site of pXM to create pXM-GCD4G and pXM-GCD4CD4.

3.2.7 Construction of pXM-GGPI

The plasmid pDAF12 contains the cDNA for human decay-accelerating factor flanked by EcoR1 sites cloned into pKSII+ (Kemble et al., 1993). The C-terminal 37 amino acids of DAF contain the signal for glycosylphosphatidylinositol addition and was cloned using PCR primers containing Xho1 and EcoR1 restriction enzyme sites 5'CTCGAGCTCGAGTCCAAATAAAGGAAGTGGA3', 5'GAATTCGAATTCTTTCTCCTTGCTCTGTTGA3'. The 288 bp fragment was digested with Xho1 and EcoR1, and ligated into pXM with the 1.4 kbp EcoR1/Xho1 ectodomain of pXM-G(AXB). The chimera was verified by sequencing with Sequenase 2.0 (United States Biochemical Corp.).

3.2.8 Construction of pBS*, pBS*G and pBS*GGPI

The plasmid pBS-SK-TΦ-N contains the N gene of VSV Indiana and a T7 terminator cloned into the pBluescript sK +/- vector from Promega (Stillman et al., 1995). The gene encoding the N protein of VSV Indiana was released from the plasmid by digested with Kpn1 and EcoRV to generate pBS-SK-TΦ with 3'overhang and a blunt end respectively.

The Kpn1 3' overhang was degraded by T7 polymerase 3'→5' exonuclease activity. The vector was blunt-end ligated together to produce the modified vector pBS*. pBS* is different from the parental vector pBSK (+/-), it contains the T7 promoter and terminator but minus enzyme sites Apa 1, Kpn1 and Xho1. The 1.7 kbp EcoR1 fragments of pXM-G(AXB) and pXM-GGPI were subcloned into the EcoR1 site of pBS*, to construct plasmids pBS*-G and pBS*-GGPI.

3.2.9 Construction of pXM-GΔ9 and pXM-GΔ9GPI

The following PCR primers

5'CGTAACTCGAGCTTTTCTCGATTGGATTTTTGGATAG3' and

5'AATGGGTCACTACTTGTG3'were designed to amplify an 1120 bp fragment of G between nucleotides 265-1365. The 1120 bp fragment of G was digested with Kpn1/Xho1 and then subcloned into pXM-G(AXB) to construct pXM-GΔ9. The sequence of this construct was verified by sequence analysis. To construct pXM-GΔ9GPI the 1.4 kbp and 300 bp EcoR1/Xho1 fragments were isolated from pXMGΔ9 and pXM-

GGPI, respectively and subcloned into the EcoR1 site of pXM. The orientation of this clone was confirmed by a Nco1 digest.

3.2.10 Construction of pXM-G10DAF and pXM-GΔ910DAF

The following complementary pair of primers,

5'TCGAGCCCAAATAAAGGAAGTGGA3' and

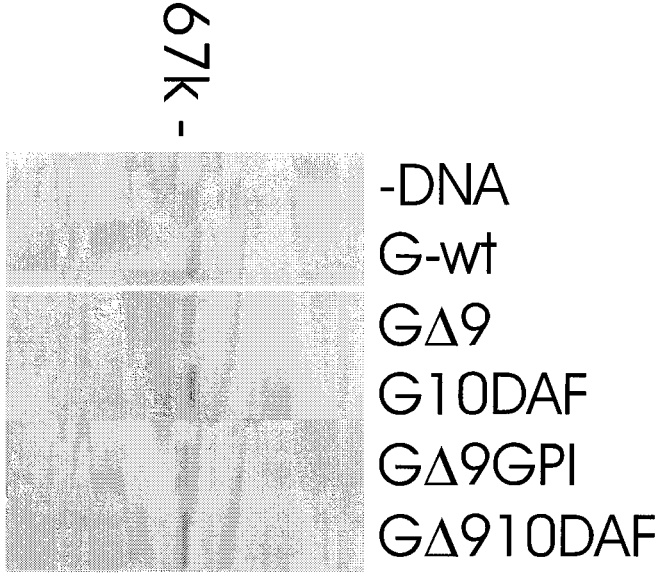
5'TCGATCCACTTCCTTTATTTGGGC3' were designed with Xho1 sticky ends to create a Xho1 linker. The Xho1 linker was cloned into the Xho1 site of either plasmid pXM-G(AXB) or pXMGΔ9 to construct pXM-G10DAF and pXM-GΔ910DAF respectively. Both constructs were verified by sequence analysis with the Sequenase 2.0 kit (United States Biochemical Corp.).

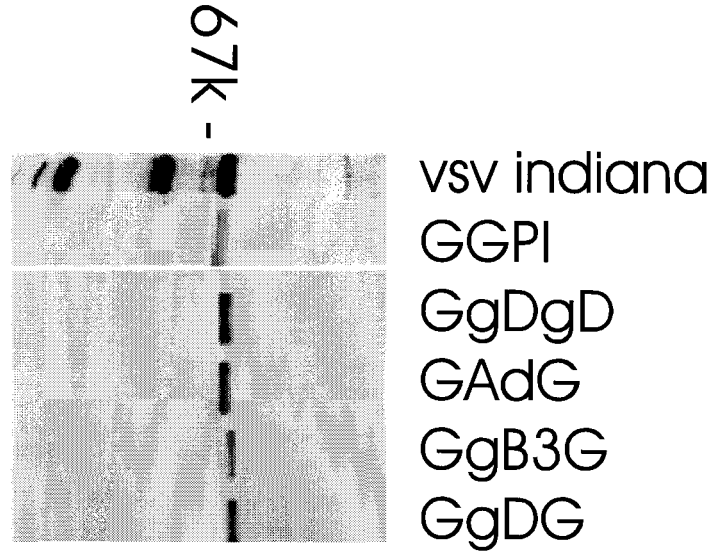
3.3 Expression of Wild-type and Chimeric G proteins

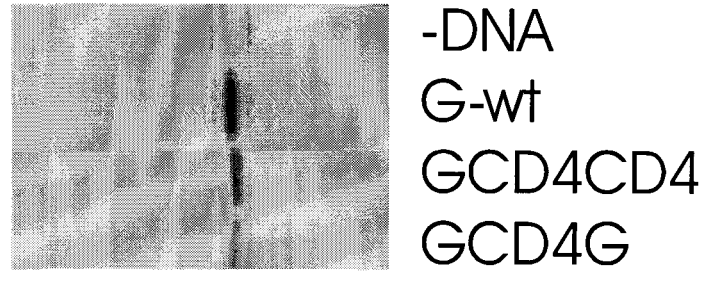
To determine if the chimeric G proteins were expressed properly, COS-1 cells were transfected with plasmids encoding the genes for wild-type or chimeric G proteins. The transfected COS-1 cells were labeled with [^{35}S]-methionine 20 hours post-transfection, and the wild-type or chimeric G proteins were immunoprecipitated with rabbit polyclonal anti-G antibody. The immunoprecipitates were analyzed by SDS-PAGE. In Fig 4, it shows that wild-type and chimeric G proteins were expressed in COS-1 cells. Chimeric G proteins were recognized by rabbit polyclonal anti-G antibody. The G chimeras and wild-type proteins migrate on a 10% SDS-PAGE gels at the expected size of 67 kDa. Chimera GGPI migrates at an approximate molecular weight of 63 kDa. This was expected since the GGPI chimera has both the transmembrane anchor and cytoplasmic tail of VSV G replaced with a glycosylphosphatidylinositol-lipid anchor, a deletion of approximately 10% of the protein compared to wild-type G. Chimera GΔ9GPI migrates as a doublet on the SDS-PAGE gel of approximately 67 kDa and 63 kDa. The 67 kDa band is less intense than the 63 kDa band, suggesting that the 63 kDa band was present at a higher levels than the 67 kDa band in COS-1 cells. The 63 kDa band is the expected size of the mature GPI-linked chimera GΔ9GPI. The 67kDa band may represent the GΔ9GPI precursor before cleavage of the DAF signal sequence and addition of the GPI lipid tail. It should be noted that the 67kDa band of GΔ9GPI migrates parallel to the control chimera GΔ910DAF on a 10% SDS-PAGE gel. The chimera

GΔ910DAF is the equivalent construct of the unprocessed GΔ9GPI precursor. All the chimeras expressed were of the expected molecular weight, suggesting that the transmembrane anchor, transmembrane and cytoplasmic tail or the amino acids at the EC-TM interface can be exchanged with equivalent domains of other viral and cellular proteins without affecting the stability or expression of the proteins.

Fig. 4. Expression of wild-type and chimeric G proteins. COS cells transfected with plasmids encoding wild-type or chimeric G proteins were labeled with [^{35}S]methionine for two hours and lysed. Cell lysates were immunoprecipitated with anti-G antibody and analyzed by SDS-PAGE (Zhang and Ghosh, 1994). The number at the left margin is molecular weight (k, thousands).


67k -
-DNA
G-wt
GΔ9
G10DAF
GΔ9GPI
GΔ910DAF


67k -
vsv indiana
GGPI
GgDgD
GAdG
GgB3G
GgDG

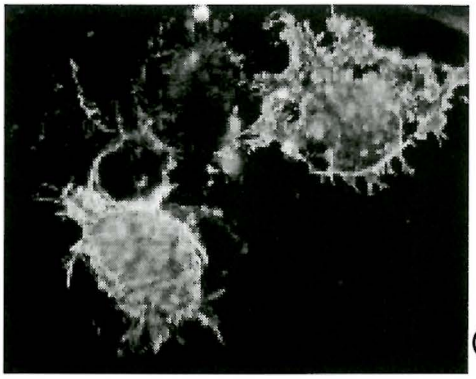

-DNA
G-wt
GCD4CD4
GCD4G

3.4 Intracellular Localization of Wild-type and Chimeric G Proteins

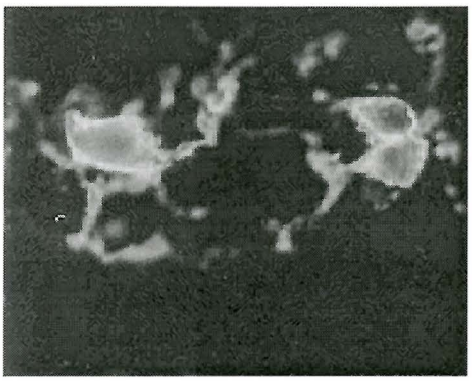
Wild-type G is expressed on the plasma membrane of VSV infected cells, the location of assembly and budding of VSV virions (Wagner and Rose, 1996). The fundamental requirement of the cell-cell fusion activity of glycoprotein G is its presence on the cell surface of infected or transfected cells. To determine if wild-type and chimeric G proteins were expressed on the cell surface, COS-1 cells were examined for indirect immunofluorescence. COS-1 cells were grown on glass coverslips and transfected with plasmids encoding genes for wild-type or chimeric G proteins. Twenty hours post-transfection the COS-1 cells were fixed to glass coverslips with paraformaldehyde, incubated with rabbit anti-G antibody followed by affinity purified Fluorescein-conjugated goat antibody to rabbit IgG. The cell surface expression of wild-type and chimeric G were visualized by epifluorescence microscopy. COS-1 cells expressing wild-type, chimeric G proteins or -DNA control were photographed and are shown in Fig. 5. Cells transfected with calcium phosphate alone (-DNA) do not show cell surface immunofluorescence. In contrast, cells expressing wild-type G protein shows strong cell surface immunofluorescence. This indicates that the glycoprotein localizes to the plasma membrane in transfected COS-1 cells. All chimeric G proteins were expressed on the surface of COS-1 cells as determined by immunofluorescence. Qualitatively, the chimeric G proteins were expressed on the surface of COS-1 cells at similar fluorescent levels to wild-type G.

Fig. 5A. Cell surface localization of chimeric G proteins by indirect immunofluorescence. COS-1 cells transfected with plasmids encoding wild-type or chimeric G proteins were fixed with paraformaldehyde and incubated with rabbit anti G-antibody and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobin G (Zhang and Ghosh, 1994).

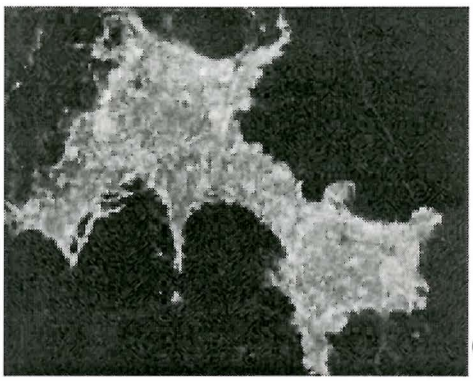
-DNA



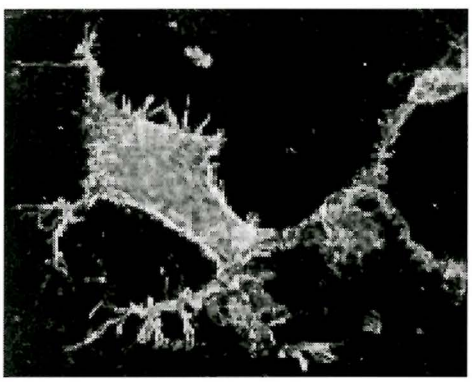
G



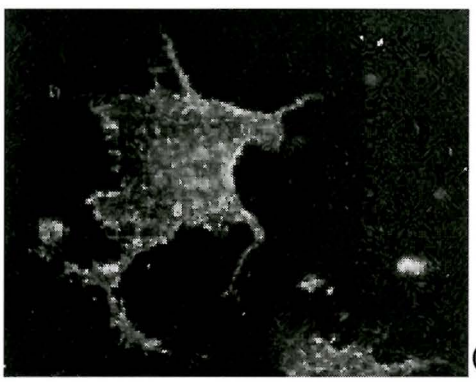
GAdG



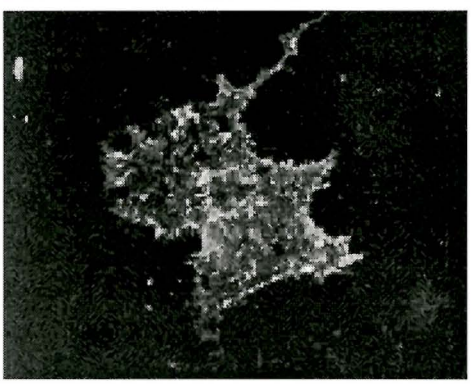
GgDG



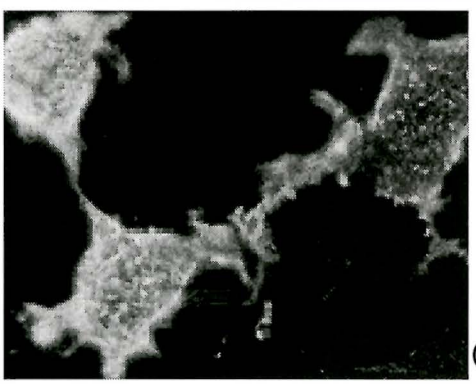
GCD4G



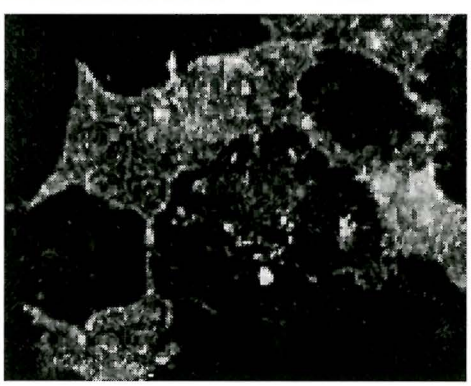
GgB3G



GgDgD

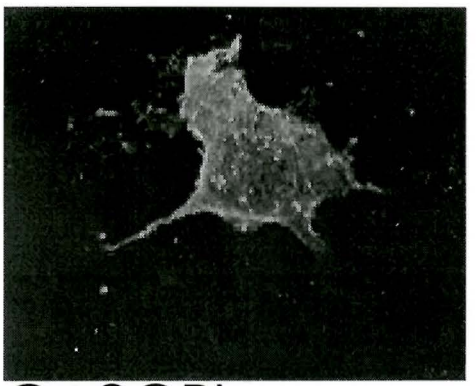


GCD4CD

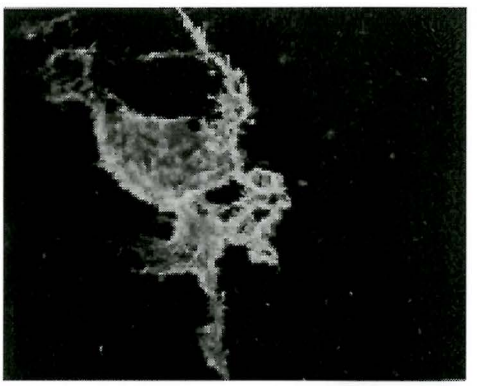


GGPI

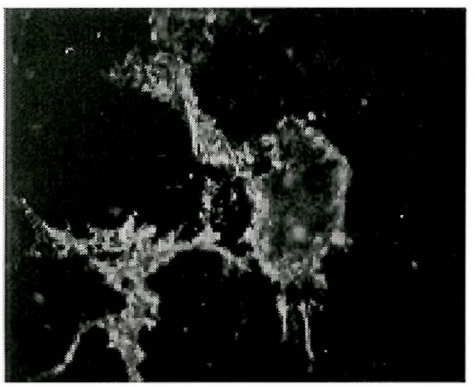
Fig. 5B. Cell surface localization of chimeric G proteins by indirect immunofluorescence. COS-1 cells transfected with plasmids encoding wild-type or chimeric G proteins were fixed with paraformaldehyde and incubated with rabbit anti G-antibody and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobin G (Zhang and Ghosh, 1994).



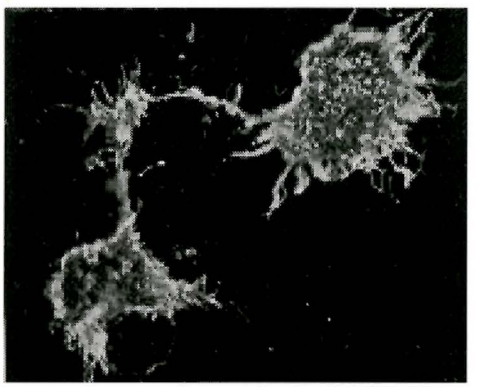
GΔ9GPI



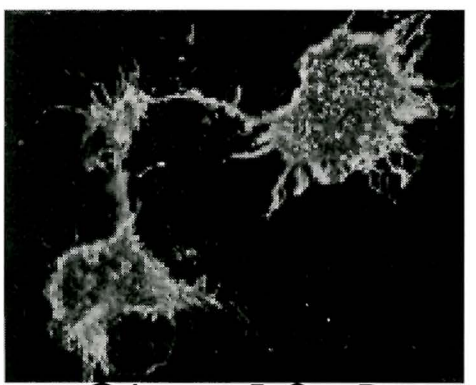
GΔ9



G10DAF



GΔ910DAF

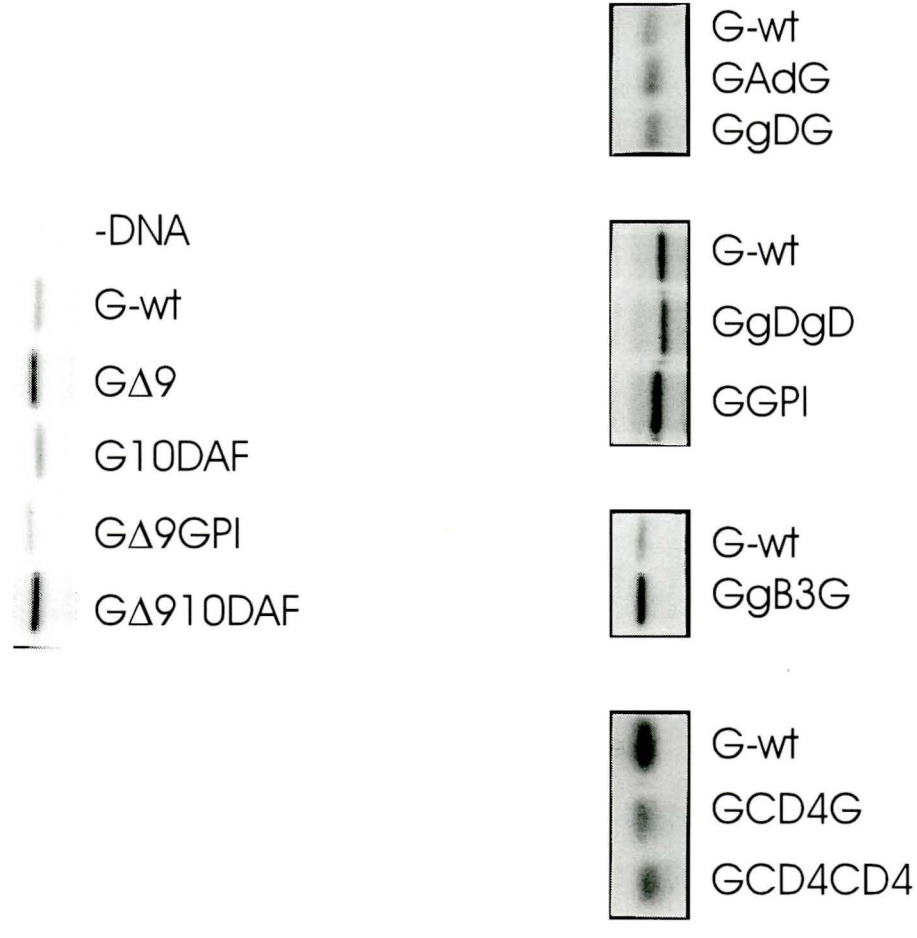


GtmΔ12gB

It was previously shown for VSV glycoprotein G and influenza HA that cell surface density of the viral fusion proteins can affect the extent of fusion (Ellens et al., 1990) (Puri et al., 1993) (Hernandez et al., 1996). VSV G has a threshold cell surface density that must exist for fusion to occur, increasing the cell surface expression of G protein above this threshold level will increase the extent of fusion. To assess the fusogenic capabilities of the chimeric G proteins compared to wild-type G, the cell surface expression should be similar. To quantitate the cell surface expression of chimeric G proteins, all cell surface proteins were iodinated with ^{125}I using a lactoperoxidase catalyzed reaction (Guan et al., 1985). COS-1 cells transfected with plasmids encoding genes for wild-type G or chimeric G proteins were iodinated at twenty hours post-transfection. The cells were lysed, wild-type and G chimeric proteins were immunoprecipitated with polyclonal anti G antibody. The immunoprecipitates were analyzed by SDS-PAGE. The 10 % SDS-PAGE gels were processed for fluorography and the bands were quantitated by densitometry scanning of audioradiographed gels, shown in Fig 6. Chimeras with the transmembrane domain exchanged alone or in conjunction with the cytoplasmic tail with equivalent domains from other viral integral membrane proteins or CD4 such as, GAdG, GgDG, GCD4G,GgB3G, GgDgD and GCD4CD4 were expressed on the cell surface relative to wild-type G at 115, 110, 70, 170, 75, and 85 %, respectively. Chimeras GGPI and GΔ9GPI have the transmembrane anchor and cytoplasmic tail replaced with a glycosylphosphatidylinositol-lipid anchor. They are expressed on the cell surface at 90 and 80 % of wild-type G, respectively.

Chimera GΔ9 has the conserved 9 amino acids flanking the extracellular side of the transmembrane anchor deleted and was expressed on the cell surface at 208 % of wild-type. Chimera G10DAF has a 9 amino acid insertion of DAF plus one serine residue and was expressed on the cell surface at 128 % of wild-type. Chimera GtmΔ12gB has 8 amino acids of the gB ectodomain plus three serine residues and the third transmembrane domain of HSV-1 gB and was expressed at 120% of wild-type G. Chimera GΔ910DAF has 9 amino acids of VSV G replaced with 9 amino acids from DAF plus one serine and was expressed on the surface at 270 % of wild-type G levels. Taken together, all chimeric G proteins are expressed on the cell surface at level that are similar to wild-type G expression (Table III).

Fig. 6. Iodination of wild-type and chimeric G proteins on the surface of COS-1 cells. COS cells transfected with constructs of wild-type G or chimeric G proteins were radioiodinated with ^{125}I in a lactoperoxidase catalyzed reaction. Cell lysates were immunoprecipitated and analyzed by SDS-PAGE. The amount of chimeric G protein expressed on the cell surface was quantitated by scanning densitometry, and surface expression of chimeric G proteins were expressed as a percent of wild-type G protein.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G-wt
GΔ9
G10DAF
GΔ9GPI
GΔ910DAF
G-wt
GAdG
GgDG
G-wt
GgDgD
GGPI
G-wt
GgB3G
G-wt
GCD4G
GCD4CD4

3.5 Transport of Chimeric G proteins and wild-type G

Wild-type G is synthesized in the ER. It has two N-linked oligosaccharides that are added cotranslationally (Doms et al., 1993) (Coll, 1995). The G protein is transported from the ER through the Golgi on its way to the plasma membrane. Measuring the acquisition of endoglycosidase H resistance is a method used to estimate the rates of transport of glycoproteins from the ER to the Golgi complex (Kornfeld and Kornfeld, 1985). To determine if the chimeric G proteins were transported from the ER to Golgi, acquisition of Endo H resistance was measured. COS-1 cells were pulse labeled with [^{35}S]-methionine for 15 minutes and chased for either 0 minutes or 60 minutes with non-radioactive methionine. The cells were lysed, immunoprecipitated with polyclonal anti-G antibody and half of the immunoprecipitates were digested with endo H overnight. Both halves of the immunoprecipitate, the endoH treated and untreated were analyzed by SDS-PAGE. As shown in Fig 7., immunoprecipitates that have been pulsed for 15 minutes with [^{35}S]-methionine, designated time 0, were sensitive to endo H digestion. This indicates that all proteins were modified with N-linked oligosaccharides. Wild-type G is transported from the ER to the Golgi complex with a half time of about 15 minutes (Rose and Bergmann, 1983). Wild-type G becomes entirely resistant to Endo H digestion after a 60 minute chase with non-radioactive methionine, suggesting that all the labeled G protein is transported from the ER to the Golgi complex. Chimeric G immunoprecipitates that were pulsed for 15 minutes with [^{35}S]-methionine followed with a 60 minute chase were either entirely resistant or almost entirely resistant to endo H

Fig. 7A. Acquisition of endo H resistance of wild-type and chimeric G proteins. COS-1 cells transfected with constructs of chimeric G proteins were labeled with [^{35}S]methionine for 15 minutes, chased with non-radioactive methionine for 0 minutes (top) or 60 minutes (bottom), and immunoprecipitated with anti-G antibody. One half of the sample was treated with endo H (+), the other half was not (-). Samples were analyzed by SDS PAGE (Zhang and Ghosh, 1994)). The number in the left margin is molecular weight (k,thousands).

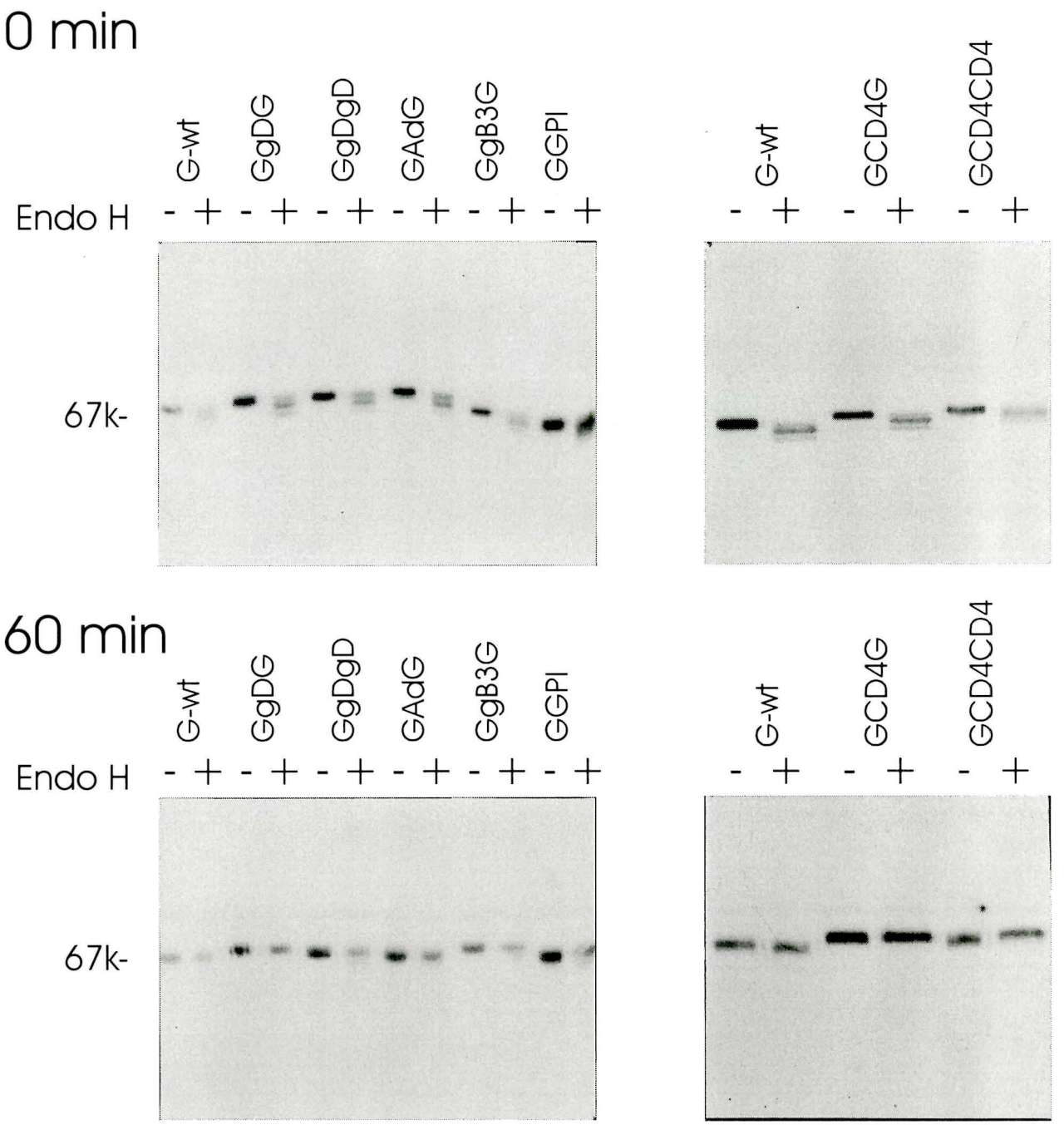

0 min
G-wt
GgDG
GgDgD
GAdG
GgB3G
GGPI
Endo H
- + - + - + - + - + - +
67k-
G-wt
GCD4G
GCD4CD4
- + - + - +
60 min
G-wt
GgDG
GgDgD
GAdG
GgB3G
GGPI
Endo H
- + - + - + - + - + - +
67k-
G-wt
GCD4G
GCD4CD4
- + - + - +

Fig. 7B. Acquisition of endo H resistance of wild-type and chimeric G proteins. COS-1 cells transfected with constructs of chimeric G proteins were labeled with [^{35}S]methionine for 15 minutes, chased with non-radioactive methionine for 0 minutes (top) or 60 minutes (bottom), and immunoprecipitated with anti-G antibody. One half of the sample was treated with endo H (+), the other half was not (-). Samples were analyzed by SDS PAGE (Zhang and Ghosh, 1994)). The number in the left margin is molecular weight (k,thousands).

0 Min
Endo H
VSV indiana
- + GΔ9 - + G10DAF - + GΔ9GPI - + GΔ910DAF
60 Min
Endo H
VSV indiana
- + GΔ9 - + G10DAF - + GΔ9GPI - + GΔ910DAF

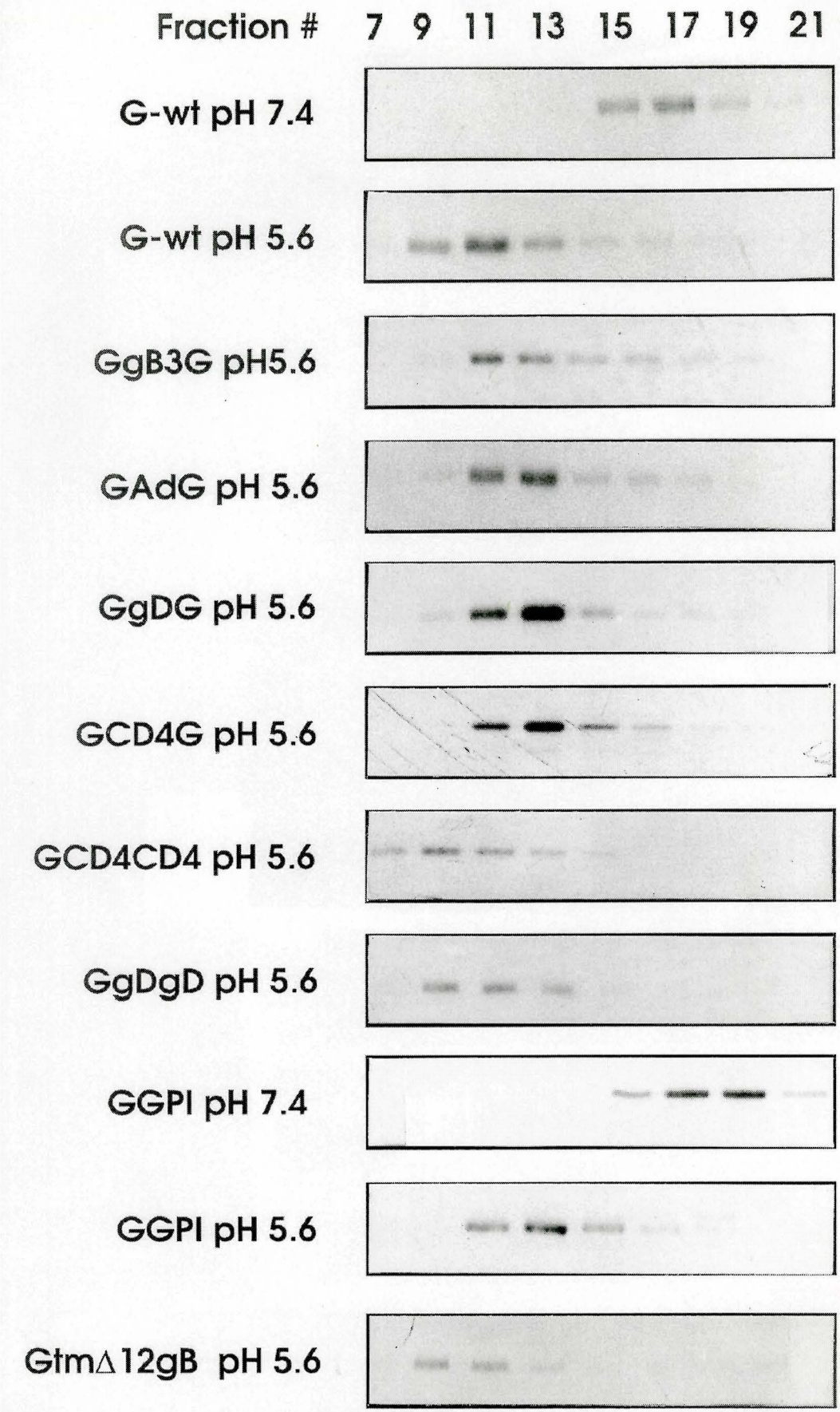
3.6 Oligomerization of Wild-type and Chimeric G Proteins

The fusion activity of VSV glycoprotein G is dependent on the ability of the G protein to fold correctly and achieve the necessary tertiary and quaternary structure. VSV glycoprotein must oligomerize to be transported out of the ER and exists in the viral membrane as a trimer (Kreis and Lodish, 1986)(Doms et al., 1988)(Dubovi and Wagner, 1990)(Lyles et al., 1990)(Doms et al., 1993)(Coll, 1995). To determine if the chimeric G proteins can also achieve the correct tertiary and quaternary structures we analyzed the sedimentation profiles of wild-type and chimeric G proteins through 5%-20% continuous sucrose gradients. This assay has been used previously to determine the oligomeric state of wild-type and mutant G proteins (Doms et al., 1987)(Doms et al., 1988)(Guan et al., 1988)(Crise et al., 1989)(Whitt et al., 1990)(Li et al., 1993)(Zhang and Ghosh, 1994)(Fredericksen and Whitt, 1995) This assay takes into account two unique properties of the VSV glycoprotein G. VSV glycoprotein G that has been solubilized by Triton X-100 at neutral pH sediments through a continuous sucrose gradient as a monomer. The VSV G trimer is not stable at neutral pH. In contrast, VSV G solubilized by Triton X-100 at pH 5.6 sediments as a trimer. The ability of G to sediment as a stable trimer at low-pH correlates closely to the pH required to activate fusion (Fredericksen and Whitt 1995). This may also suggest that the G chimeras are capable of a low-pH induced conformational change.

COS-1 cells were transfected with plasmids encoding the genes for wild-type and chimeric G proteins. Twenty hours post-transfection COS-1 cells were labeled with [^{35}S]-methionine and lysed with 1% Triton X-100 lysis buffer at pH 7.4 or pH 5.5. The cell lysates were centrifuged through a 5-20% sucrose gradient overnight and fractionated from bottom to top into 24 fractions. Since the G protein sediments between fractions 9 and 19 only fractions 7 to 21 were immunoprecipitated. Fractions 7 (bottom) to 21 (top) were immunoprecipitated with polyclonal anti-G antibody and analyzed by SDS-PAGE. The results are shown in Fig. 8. The sedimentation profile of wild-type G indicates that G sediments in fractions 15-17 at pH 7.4 and fraction 9-13 at pH 5.6 as a monomer and trimer, respectively. This confirms previously published results from our lab (Li et al, 1993) (Zhang and Ghosh, 1994) and other labs (Doms et al., 1985) (Crise et al., 1989) (Fredericksen and Whitt, 1995). Aldolase and BSA markers confirm the wild-type G sedimentation profile, Aldolase (8S) sediments as a marker for the trimer in fraction 11 and the (4S) BSA sediments as a marker for the monomer in fraction 17. All chimeras tested for oligomerization at pH 5.6 appeared in the range of fractions 9-13 and therefore have similar sedimentation profiles to wild-type G at pH 5.6. This indicates that all the chimeras tested for oligomerization could form oligomers at pH 5.6. This also suggests that the chimeric G proteins tertiary and quaternary structures were not significantly altered. Chimeras GΔ9, G10DAF, GΔ910DAF and GΔ9GPI were not tested for oligomerization. Since trimerization of G is essential for transport out of the ER (Doms et al., 1987), the presence of these chimeras on the cell surface indirectly suggests that all

four chimeras can oligomerize correctly. The sedimentation profile of GGPI at pH 5.6 confirms previous results that the ectodomain of VSV G contains all the necessary structural information for oligomerization (Crise et al., 1989).

Fig. 8. Analysis of oligomer formation of wild-type and chimeric G proteins by sucrose density gradient centrifugation. COS-1 cells transfected with wildtype or chimeric G genes were labeled with [^{35}S]methionine for 30minutes and chased with non-radioactive methionine for 90 minutes. Cell lysates in 1% Triton X-100 at pH 5.6 or pH 7.4, were centrifuged through a 5 to 20% sucrose gradient buffered at the same pH as the cell lysate. Twenty four fractions were collected from the bottom to the top, and fractions 7 to 21 were immunoprecipitated with anti-G antibody and analyzed by SDS-PAGE (Zhang and Ghosh,1994). The bottom fraction is to the left. 8S (aldolase) and 4S (bovine serum albumin) markers sediment in fractions 11 and 17 respectively. Chimeras GΔ9, GΔ910DAF, GΔ9GPI, and G10DAF were not tested for oligomerization.


Fraction # 7 9 11 13 15 17 19 21
G-wt pH 7.4
G-wt pH 5.6
GgB3G pH5.6
GAdG pH 5.6
GgDG pH 5.6
GCD4G pH 5.6
GCD4CD4 pH 5.6
GgDgD pH 5.6
GGPI pH 7.4
GGPI pH 5.6
GtmΔ12gB pH 5.6

3.7 Polykaryon Formation of Chimeric G Proteins

Cells transfected with a cDNA of VSV G can be induced to form syncytia or polykaryons when exposed to acidic media. Polykaryons or syncytia are large multinucleated cells resulting from the induced fusion of cells expressing G protein on the cell surface with surrounding cells. Polykaryons can have as many as 50 nuclei (Zhang and Ghosh, 1994). It was previously shown that mammalian cells expressing a cDNA clone of a viral glycoprotein can form polykaryons or syncytia in response to low-pH (White et al.,1982)(Kondor-Koch et al., 1983)(Florkiewicz and Rose, 1984)(Reidel et al.,1984). This demonstrates that viral fusion proteins can mediate fusion in the absence of any viral gene products. In the case of VSV G protein, it is sufficient to cause cell fusion in the absence of other viral proteins (Florkiewicz and Rose, 1984)(Reidel et al., 1984)(Puri et al., 1993). The syncytia assay was used to quantify the extent fusion caused by wild-type G and chimeric G proteins (Florkiewicz and Rose, 1984). COS-1 cells transfected with the genes encoding wild-type G or chimeric G proteins were exposed to pH 5.6 or pH 5.2 fusion buffer for 1 minute to induce cell-cell fusion as shown in Fig. 9. Fusion was quantitated by counting the number of polykaryons formed in 25 fields from 2 independent experiments, and compared against the number of polykaryons formed at pH 5.6 for wild-type G. The fusion optimum for VSV G cell-cell fusion was previously determined at pH 5.6, above or below this pH fusion is reduced for wild-type G (Florkiewicz and Rose, 1984)(Zhang and Ghosh, 1994)(Fredericksen and Whitt, 1995)(Fredericksen and Whitt, 1996). As shown in Fig. 9., COS-1 cells expressing wild-

type G protein on the cell surface form extensive syncytia 100% and 90% at both pH 5.6 and pH 5.2, respectively. In contrast, cells that were not transfected with plasmid DNA do not induce polykaryons. Chimeric proteins in which the transmembrane anchor alone or in conjunction with the cytoplasmic tail were exchanged with the equivalent domain of other integral membrane proteins were able to mediate fusion.

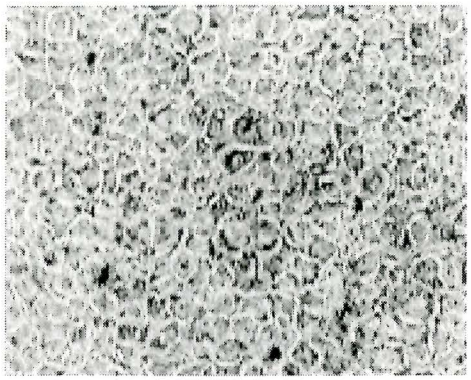
Chimeras GAdG and GgB3G showed wild-type levels of fusion at both pH 5.6 and pH 5.2. Chimera GgDG showed 70% fusion at pH 5.6 and 90% fusion at pH 5.2. Chimera GCD4G showed 90% fusion at pH 5.6 and 75% fusion at pH 5.2. The transmembrane hybrids showed similar extents of fusion to wild-type, suggesting that the sequence of the transmembrane anchor of VSV G was not essential for fusion (Table III).

Hybrids with foreign TM and CT domains showed reduced amounts of fusion compared to wild-type. Chimera GCD4CD4 showed 30% fusion at both pH 5.6 and 5.2, while GgDgD showed 15 % fusion at pH 5.6 and 35% at pH 5.2 (Table III). This suggests that the amino acid sequences of the transmembrane anchor and cytoplasmic of VSV G were also not required for fusion, although the CT of VSV G may regulate fusion. Recently it has been shown that increasing the level of surface expression of chimeras GgDgD (132%), GCD4G (162%), GCD4CD4(116%) and GGPI(192) to levels significantly above wild-type G did not increase their fusion levels (Odell et al., 1997). This indicates that chimeric G proteins expressed in this study were at levels sufficient to cause maximum fusion. It was also shown that both the threshold and pH optimum of GgDgD and GCD4CD4 were shifted to more acidic values (Odell et al., 1997),

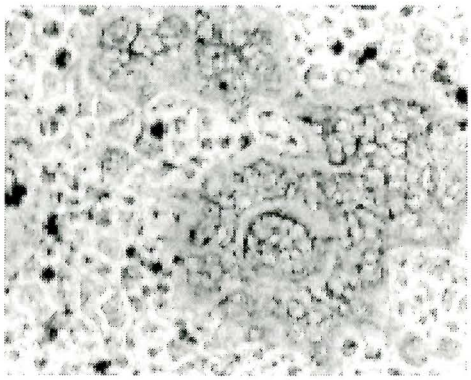
suggesting the CT of G protein may have a role in modulating the fusion activity of VSV G.

Chimeras GGPI, GΔ9GPI, GΔ9, G10DAF, GΔ910DAF and GtmΔ12gB were all non fusogenic at pH 5.6 and pH 5.2. Since it has been shown previously that mutations in the G protein can cause the pH required for fusion to be shifted to lower pH (Li et al., 1993) (Zhang and Ghosh, 1994) (Fredericksen and Whitt, 1995)(Fredericksen and Whitt, 1996), the GGPI chimera was also assayed for fusogenic activity as low as pH 5.0, without detecting any fusogenic activity (data not shown). The results demonstrate that the fusion activity of G is sensitive to deletion or replacement of the 9 juxtamembrane amino acids and insertion of 10 amino acids at the transmembrane ectodomain junction.

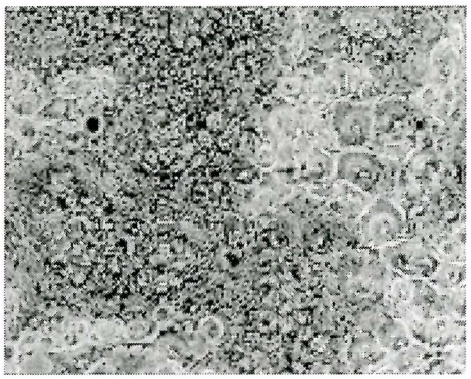
Fig. 9A. Polykaryon formation induced by wild-type and chimeric G proteins. COS-1 cells transfected with plasmids encoding wild-type or chimeric G constructs were exposed to fusion media at pH 5.6 for 60 seconds then incubated for 2.5 hr with regular media at 37°C. The fusion shock was repeated, cells were incubated in regular media for a further 2 hrs, fixed, stained and photographed (Zhang and Ghosh, 1994).



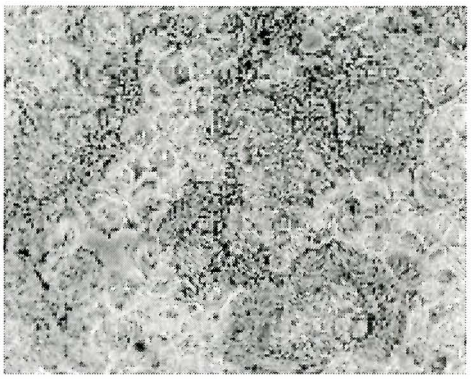
-DNA



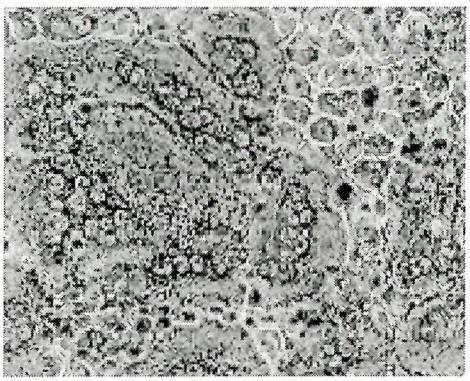
G



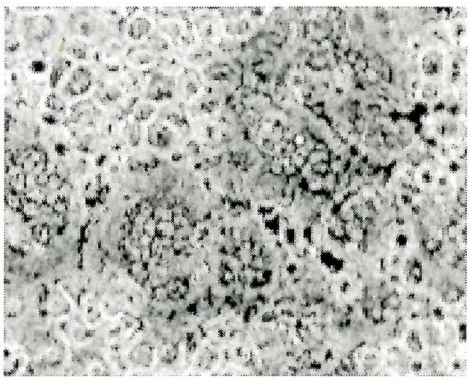
GAdG



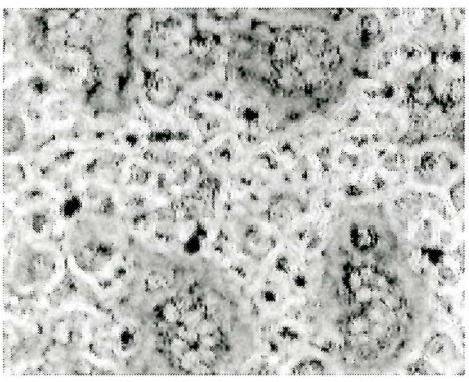
GgDG



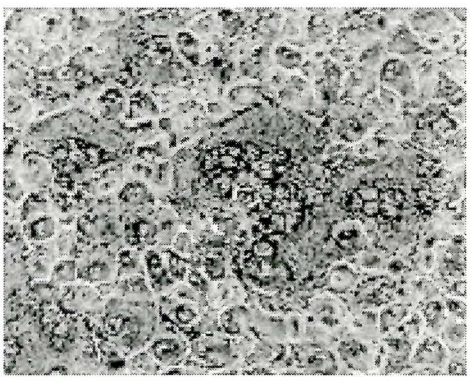
GCD4G



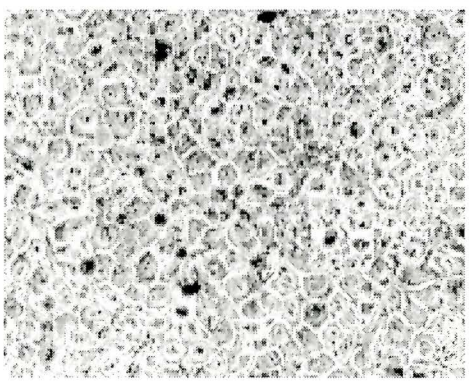
GgB3G



GgDgD

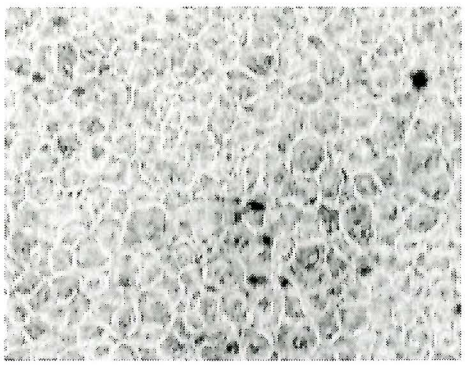


GCD4CD

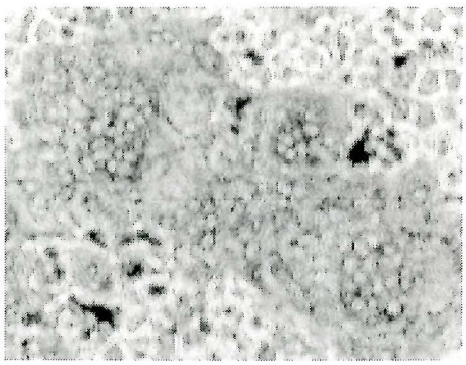


GGPI

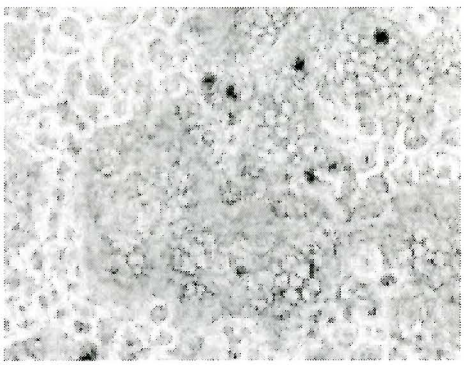
Fig. 9B. Polykaryon formation induced by wild-type and chimeric G proteins. COS-1 cells transfected with plasmids encoding wild-type or chimeric G constructs were exposed to fusion media at pH 5.2 for 60 seconds then incubated for 2.5 hr with regular media at 37°C. The fusion shock was repeated, cells were incubated in regular media for a further 2 hrs, fixed, stained and photographed (Zhang and Ghosh, 1994).



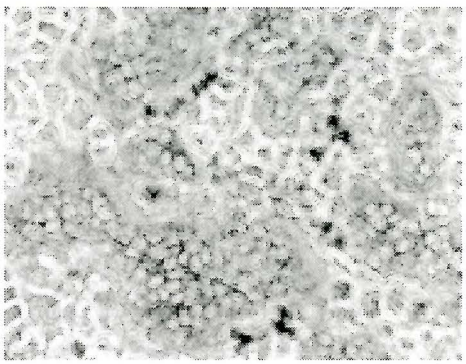
-DNA



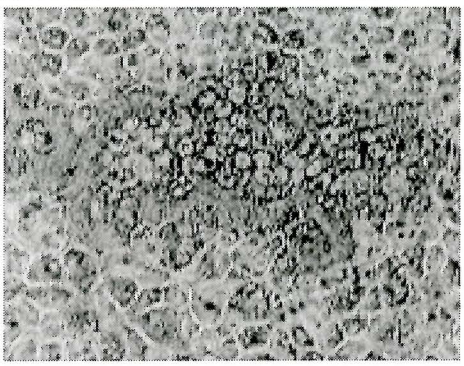
G



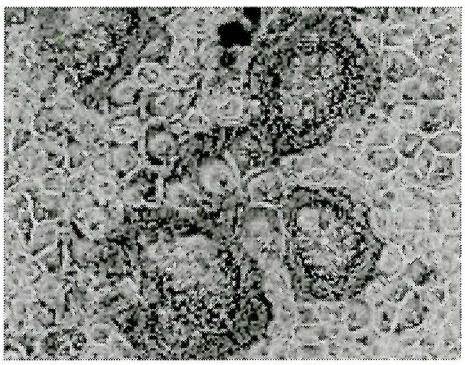
GAdG



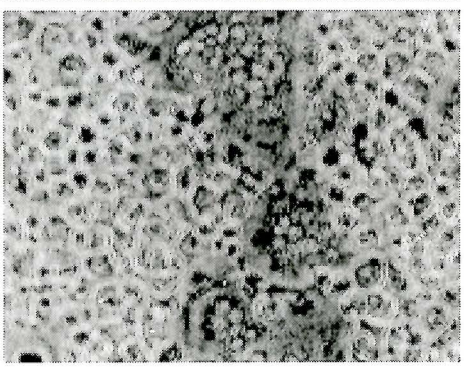
GgDG



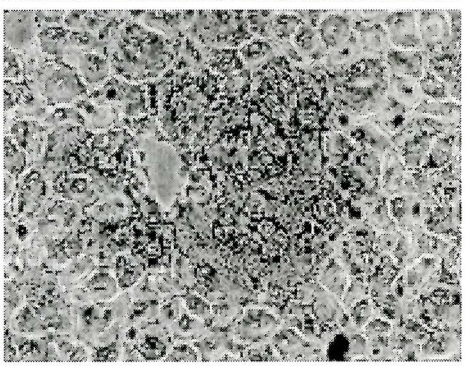
GCD4G



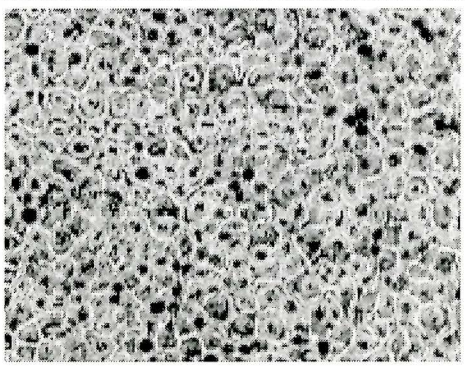
GgB3G



GgDgD

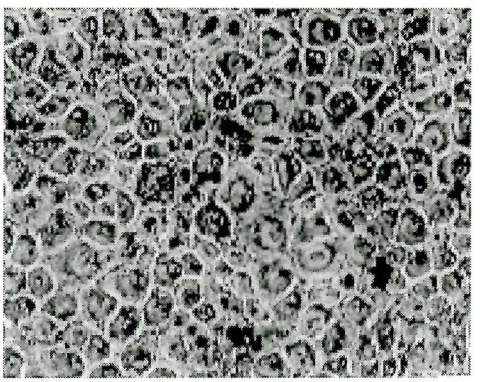


GCD4CD

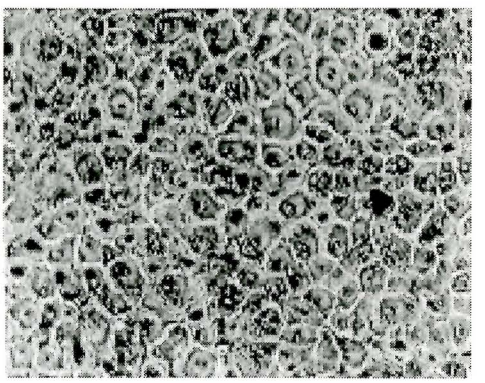


GGPI

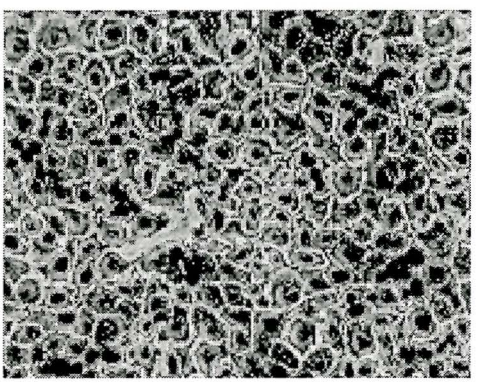
Fig. 9C. Polykaryon formation induced by wild-type and chimeric G proteins. COS-1 cells transfected with plasmids encoding wild-type or chimeric G constructs were exposed to fusion media at pH 5.6 for 60 seconds then incubated for 2.5 hr with regular media at 37^{o}C. The fusion shock was repeated, cells were incubated in regular media for a further 2 hrs, fixed, stained and photographed (Zhang and Ghosh, 1994).



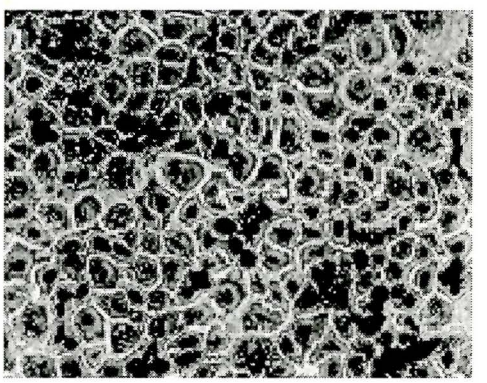
GΔ9GPI



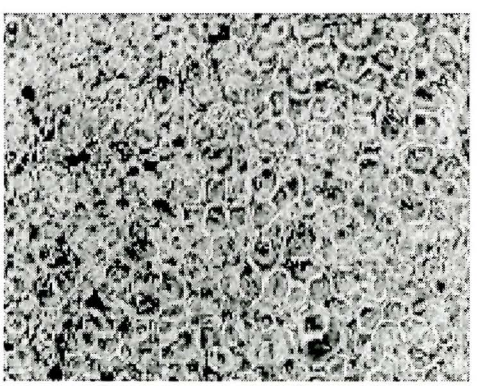
GΔ9



G10DAF

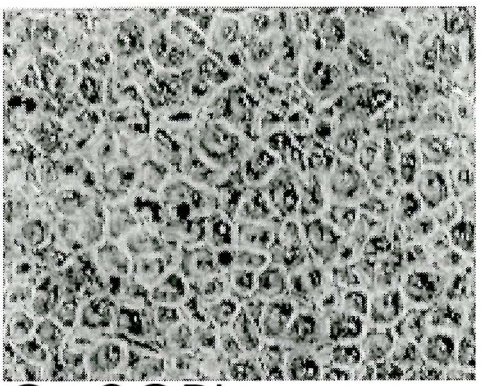


GΔ910DAF

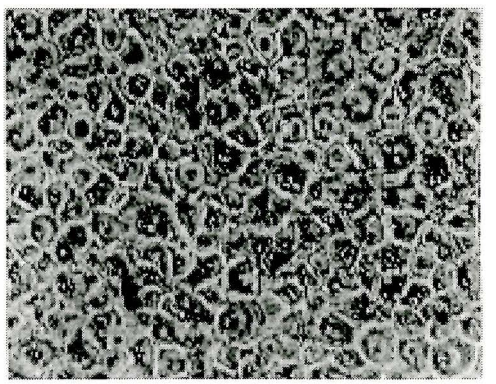


GtmΔ12gB

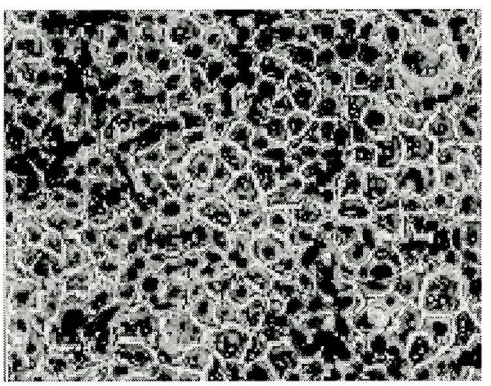
Fig. 9D. Polykaryon formation induced by wild-type and chimeric G proteins. COS-1 cells transfected with plasmids encoding wild-type or chimeric G constructs were exposed to fusion media at pH 5.2 for 60 seconds then incubated for 2.5 hr with regular media at 37°C. The fusion shock was repeated, cells were incubated in regular media for a further 2 hrs, fixed, stained and photographed (Zhang and Ghosh, 1994).



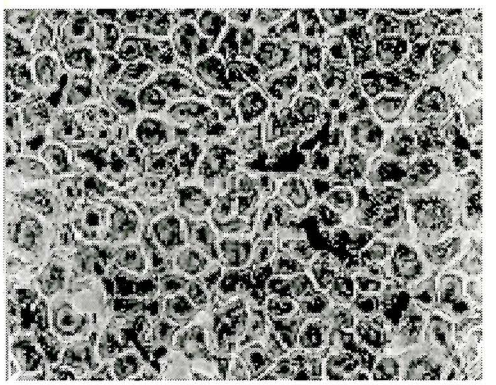
GΔ9GPI



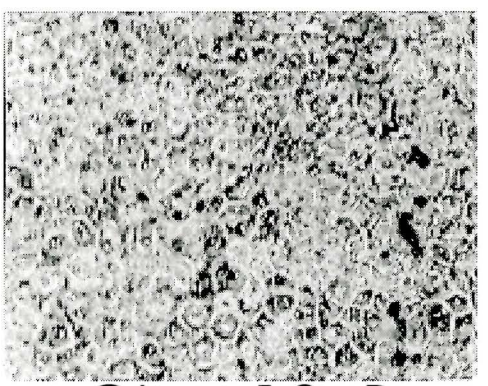
GΔ9



G10DAF



GΔ910DAF



GtmΔ12gB

3.8 Trypsin Sensitivity of Chimeric G Proteins

Many viral fusion proteins undergo a low-pH induced conformational change that shifts the viral fusion protein from a native conformation to the fusion active state with a change from neutral to low-pH respectively(White, 1990)(White, 1992)(Gaudin et al., 1995)(Hughson1995)(Coll, 1995)(Hernandez et al., 1996). Assays have been developed for rabies glycoprotein G, influenza HA and Semliki Forest virus E protein that detect changes in the viral fusion protiens conformation by either detecting changes in protease sensitivity of the viral proteins with respect to low-pH (Kielian and Helenius, 1985)(Wiley et al., 1987)(Gaudin et al., 1991)(Gaudin et al.,1995) or with conformational specific monoclonal antibodies (White and Wilson, 1987)(Gaudin et al., 1993). The conformational change of VSV has been suggested on the basis of fusion kinetics data (Puri et al., 1990)(Clague et al., 1990)(Puri et al., 1992). Recently, an assay has been developed that detects the increasing resistance of VSV G protein to tryptic digestion with decreasing pH (Fredericksen and Whitt, 1996). The trypsin sensitivity profile of wild-type G with respect to pH directly demonstrates the ability of the wild-type G protein to undergo a low-pH induced conformational change. As shown in Fig.10, the wild-type G protein is completely sensitive to trypsin digestion at pH 7.4 and becomes completely resistant to trypsin digestion at pH 6.5 and below. With the exception of GΔ9GPI, GGPI and GtmΔ12gB, all chimeric G proteins have similar tryptic digestion profiles to wild-type G. A control sample (labeled C) with wild-type or chimeric proteins denatured with 0.3% SDS and trypsin at pH 5.6, demonstrates that trypsin is completely

active over the pH range 7.4 to 5.6. Therefore replacing the transmembrane anchor alone or in conjunction with the cytoplasmic tail of VSV G with the equivalent domains from other viral integral membrane proteins or CD4 does not affect the VSV G low-pH induced conformational change.

The G protein can also tolerate a deletion (GΔ9), insertion (G10DAF) or replacement (GΔ910DAF) of the 9 conserved extracellular juxtamembrane amino acids with 9 amino acids of DAF without affecting tryptic profile of the chimeras. Chimera GΔ9 has 9 exoplasmic juxtamembrane amino acids of VSV G deleted (a.a 453-461), and it shows a similar tryptic digestion profile to wild-type G. This suggests that those 9 amino acids of VSV G may not be essential for its acid induced conformational change, although this region may be important for VSV G fusion. However, another chimera GtmΔ12gB has an altered conformationl change profile and is also fusion defective. Deletion of the 11 extra gB amino acids of GtmΔ12gB to create GgB3G results in a protein with wild-type properties.

Both chimeras GGPI and GΔ9GPI have the transmembrane anchor and cytoplasmic tail of G replaced with a glycosylphosphatidylinositol (GPI) anchor and both chimeras require a lower pH than wild-type to induce a conformational change. This suggests that anchoring to the membrane via the GPI-lipid anchor may affect the conformational change of G protein. Since chimeras G10DAF and GΔ910DAF have trypic digestion profiles similar to wild-type, the altered tryptic digestion profiles are a result of the GPI lipid tails and not the 10 amino acids of DAF. This may suggest that the

GPI-lipid anchor of GGPI and GΔ9GPI shifts the pH required to induce a conformational change to lower pH values.

Fig. 10A. pH-dependent resistance of wild-type and chimeric G proteins to trypsin digestion. COS-1 cells transfected with wild-type or chimeric G genes were labeled with [^{35}S]methionine for 30 min, chased with non-radioactive methionine for 60 minutes. Labeled cells were lysed in the presence of 1% Triton X-100 in a buffer containing 2xMNT at the indicated pH. Cell lysates were incubated with or without 10 μg of TPCK-trypsin for 30 min at 37^{o}C. In lane C samples digested at pH 5.6 were incubated with 0.3% SDS. Lysates were immunoprecipitated with anti-G antibody and analyzed by SDS-PAGE. Bands were quantitated by scanning densitometry. Percent trypsin resistance was calculated by quantitating the band-intensity of trypsin digested samples compared to non-trypsin digested samples at the same pH (Fredericksen and Whitt, 1996).

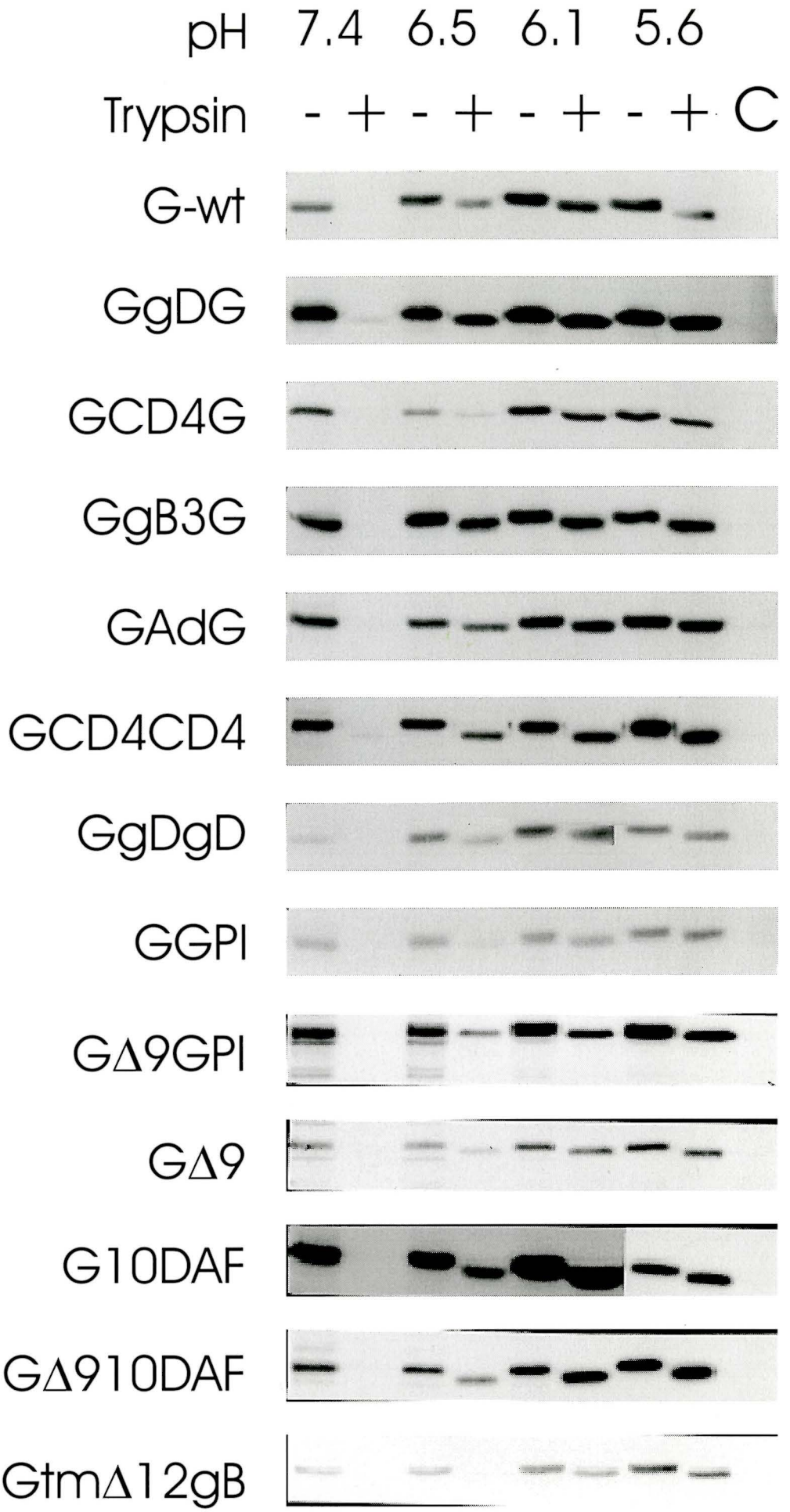
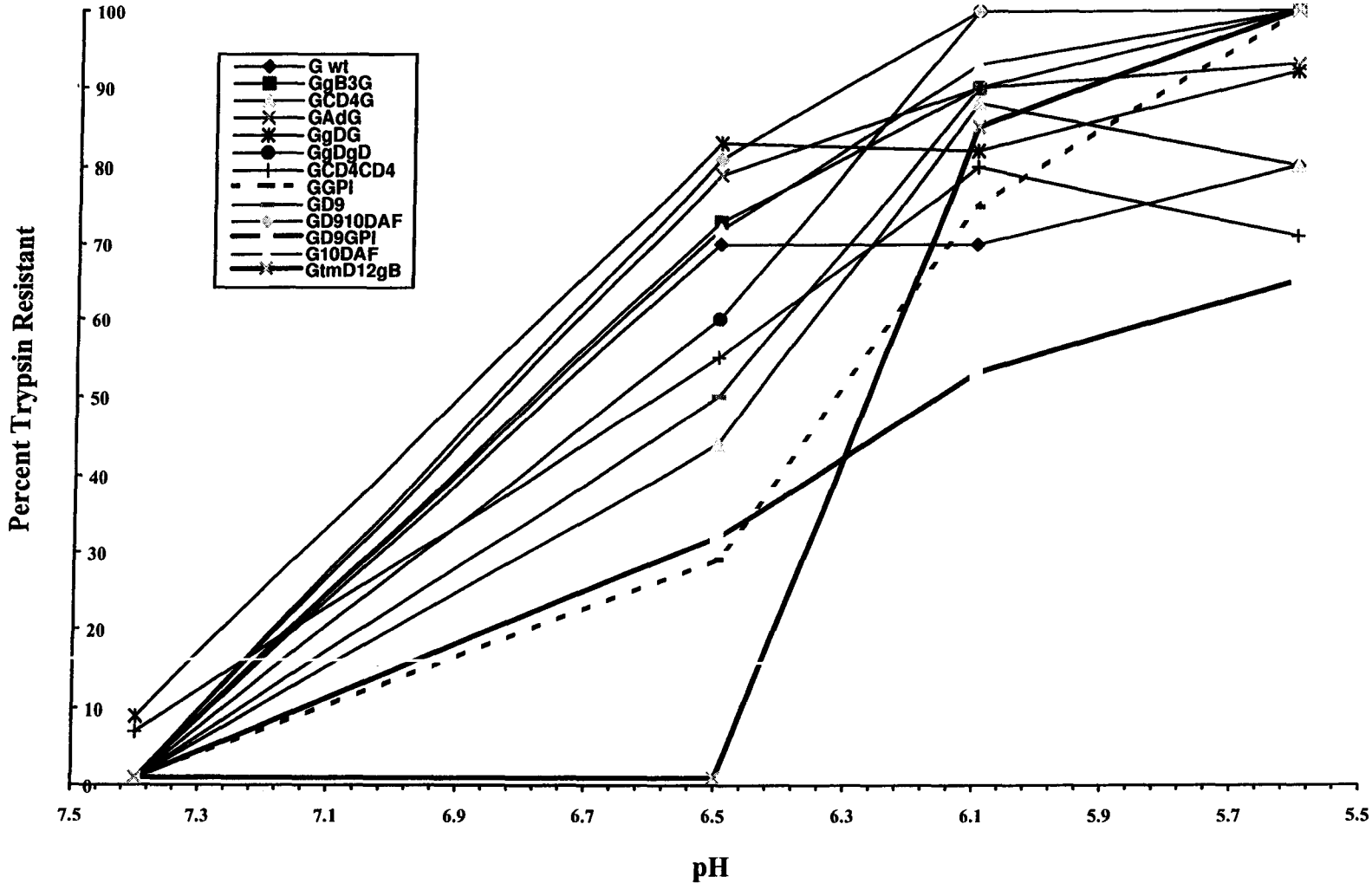

pH 7.4 6.5 6.1 5.6
Trypsin - + - + - + - + C
G-wt
GgDG
GCD4G
GgB3G
GAdG
GCD4CD4
GgDgD
GGPI
GΔ9GPI
GΔ9
G10DAF
GΔ910DAF
GtmΔ12gB

Fig. 10B. pH-dependent resistance of wild-type and chimeric G proteins to trypsin digestion. The intensity of tryptic digested bands on autoradiographed gels were quantitated using a scanning densitometer. Percent resistane to trypsin was estimated by determining the percent intensity of a trypsin digeted band against the undigested band for each pH of the Chimera.


Percent Trypsin Resistant
100
90
80
70
60
50
40
30
20
10
0
7.5
7.3
7.1
6.9
6.7
6.5
6.3
6.1
5.9
5.7
5.5
pH
G wt
GgB3G
GCD4G
GAdG
GgDG
GgDgD
GCD4CD4
GGPI
GD9
GD910DAF
GD9GPI
G10DAF
GtmD12gB

3.9 Lipid Mixing of R18-labeled PMV

Hemifusion is a putative fusion intermediate in which the outer leaflets of two plasma membranes mix while the contents of the two cells or vesicles remain separated. It has been shown previously that a GPI-linked hemagglutinin (GPI-HA) molecule of influenza was fusion defective in a syncytia forming assay, but was able to mediate hemifusion when analyzed for lipid mixing (Kemble et al., 1994)(Melikyan et al., 1995). Recently it was shown that truncation of the carboxy tail of paramyxovirus SV5 fusion protein resulted in a fusion defective protein that could also induce hemifusion (Bagai and Lamb, 1996). Chimera GGPI contains the ectodomain of VSV G linked to a GPI lipid anchor was also fusion defective. To determine the ability of GGPI to mediate hemifusion, a modified octadecylrhodamine (R18) lipid mixing assay was used (Puri et al., 1993). The R18 probe has two properties that make it ideal for monitoring fusion and lipid mixing activity. The R18 probe is hydrophobic and readily partitions into membranes. The probes fluorescence is also quenched at high surface density, while the probes fluorescence increases as the probe is diluted during fusion or lipid mixing. BHK cells were infected at low m.o.i with a recombinant vaccinia virus that expresses T7 RNA polymerase. The BHK cells were simultaneously transfected with plasmids encoding the genes for wild-type G and GGPI under the control of the T7 promoter. Twenty hours post-transfection, R18-labeled plasma membrane vesicles were bound to BHK cells. The plasma membrane vesicles (PMV's) were made from VERO cells that had previously

been used as target membranes for VSV fusion (Puri et al., 1993). The cells were scraped off the tissue culutre dishes and into a HEPES buffer, and low-pH induced lipid mixing was monitored by measuring increased fluorescence (dequenching) using an Aminco-Bowman Series 2 Luminescence Spectrometer. Fusion was induced by lowering the pH to 5.5 with 100 µL 0.5 M MES at the 100 second time point. Full dequenching was determined by the addition of 250 µL 1% Triton X-100. As shown in Fig.11, BHK cells that were not transfected (-DNA) do not show any significant increase in fluorescence in response to low-pH. BHK cells expressing wild-type protein had an increase in fluorescence of 24.8% and 22.5% at pH 5.5 and pH 5.2, respectively. The amount of fluorescence dequenching for wild-type G was consistent with previously published values for VSV G and other viral systems (Puri et al., 1993)(Kemble et al, 1994). BHK cells expressing GCPI showed a slow increase in fluorescence with maximal dequenching of 5.8% and 2.1% at pH 5.6 and pH 5.2, suggesting the GPI chimera was defective at fusion and lipid mixing activity as compared to wild-type G. Therefore unlike a GPI-linked HA protein, a GPI-linked VSV G protein does not appear to promote hemifusion.

Fig.11A. Lipid-mixing activity of wild-type G and chimeric G proteins as measured by the R18 assay at pH 5.6. BHK cells are infected with a recombinant vaccinia virus that expresses the T7 RNA polymerase. The BHK cells are subsequently transfected with plasmids encoding wild-type G or GGPI under the control of the T7 promoter and terminator of pBS*. Twenty hours post-transfection, the R18-labeled PMV's were bound to BHK monolayers at 4°C. The cells were washed and scraped into 0.5 ml DPBS buffer. Fusion was monitored with 100 µl aliquots of R18-labeled PMV-BHK cell suspensions in 2 ml HEPES-NaCl pH 7.4 preequilibrated to 37°C. Fluorescence was monitored using an Aminco-Bowman Series 2 Luminescence Spectrophtometer at 1s time resolution and 560nm and 585 nm excitation and emission wavelength, respectively. Fusion was induced with 0.5 M MES pH 5.0 at 100 seconds. Full dequenching was determined by the addition of 1% Triton X-100. Percent dequenching was determined taken as a percent of full (Triton X-100) dequenching.

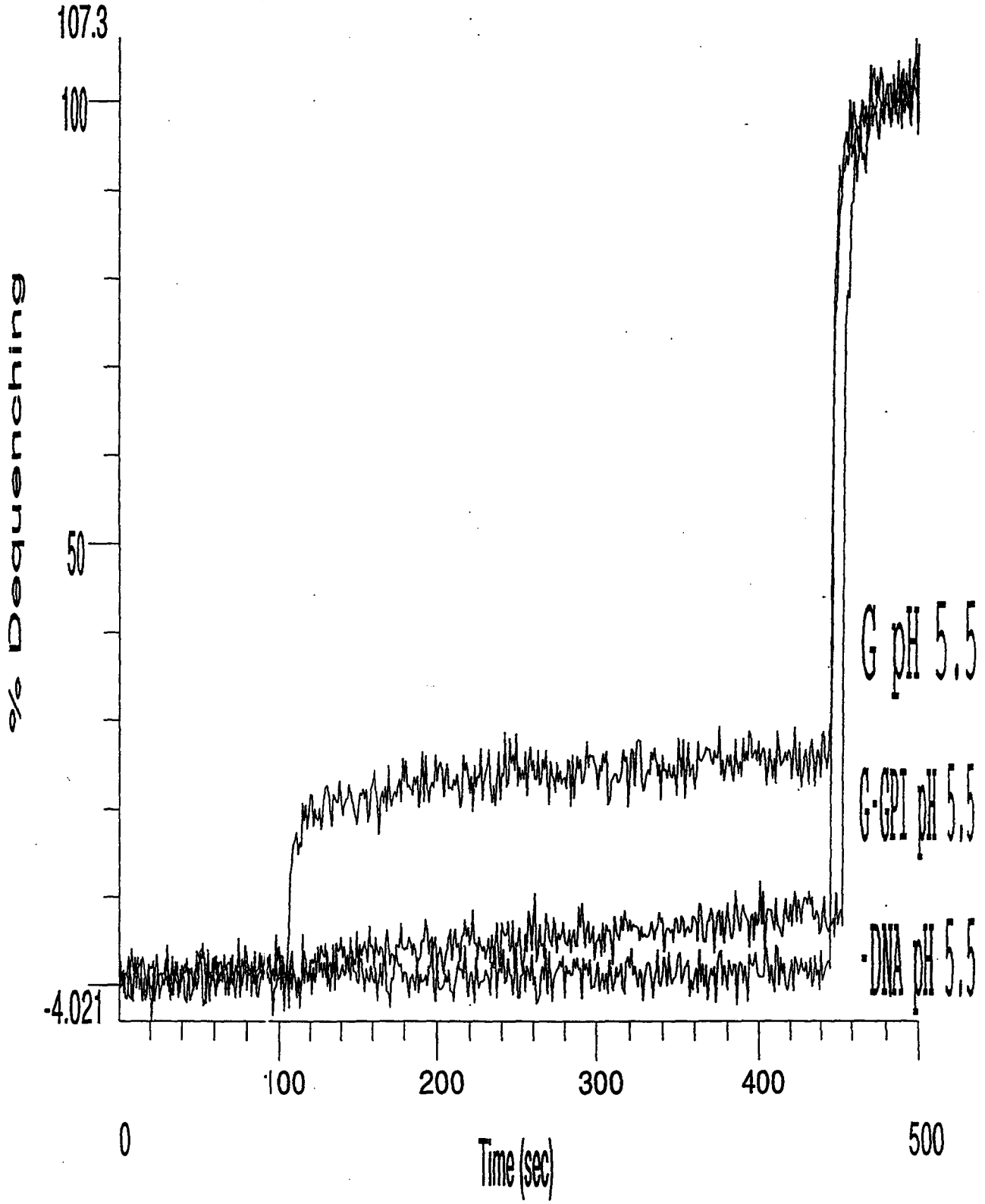

107.3
100
50
-4.021
% Dequenching
G pH 5.5
G-GPI pH 5.5
-DNA pH 5.5
0
100
200
300
400
500
Time (sec)

Fig.11B. Lipid-mixing activity of wild-type G and chimeric G proteins as measured by the R18 assay at pH 5.2. BHK cells are infected with a recombinant vaccinia virus that expresses the T7 RNA polymerase. The BHK cells are subsequently transfected with plasmids encoding wild-type G or GGPI under the control of the T7 promoter and terminator of pBS*. Twenty hours post-transfection, the R18-labeled PMV's were bound to BHK monolayers at 4°C. The cells were washed and scraped into 0.5 ml DPBS buffer. Fusion was monitored with 100 µl aliquots of R18-labeled PMV-BHK cell suspensions in 2 ml HEPES-NaCl pH 7.4 preequilibrated to 37°C. Fluorescence was monitored using an Aminco-Bowman Series 2 Luminescence Spectrophtometer at 1s time resolution and 560nm and 585 nm excitation and emission wavelength, respectively. Fusion was induced with 0.5 M MES pH 5.0 at 100 seconds. Full dequenching was determined by the addition of 1% Triton X-100. Percent dequenching was determined taken as a percent of full (Triton X-100) dequenching

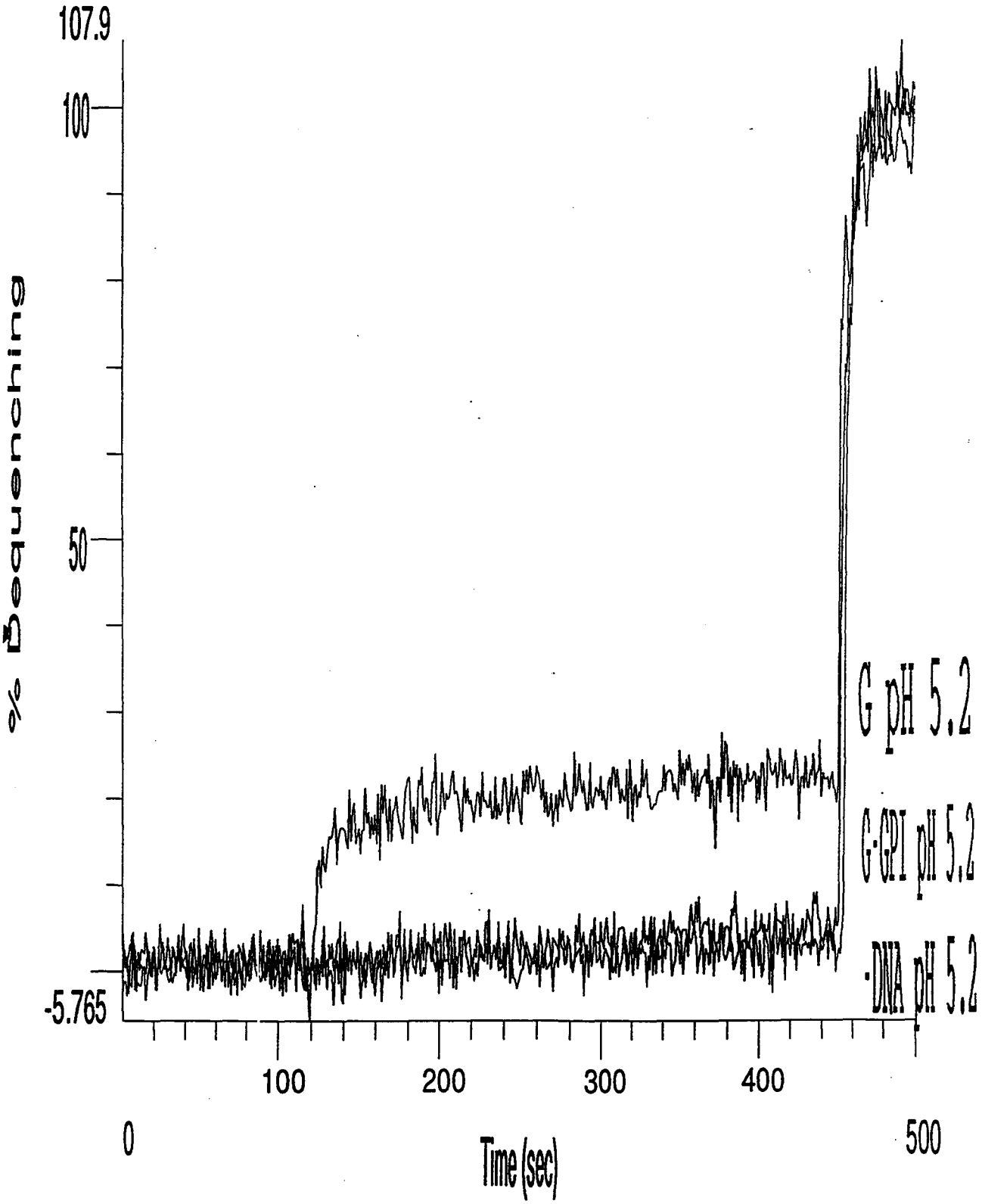

107.9
100
50
-5.765
% Bequenching
0
100
200
300
400
500
Time (sec)
G pH 5.2
G-GPI pH 5.2
-DNA pH 5.2

Table III: Summary of the properties of wild-type G and chimeric G proteins

Chimera	Cell Surface Expression: Immuno -fluorescence	Cell Surface Expression: Iodination (%)	Cell Fusion pH 5.6 (%)	Cell Fusion pH 5.2 (%)	Oligomer	% Trypsin Resistant at pH 6.5
wild-type	+	100	100	90	+	70
Transmembrane Hybrids						
GgB3G	+	170	100	100	+	73
GCD4G	+	70	90	75	+	44
GAdG	+	115	95	100	+	79
GgDG	+	110	70	90	+	83
Transmembrane-Cytoplasmic tail hybirds						
GgDgD	+	75	15	35	+	60
GCD4CD4	+	85	30	30	+	55
GPI Hybrids						
GGPI	+	90	<1	<1	+	29
GΔ9GPI	+	80	<1	<1	NT*	32
EC-TM Junction Hybrids						
G10DAF	+	128	<1	<1	NT*	72
GΔ9	+	208	<1	<1	NT*	50
GΔ910DAF	+	270	<1	<1	NT*	81
GtmΔ12gB	+	120	<1	<1	+	1

*NT is the abbreviation for Not Tested

4. Discussion

In comparison to other viral fusion systems, relatively little is known about the VSV G fusion mechanism. G protein is the single type 1 integral membrane protein embedded in the envelope of VSV. The glycoprotein has three main domains, the ectodomain containing the fusion peptide, the hydrophobic membrane anchor and the cytoplasmic tail. Mutagenesis of the VSV G protein fusion peptide can either abolish fusion or shift the pH required for fusion to more acidic values (Zhang and Ghosh, 1994)(Fredericksen and Whitt, 1995). Similar results have been reported for other viruses, such as SIV (Bosch et al., 1989), HIV-1 (Freed et al.,1990)(Freed et al., 1995), HIV-2 (Freed et al., 1992), influenza (Gething et al., 1986), SFV (Levy-Mintz et al., 1992) and NDV (Sergel-Germans et al.,1994). However, only the fusion peptide of VSV G has been identified as a domain in the VSV G fusion mechanism (Li et al., 1993)(Zhang and Ghosh, 1994) (Fredericksen and Whitt, 1995). To better understand the mechanism of VSV G mediated fusion it is essential to identify the domains in the VSV glycoprotein, other than the fusion peptide, that may be involved in the fusion mechanism.

The transmembrane anchor has been suggested to be an important domain in the fusion mechanism for HIV-1 gp 160, influenza HA, SFV E1 and Moloney murine leukemia virus envelope TM protein. Soluble forms of viral spike proteins such as influenza HA (White et al., 1982)(Wiley et al., 1987), SFV E1 (Klimjack et al., 1994), VSV G (Florkiwicz and Rose, 1984) and HIV gp 160 (Weiss and White, 1993),

containing only the ectodomain and fusion peptide were not fusogenic as tested in a syncytia forming assay. This suggests a requirement for membrane anchoring the viral spike proteins in order for the viral spike proteins to promote cell-cell fusion. However, the requirements for membrane anchoring on the VSV G fusion memchanism have not been clearly defined.

Studies involving chimeras of HIV gp160 or rabies G in which the transmembrane anchor and cytoplasmic tail were exchanged with the equivalent domain of VSV G were not fusogenic, suggesting an important role of either the transmembrane anchor or cytoplasmic tail (Whitt et al., 1991)(Owens et al., 1993). When the charged amino acids of the HIV-1 gp 160 transmembrane anchor were mutagenized, the fusion activity of gp160 was abolished(Helseth et al., 1990)(Owens et al., 1994)(Freed and Martin, 1995). The charged residues of the HIV-1 gp 160 transmembrane domain, Lysine 689 and arginine 696, may be important to the fusion mechanism of HIV-1 gp160(Helseth et al., 1990). Also, the transmembrane anchor of both Moloney murine leukemia virus and Murine coronavirus spike protein were suggested to be important for its fusion mechanism (Ragheb et al., 1994)(Evelyne et al.1995). A deletion mutant of the Moloney murine leukemia virus envelope TM protein demonstrated that only 8 amino acids of the membrane spanning peptide segment was sufficient for membrane anchoring of the protein but not sufficient for membrane fusion (Ragheb et al., 1994) Therefore it appears that the transmembrane anchor may be an essential domain of viral fusion proteins and is required for the fusion mechanism of some viral fusion proteins.

To study the role of the VSV G transmembrane anchor, we constructed chimeric proteins in which the transmembrane anchor was exchanged with the equivalent domains of other integral membrane proteins such as GgDG, GCD4G, GgB3G and GAdG. All the transmembrane anchor chimeras were found to be expressed, glycosylated, form oligomers and underwent a low-pH induced conformational change similar to wild type G protein. When assayed for fusion activity at pH 5.6 and pH 5.2 all the transmembrane chimeras were able to induce a similar number of syncytia as compared to wild-type G at the same pH. This suggests that the transmembrane anchor of VSV G can be replaced with an equivalent domain from other viral spike proteins and remain fusion competent. Even the T-cell surface protein CD4, can substitute for the VSV G transmembrnane domain and the GCD4G chimera retains wild-type fusogenic activity. Therefore the data suggests that the specific amino acid sequence of the VSV G transmembrane anchor is not required for its fusion activity.

Similar results have been published for the gp160 protein of HIV-1, in which the transmembrane anchor was exchanged with an equivalent domain from CD22 (Wilk et al., 1996) The infectivity of the recombinant HIV-1 virus expressing the gp160-CD22 chimera was similar to wild-type gp 160 (Wilk et al., 1996). Suggesting that the specific amino acid sequence of the HIV gP160 transmembrane anchor, like VSV G protein, is not required for their fusogenic activity. The transmembrane anchor of the fusogenic env protein of human T cell leukemia virus Type 1 can also be replaced with the equivalent

domain from Friend murine leukemia virus and the chimera remains fusogenic as tested in a cell-cell fusion assay (Denesvre et al., 1995).

The cytoplasmic tail of some viral fusion proteins can exert both a positive and negative effect on viral fusion. Removal of a segement of the long cyoplasmic tails from the fusogenic glycoproteins of human and simian immunodeficiency virues and murine leukemia virus increases the fusogenic activity of these proteins (Ritter et al., 1993)(Ragheb and Anderson, 1994)(Freed and Martin, 1995)(Yang and Compans, 1996). In contrast, specific sequences in the cytoplasmic tail of Newcastle disease virus are required for its fusogenic activity (Sergel and Morrison, 1995). Deletion of amino acids 540-553 of the cytoplasmic tail of NDV resulted in loss of its fusion activity (Sergel and Morrison, 1995). Also, replacing both the transmembrane anchor and cytoplasmic tail of rabies G with the equivalent domain from VSV G results in a protein that was completely fusion defective in a cell-cell fusion assay (Whitt et al., 1991) Recently it was demonstrated that truncation of the cytoplasmic tail of simian virus 5 F fusion protein abolishes the proteins fusion activity, but not lipid mixing activity (Bagai and Lamb, 1996). In contrast, truncation of either the cytoplasmic tail of influenza HA or parainfluenza virus 2 F protein do not affect the fusion activity of either viral protein (Simpson et al., 1992),(Yao and Compans, 1995).

To study the role of the VSV G transmembrane domain in conjunction with the cytoplasmic domain we constructed chimeric proteins with both the transmembrane and cytoplasmic domain of VSV G exchanged with the equivalent domains from HSV-1 gD

and the cellular protein CD4. Both chimeras, GgDgD and gCD4CD4 were expressed, glycosylated, formed oligomers and underwent a low-pH induced conformational change similar to wild type G protein. When assayed for polykaryon formation at pH 5.6 and pH 5.2, both constructs showed reduced levels of syncytia formation as compared to wild-type G at the same pH. The reduced fusogenic activity of the chimeras was not due to low levels of protein expression as both chimeras were expressed on the cell surface of COS-1 cells at levels similar to wild-type G protein. Recently, both GCD4CD4 and GgDgD were over expressed on the surface of COS-1 cells at 110 % and 132 % of wild-type G levels without significantly increasing the fusogenic activity of either chimera (Odell et al., 1997). Therefore replacement of the transmembrane in conjunction with the cytoplasmic tail of VSV G protein with equivalent domains of either a viral spike protein HSV-1 gD or T cell surface protein CD4 results in VSV G chimeras with significantly reduced fusion activities.

Recently it has been found that the GgDgD and GCD4CD4 chimeras also have altered pH induced fusion profiles. Wild-type G polykaryon formation is initially detected below pH 6.3,maximal at pH 5.6 and decreases at pH values lower than pH 5.6 (Zhang and Ghosh, 1994) (Fredericksen and Whitt, 1995). In this study polykaryon formation was detected at pH 5.6 and increased at pH 5.2 for chimeras GgDgD and GCD4CD4. Recently it was determined that chimeras GgDgD and GCD4CD4 polykaryon formation is initially detected at pH 5.6 and was maximal at pH 5.0 (Odell et al., 1997). Taken together, the data suggests that replacement of the transmembrane

anchor and cytoplasmic tail of VSV G resulted in hybrid proteins with reduced fusion activity and required lower pH to induce fusion (Odell et al., 1997). This may suggest that the cytoplasmic tail of VSV G can modulate the fusion activity of the protein. Studies involving parainfluenza virus type 3 show that a deletion mutant of its cytoplasmic tail reduces the F proteins fusogenic activity (Yao and Compans, 1995). The cytoplasmic tail of VSV G alone could regulate the fusogenic activity of G protein by affecting the conformational change induced by acidic pH. Studies involving simian immunodediciency virus envelope glycoprotein show that removal of a cytoplasmic tail peptide, previously shown to enhance fusion, affects the conformation of the ectodomain of the envelope glycoprotein (Spies et al., 1994). Alternatively the transmembrane anchor in conjunction with the cytoplasmic tail may form a structural entity that modulates the fusion activity of VSV G.

All viral fusion proteins characterized to date are type 1 integral membrane proteins (White, 1990)(White, 1992) (Hernandez et al., 1996) suggesting a requirement for anchoring the viral fusion protein to the membrane. Since the specific amino acid sequence of the VSV G transmembrane anchor was not essential for its fusion, we investigated the ability of VSV G to mediate fusion with a lipid anchor. To determine the requirements of a peptide anchor on VSV G mediated fusion, we replaced the transmembrane anchor and cytoplasmic tail of G with the signal sequence of DAF to produce the chimera GGPI. Previously, a GPI-linked influenza HA protein was shown to be expressed on the cell surface, formed oligomers, underwent a low-pH conformational

change, exposed its fusion peptide similar to wild-type HA, but the GPI linked HA was not fusogenic (Kemble et al., 1993)(Kemble et al.,1994). However,the GPI-linked HA chimera was able to promote lipid mixing or hemifusion. Hemifusion is the mixing of the exoplasmic leaflets of two lipid bilayers, but not the contents of the cytoplasms (Kemble et al., 1994) This suggests an important role for the transmembrane anchor of HA in its fusion mechanism. The HA molecule can mediate the preparatory steps for fusion when anchored via a GPI-linkage to the plasma membrane (Kemble et al., 1994)(Melikyan et al., 1995). However, the transmembrane anchor of HA is required to promote fusion (Kemble et al., 1994)(Melikyan et al., 1995). It is still unclear what specific contributions the HA transmembrane anchor adds to the fusion mechanism. The requirement for a specific amino acid sequence of the influenza hemagglutinin transmembrane anchor have not been determined. In general, the HA ectodomain, that contains the fusion peptide, is sufficient to establish lipid continuity betwen two membranes, but a transmembrane anchor is required to establish cytoplasmic continuity (Kemble et al., 1994)(Melikyan et al., 1995).

To determine the role of a peptide anchor on VSV G fusion, the transmembrane and cytoplasmic domains of VSV G protein were replaced with a signal sequence from DAF. DAF encodes a signal sequence for the addition of a glycosylphosphatidyl-inositol lipid anchor (Caras et al., 1987). This hybrid, GGPI, was expressed, glycosylated and formed oligomers like wild-type G, but was fusion defective at both pH 5.6 and pH 5.2. It has been shown previously that mutations in the fusion peptide of VSV G protein can

alter the threshold pH required to induce fusion (Li et al., 1993)(Zhang and Ghosh, 1994)(Fredericksen and Whitt, 1995)(Fredericksen and Whitt, 1996). Therefore in this study the GGPI chimera was also assayed at pH 5.0 without detecting polykaryon formation (data not shown). In fact the GGPI chimera was found to be fusion negative over a pH range of pH 6.4 to as low as pH 4.8 (Odell et al., 1997). The lack of fusogenic activity of GGPI was similar to the results of other hybrids of GPI-linked viral spike proteins, such as influenza HA (Kemble et al., 1994)(Melikyan et al., 1995), human immunodeficiency virus-1 gp160 (Salzwedel et al., 1993)(Weiss and White, 1993), herpes simplex virus-1 gB (Li et al., 1997) and Moloney murine leukemia virus envelope protein (Ragheb and Anderson, 1994), all were defective for synctium formation.

The data suggests that a GPI-lipid linked VSV G hybrid is fusion defective and may require a transmembrane anchor to mediate fusion. Since the lipid linked GGPI hybrid also retains 9 amino acids from the signal of decay accelerating factor, the lack of fusion activity of GGPI could in fact be contributed by either the 9 retained amino acids of DAF in the hybrid or replacement of the transmembrane anchor and cytoplasmic tail with a lipid anchor. To assess the effect of 9 amino acids from DAF on the fusion of VSV G, a hybrid G10DAF was constructed. This hybrid has 9 amino acids of DAF inserted at the VSV ectodomain-transmembrane interface. This hybrid was expressed, glycosylated and underwent a low-pH conformational change similar to wild-type G, but was fusion negative in a cell-cell fusion assay. The results suggest that 9 extra amino acids of DAF alone at the EC-TM interface are sufficient to abolish the fusion activity of

VSV G. This is in contrast to a GPI lipid linked HA chimera, where replacing the 9 juxtamembrane amino acids of HA with 9 amino acids of DAF does not abolish its fusion activity (Kemble et al., 1994). Another VSV G hybrid GtmΔ12gB, that contains 8 extra amino acids of the gB ectodomain inconjuction with the gB transmembrane domain is also defective at fusion. However deletion of the 11 amino acids at the gB ectodomain-transmembrane anchor interface results in a fusion competent chimera GgB3G.

The biological role of the 9 amino acids (453 to 461 of VSV G indiana) at the EC-TM interface is not known. However, amino acids L453, G456, W457, F458 and W 461 of VSV G indiana are consrved among all five VSV serotypes. This may indicate an important biological role for this region of the G protein. To investigate the role of this domain on VSV G low-pH induced fusion the following chimera were constructed G10DAF, GΔ9 and GΔ910DAF. The sensitivity of VSV G to a 9 amino acid insertion at the EC-TM region was exemplified previously by G10DAF. Two other hybrids GΔ9 and GΔ910DAF, both are VSV G hybrids that have either a deletion (GΔ9) or replacement (GΔ910DAF) of the 9 conserved ectodomain amino acids -juxtaposed to the transmembrane anchor. Both hybrids were expressed, glycosylated and underwent a low-pH conformational change similar to wild-type G, but were fusion negative in a cell-cell fusion assay.

The data may suggest that the EC-TM specific amino acids of VSV G interface are required for the fusion activity of the G protien. In order to test this hypothesis the conserved amino acid sequence of the EC-TM interface would have to be mutated by site-

directed mutagenesis and the mutant G proteins assayed for loss of fusion activity. Identifying an amino acid motif required for fusion would be an interesting future project. An alternate scenario is that a defined spatial distance of the VSV G ectodomain to the viral membrane is required for fusion to occur. Therefore an insertion or deletion of 9 amino acids of DAF at the EC-TM interface of VSV G abolishes its fusion activity while a two serine amino acid insetion at the EC-TM interface of VSV G does not affect its fusogenic activity (Li et al., 1993). It should be noted that it is not unusual for small insertions to disrupt the fusion mechanism of G, as small two amino acid insertions in three different regions of the ectodomain of VSV G (H2, H5 and H10) abolish its fusion activity (Li et al., 1993). Since all three G hybrids, G10DAF, GΔ9 and GΔ910DAF were fusion negative it may suggest a combination of both hypothesis. The 9 amino acids of the VSV G EC-TM junction and its proximity to the membrane may be important for the VSV G fusion mechanism. Future work in this area is required to prove or disprove this hypothesis.

The lipid linked GGPI was not fusogenic as tested in a cell-cell fusion assay. Previous studies have shown that a influenza HA anchored to the membrane via GPI lipid linkage could not induce fusion, but could promote lipid mixing (Kemble et al., 1994) (Melikyanet al., 1995). To determine if a VSV G hybrid linked to the membrane through a GPI lipid linkage could also promote lipid mixing, BHK cells expressing both wild type and GPI were assayed for lipid mixing using a R18 fluorescent dequenching assay. Wild type G showed 24.8% and 22.5% fluorescent dequenching at pH 5.6 and pH 5.2

respectively. In contrast to wild-type G, GGPI could mediate only 5.8 % and 2.1 % fluorescent dequenching at pH 5.6 and pH 5.2 respectively. This suggests that unlike a GPI-linked hemagglutinin, the GGPI hybrid is defective at lipid mixing. The lack of fusion and lipid mixing activity of GGPI hybrid may be due to an altered conformation of the G protein ectodomain or conformational change due to the GPI lipid linkage. Both the 9 extra amino acids of DAF and the GPI lipid anchor are candidates for disrupting the lipid mixing activity. It should be noted that other viral proteins linked through a GPI linkage such as the HSV-1 gB protein (Li et al., 1996), HIV gp 160 protein (Salzwedel et al., 1993)(Weiss and White, 1993) and Moloney murine leuemia virus envelope protein (Ragheb and Anderson, 1994), were also fusion negative, although they were never tested for lipid mixing activity (Li et al., 1996), (Ragheb and Anderson, 1994), (Salzwedel et al., 1993), (Weiss and White, 1993).

It has been shown previously that replacement of the transmembrane anchor of influenza hemagglutinin with a GPI-linked lipid anchor can affect both the structure of the ectodomain and conformational change of hemagglutinin HA (Kemble et al., 1993). A hybrid GPI-HA resulted in a protein that underwent the preparatory steps for fusion, low-pH conformational change and exposure of the fusion peptide 0.4 and 0.2 units higher than wild-type HA (Kemble et al., 1993). This same hybrid lost receptor binding activity, even though the sialic acid binding pocket is at the distal tip of the hemagglutinin molecule (Kemble et al., 1993). Suggesting that the replacement of the transmembrane anchor with a GPI-lipid anchor can affect the structure of the chimeras ectodomain, even

at significant distance from the membrane (Kemble et al., 1993). Both VSV G chimeras, GGPI and GΔ9GPI have altered tryptic digest profiles compared to wild type G. Suggesting that the GPI lipid anchor, or lack of a transmembrane anchor and cytoplasmic tail affects the chimeras at the level of conformational change. This altered conformational change may affect the ability of GGPI to mediate lipid mixing. It would be interesting for future work to test the ectodomain-transmembrane junction hybrids for lipid mixing activity. These hybrids differ from wild-type only in fusion promotion, and are likely candidates for a hemifusion intermediate.

In summary, the specific amino acid sequence of the VSV G glycoprotein transmembrane domain is not required for its low-pH induced fusion mechanism and any integral protein may substitute with out affecting its fusogenic activity. Replacing the transmembrane anchor and cytoplasmic tail results in a hybrid with reduced fusogenic properties, indicating that the cytoplasmic tail alone or in conjunction with the transmembrane anchor may regulate the fusion activity of VSV G. The fusion activity is also sensitive to insertions, deletions and replacement of the 9 conserved amino acids at the ectodomain-transmembrane anchor interface. Although GGPI is not fusogenic, it appears that unlike a GPI-linked hemagglutinin model for hemifusion, a GPI-linked ectodomain of VSV G can not mediate hemifusion.

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