Molecular Architecture of the Bipartite Fusion Loops of Vesicular Stomatitis Virus Glycoprotein G, a Class III Viral Fusion Protein

The glycoprotein of vesicular stomatitis virus (VSV G) mediates fusion of the viral envelope with the host cell, with the conformational changes that mediate VSV G fusion activation occurring in a reversible, low pH-dependent manner. Based on its novel structure, VSV G has been classified as class III viral fusion protein, having a predicted bipartite fusion domain comprising residues Trp-72, Tyr-73, Tyr-116, and Ala-117 that interacts with the host cell membrane to initiate the fusion reaction. Here, we carried out a systematic mutagenesis study of the predicted VSV G fusion loops, to investigate the functional role of the fusion domain. Using assays of low pH-induced cell-cell fusion and infection studies of mutant VSV G incorporated into viral particles, we show a fundamental role for the bipartite fusion domain. We show that Trp-72 is a critical residue for VSV G-mediated membrane fusion. Trp-72 could only tolerate mutation to a phenylalanine residue, which allowed only limited fusion. Tyr-73 and Tyr-116 could be mutated to other aromatic residues without major effect but could not tolerate any other substitution. Ala-117 was a less critical residue, with only charged residues unable to allow fusion activation. These data represent a functional analysis of predicted bipartite fusion loops of VSV G, a founder member of the class III family of viral fusion proteins.

Vesicular stomatitis virus (VSV) is a prototypic virus in the Rhabdoviridae, which includes many important human, animal, and plant pathogens, including Rabies virus (1). VSV is an enveloped virus that is well known to infect cells via a low pH-dependent fusion reaction within endosomes (2–4). The virus contains a single envelope protein, termed the glycoprotein (G), which mediates both attachment to host cells and fusion between the virus envelope and the host cell membrane. This fusion event delivers the VSV genome into the host cell for viral replication. In addition to being a critical determinant of viral pathogenesis, VSV G has also served as an important model for protein folding and transport through the secretory pathway of cells (5). VSV G is used extensively in pseudotyped virus systems and as a delivery system for gene therapy applications (6), and, due to the fact that VSV preferentially replicates and destroys immortalized or tumorigenic cells, the virus has been of great interest as an oncolytic agent in anticancer treatment (7).

The mature VSV G protein is an ~65-kDa type I transmembrane protein containing 511 amino acids that oligomerizes into a homotrimer during transport to the cell surface, where the trimer is then assembled into the viral particle (8). Unlike many other viral glycoproteins (9), VSV G is not subject to proteolytic priming for fusion activation. The fusogenic ability of VSV G has been of significant interest, because, unlike the proposed “spring-loaded” and essentially irreversible metastable state for the pre-fusion state of other fusion proteins such as influenza HA strain X-31 (10), VSV G is apparently fully reversible for fusion activation; it exists in a dynamic equilibrium between the pre-fusion and post-fusion states (11). In addition, whereas most viral fusion proteins can be categorized into an obvious structural group, either class I or class II (12, 13), the distinct structural features of VSV G (14, 15) have resulted in it being considered a novel “class III” fusion protein (16).

A critical feature of any viral fusion protein is the so-called “fusion peptide” (17), which inserts into the target membrane and is instrumental in initiating the merging of the two lipid bilayers (18). In class I fusion proteins (e.g. influenza hemagglutinin and paramyxovirus F), the fusion peptide is a linear sequence that, in the case of influenza HA, is externalized by proteolytic cleavage and comprises a short kinked α-helix (19). In class II fusion proteins, e.g. Semliki Forest virus and tick-borne encephalitis virus, the fusion peptide comprises an internal loop at one end of the fusion domain (20). Prior to determination of its x-ray structure, the identification of the VSV G fusion peptide was challenging. Initial attempts to understand membrane fusion utilized hydrophobic photolabeling and demonstrated that VSV was able to interact with the host cell membrane in response to low pH, and that residues 59–221 of the G protein were in close proximity to the membrane during this process (21). Mutational analysis of VSV G demonstrated that modification of a highly conserved region (residues 118–
139) abolished fusion activity or modified the pH of fusion activation (22–24); in particular, the mutations G124A, P127G/L, and A133K dramatically decreased cell-cell fusion activity. Other mutations at this region, such as F125Y and D137N, shifted the optimum pH for G protein-mediated cell-cell fusion.

Overall, the region between amino acids 118 and 139 was generally considered to represent an internal fusion peptide for VSV G (17). However, other studies demonstrated that amino acids 395–418 (the membrane-proximal region) have an significant influence on fusion (25), and additional studies identified region 145–164, termed the p2-like peptide, as being a pivotal domain in facilitating glycoprotein G-mediated membrane fusion (26, 27). A synthetic p2 peptide was shown to mediate liposome-liposome fusion in a low pH- and phosphatidylserine-dependent manner (27). Further studies revealed that the action of the p2-like peptide was specifically facilitated by electrostatic interactions between phosphatidylserine and two histidine residues within this region (26, 28). Unlike for some other viral fusion proteins, notably Semliki Forest virus E1 (29), there is no apparent divergence rhabdoviruses (Fig. 1). This arrangement of hydrophobic residues exposed at the end of the loops (Trp-72, Tyr-73, Tyr-116, and Ala-117) (Fig. 1). The hydrophobic nature of this bipartite fusion loop is conserved across a wide range of divergent rhabdoviruses (Fig. 1). This arrangement of hydrophobic loops is highly reminiscent of the fusion peptide (or fusion loop) of a class II fusion protein (20). However, a significant difference for VSV G is that the hydrophobic amino acids are shared over two non-contiguous loops, whereas for class II fusion proteins the key amino acids are on a contiguous stretch of primary sequence. In support of the suggestion that this bipartite loop comprises a critical fusion domain, selection of fusion-defective mutants previously identified one of these amino acids (Ala-117 in the second loop region) as having a critical function in VSV G-mediated membrane fusion (22). This mutation was previously referred to as an A133K substitution due to different numbering of amino acids in the G protein sequence.

Based on the available x-ray structure of VSV G, here we systematically mutated the proposed bipartite fusion loop of VSV G (residues Trp-72, Tyr-73, Tyr-116, and Ala-117) and characterized how these amino acids modulate membrane fusion activity. These data represent an analysis of the molecular architecture of the fusion-active loops of VSV G, a founder member of the class III family of viral fusion proteins.

**MATERIALS AND METHODS**

**Cell Culture**—Vero E6 cells and 293T cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium (Cellgro) containing 10% fetal bovine serum, 100 units/ml penicillin, and 10 μg/ml streptomycin.

**Plasmids and Site-directed Mutagenesis**—Plasmid phCMV-VSV G encoding the vesicular stomatitis virus Indiana glycoprotein (VSV G) (NCBI accession number CAC47944) (32), was kindly provided by Dr. Jean Dubuisson (Institut Pasteur de Lille, Lille Cedex, France). Site-directed mutagenesis using phCMV-VSV G as a template was performed using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). According to the manufacturer’s protocol, pairs of complementary oligonucleotides were designed to introduce the desired mutations. Mutations were then confirmed by sequencing using an Applied Biosystems Automated 3730 DNA Analyzer at the Cornell University Life Sciences Core Laboratories Center.

**Biotinylation of Surface Protein and Immunoprecipitation**—Vero E6 cells grown on 6-well plates were transfected with 1 μg of wild-type or mutant VSV G-expressing plasmid, using Lipo-

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**FIGURE 1. Structural localization of the fusion loops of VSV G.** A model of the post-fusion trimer of VSV G (PDB accession number 2CMZ) is shown in both surface (a) and schematic form (b). The location of proposed bipartite fusion loops is colored green in a. Panel c shows an enlarged image of the fusion loops, with the side chains of the proposed fusion loops (residues Trp-72, Tyr-73, Tyr-116, and Ala-117) shown in stick representation. Images were generated with MacPyMOL software (DeLano Scientific). Panel d shows an alignment of the putative fusion loops across the *Rhabdoviridae* and is modified from Refs. 14 and 59.
Mutational Analysis of the VSV G Fusion Loops

fectamine 2000 (Invitrogen) according to the manufacturer’s instructions, for 24 h at 37 °C, or for 36 h at 32 °C. For cell-surface biotinylation, the transfected cells were washed twice with ice-cold phosphate-buffered saline (PBS), and then cells were labeled with 250 μg/ml Sulfo-NHS-SS-biotin (Pierce) for 30 min on ice. The biotin-labeled cells were then added to 150 mM ice-cold glycine solution for 20 min to quench unlabeled free biotin followed by an ice-cold PBS wash. The cells were then lysed in 500 μl of radioimmune precipitation assay buffer (100 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholic acid, pH 7.4), including complete protease inhibitor mixture (Roche Applied Science), and the cell lysates were affinity-purified using immobilized NeutrAvidin beads (Pierce) overnight at 4 °C. Finally, the NeutrAvidin beads were washed with radioimmune precipitation assay buffer followed by the addition of SDS-PAGE Laemmli sample loading buffer containing 50 mM dithiothreitol. The surface-biotinylated VSV G protein was analyzed by Western blot using the anti-VSV G monoclonal antibody P5D4 (kindly provided by Dr. Ari Helenius, ETH-Zurich), and images were obtained from LAS-3000 mini Fujifilm imaging system (Fuji Photo Film Co., Ltd). The biotinylation assay was repeated three times, and the results obtained in the Western blot were quantified using IP Lab software (Scanalytics) and plotted in Sigma Plot 9.0 (Systat Software).

Syncytium Formation Assay and Luciferase Reporter Gene Fusion Assay—Subconfluent Vero cells in 24-well plates were transfected with pcMV-VSV G plasmids encoding either wild-type or mutant G protein, using Lipofectamine 2000, for 24 h at 37 °C or 32 °C before induction of syncytium formation. To examine low pH-induced syncytia formation, the transfected cells were rinsed once with PBS and then incubated with HMSS fusion buffer (5 mM HEPES, 5 mM MES, 5 mM sodium succinate 150 mM NaCl, adjusted to the indicated pH with HCl, stored at room temperature) for 1 min. The cells were then washed with PBS and incubated with fresh culture medium for another 3 h after fusion induction. Finally, the cells were fixed with 3% paraformaldehyde, and without being permeabilized, the cells were processed for indirect immunofluorescence microscopy using the anti-VSV G ectodomain-specific monoclonal antibody I14 (clone 1E9F9) (33) (a kind gift from Dr. Ari Helenius, ETH-Zurich) and Alexa 488-labeled anti-mouse secondary antibody (Invitrogen-Molecular Probes). Using a 20× Plan Apo objective (numerical aperture, 0.75). Images were captured with a Sensicam EM camera (Cooke Corp.) using IP Lab software (Scanalytics).

For quantification of syncytium formation, the percentage of cells involved in syncytia was determined by counting the ratio of cells in syncytia containing three or more nuclei to total cells in the field. The experiments were repeated three times, and >500 cells from at least 5 different fields were counted in each experiment.

Cell-cell fusion mediated by G protein was also quantified by a luciferase reporter gene assay, with plasmids kindly provided by Dr. Thomas Gallagher (Loyola University, Chicago, IL). In brief, 293T cells in each well of a 24-well plate were co-transfected with 125 ng of plasmid encoding luciferase cDNA under control of T7 promoter and 125 ng of VSV G wild-type or mutant plasmids, using 1 μl of Exgen 500 (Fermentas, Ontario, Canada) at either 37 °C or 32 °C depending on the mutants used for transfection. 24 h post-transfection, transfected 293T cells were overlaid at a 1:3 ratio with 293T cells, which had been previously transfected with a plasmid encoding T7 polymerase. The 293T cell mixtures were cultured for 1 h to allow cells to adhere to the plate. Cell-cell fusion was induced with low pH buffer as described above. The cells were lysed 4 h post-fusion, and the supernatants were measured for luciferase activity using the Lucerfaase Assay System (Promega, Madison WI), according to the manufacturer’s instructions. Light emission was measured using a Glomax 20/20 luminometer (Promega, Madison, WI).

Production and Transduction of VSV G-pseudotyped Virions—VSV G protein-pseudotyped particles were generated from a murine leukemia virus (MLV)-based transfer vector system as described previously (34, 35) with plasmid-encoding luciferase flanked by retroviral packaging sequences and an MLV Gag-Pol construct were kindly provided by Dr. Jean Dubuisson (Institut Pasteur de Lille, Lille Cedex, France). The pCMV-VSV G wild-type or mutant plasmids, together with the plasmids encoding luciferase, murine leukemia virus Gag-Pol, were transfected into 293T cells using Exgen 500 (Merckata) according to the manufacturer’s instructions. The transfected cells were then cultured at 37 °C for 48 h or 32 °C for 72 h depending on the VSV G mutants used in the transfection. The supernatants containing pseudotyped particles were harvested 48 h post-transfection and filtered through 0.45-μm-pore-sized membranes before used for infection assay. To analyze G protein incorporation into pseudotyped particles, the viral particles were concentrated by ultracentrifugation. Briefly, 700 μl of viral supernatants was layered on the top of 300 μl of 30% sucrose cushion and subject to ultracentrifugation at 50,000 rpm for 2 h in a TLA55 rotor (Beckman), before the samples were analyzed by Western blot. The monoclonal antibodies P5D4 and R187 (American Type Culture Collection), recognizing the G protein and the MLV Gag protein, respectively, were used to detect VSV G and the retroviral Gag protein in the Western blot. Transduction of pseudoparticles was performed using Vero E6 cells. For a typical infection assay, 100 μl of supernatant containing either wild-type or mutant G protein-pseudotyped MLV particles was used to infect Vero E6 cells for 72 h. The cells were lysed 72 h post-infection, and luciferase activity in the lysates was measured using the same method as described above for the luciferase-based cell-cell fusion assay.

RESULTS

Mutagenesis of the VSV G Fusion Loops and Cell Surface Expression—To determine the functional properties underlying the molecular architecture of the VSV G fusion loops, we performed a systematic mutagenesis study of the four residues predicted to comprise the bipartite fusion loop (Trp-72, Tyr-73, Tyr-116, and Ala-117). In each case we substituted a variety of residues (e.g. Phe, Tyr, and Trp), polar uncharged residues (e.g. Asn), non-polar aliphatic residues (e.g. Val and Ala), and both positively and negatively charged residues (e.g. Arg and Asp). These mutants are shown in Table 1, along with a qualitative
measure of their surface expression, using immunofluorescence microscopy of the G protein in non-permeabilized Vero cells. Mutants were qualitatively scored as being surface-expressed at medium or high levels (at least half of the level of expression of wild type), or at low or very low levels (less than half of the level of expression of wild type) (see Table 1). At 37 °C, many mutants showed low, or very low, levels of surface expression. However, in these cases surface expression could often be rescued by lowering the temperature to 32 °C. All mutants showed high expression at both 37 °C and 32 °C when total G protein was detected by immunofluorescence microscopy of permeabilized Vero cells (data not shown). Mutants with low or very low surface expression had extensive localization to an intracellular compartment (data not shown). Only those mutants that showed a qualitatively medium or high level of cell surface expression at 32 °C were considered suitable for subsequent fusion and entry assays. These mutants were tested further by quantitative assays, using a combination of cell surface biotinylation and streptavidin pull down of total cell surface biotinylated G protein. All of those mutants showed a high level or wild type at 32 °C and 3 °C when total G protein was detected by immunofluorescence microscopy of permeabilized Vero cells (data not shown). Mutants with low or very low surface expression had extensive localization to an intracellular compartment (data not shown). Only those mutants that showed a qualitatively medium or high level of cell surface expression at 32 °C were considered suitable for subsequent fusion and entry assays. These mutants were tested further by quantitative assays, using a combination of cell surface biotinylation and streptavidin pull down of total cell surface biotinylated G protein. All of those mutants showed a high level or wild type at 32 °C and 3 °C when total G protein was detected by immunofluorescence microscopy of permeabilized Vero cells (data not shown). The surface expression of wild type was approximately 25–50% of wild type; +++, approximately 50–75% of wild type; +++++, approximately 75–100% of wild type. Wild type = ++++++

**TABLE 1**

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* NT, not tested.

In contrast, substitutions of Tyr-73 and Tyr-116 to Trp or Phe did not dramatically affect cell-cell fusion at pH 5.7, however much more limited fusion than wild-type was apparent at pH 6.1. Substitutions of Tyr-73 and Tyr-116 to Val or Ala completely abolished fusion at both pH 6.1 and 5.7. Substitutions to other residues had a marked effect on membrane fusion (Fig. 4A). At pH 5.7, only substitution of Trp-72 with phenylalanine (W72F) resulted in fusion activity (27% of the wild-type fusion level). In this case, fusion was rescued by lowering the pH, with fusion at pH 5.1 being close to the wild-type level. The substitution of tyrosine for tyrosine (Tyr-72Y) also gave a marked pH shift in fusion activity, however in this case fusion at pH 5.1 was only 54% of the wild-type level. For the other mutants tested (W72V and W72A), membrane fusion was at background levels for all pH values tested. Mutation of tyrosine 73 also markedly affected membrane fusion (Fig. 4B). Both phenylalanine and tryptophan substitutions gave a pH-shifted fusion phenotype; at pH 5.1,
FIGURE 2. Syncytium formation in cells expressing wild-type or mutant VSV G protein. Vero E6 cells were transfected with plasmids encoding either wild-type or mutant G protein as for Fig. 2. The cells were then treated with fusion buffer at pH 5.7, 6.1, or 6.6 for 1 min at room temperature and were reincubated with fresh culture medium following PBS washing. 3 h post-fusion induction, the cells were fixed and, without permeabilization, were processed for immunofluorescence microscopy using anti-VSV G monoclonal antibody I 14 (shown in green). Syncytium formation mediated by wild-type or mutated G protein at position Trp-72 (A), Tyr-73 (B), Tyr-116 (C), and Ala-117 (D) is shown. Cell nuclei were counterstained with Hoechst 33258 and colored blue.
Y73F and Y73W allowed efficient membrane fusion, although fusion activity at pH 5.7 was only 31 and 29%, respectively, of the level of the wild type. For the other mutants tested (Y73V and Y73A), membrane fusion was at background levels for all pH values tested. Although in this case we cannot exclude the possibility that the lack of fusion was due to limited cell surface expression (see supplemental Fig. S1 and Table 1). Mutation of tyrosine 116 gave essentially similar results to Tyr-73 (Fig. 4C). The Y116V and Y116A mutations resulted in background fusion, however substitution with aromatic residues was generally better tolerated; the Y116F mutation behaved essentially as wild-type, and the Y116F mutation had 66% of the fusion activity of wild type at pH 5.7. Interestingly, fusion of the Y116F mutant could not be rescued to wild-type levels by lowering the pH; as such, Y116F does not have a pH-shifted phenotype. Alanine 117 could tolerate a wider range of amino acid substitution in terms of fusion activity, although fusion was usually substantially below wild-type levels, and with a low pH-shifted phenotype (Fig. 4D). Alanine 117 could be replaced with a polar uncharged residue (A117N) without major effect, or with an acidic residue (A117D) to give very limited fusion at lowered pH. Surprisingly, the A117F mutation showed quite limited fusion ability in cell-cell fusion assays, across a range of pH values. Ala-117 could not be replaced with a basic residue (Lys, Arg, or His); these substitutions were unable to activate membrane fusion at any pH value tested.

**Analysis of Fusion Activity in VSV G-expressing Viral Particles**—Although a cell-cell fusion assay is a simple and direct way to evaluate protein fusogenic activity, it does not necessarily reflect viral fusion in vivo, where the fusion protein is assembled into the virus envelope. To better understand VSV G-mediated fusion in vivo and assess the biological effects of mutations in the fusion loops, we co-transfected 293T cells with a MLV Gag-Pol and luciferase plasmid, together with VSV G protein, to generate VSV G-pseudotyped viral particles. We examined wild-type G, in comparison to those mutants previously shown to exhibit efficient cell surface expression (see supplemental Fig. S1 and Table 1), first confirming the assembly of the wild-type and mutant VSV G into viral particles at either 37 °C or 32 °C. As shown in supplemental Fig. S2, both the G protein and the MLV-gag protein could be readily detected in the released pseudovirion particles. Consistent with our results from cell surface expression experiments, some of the mutants tested incorporated lower levels of the G protein, but never
Mutational Analysis of the VSV G Fusion Loops

lower than 40% of the wild-type. Pseudotyped virions were then used to infect Vero cells for 72 h, and luciferase activity was measured in cell lysates as an indicator of viral infectivity. To ensure that we had comparable amounts of virions for infection for wild-type and the mutants, we adjusted the volume of supernatant used to infect cells to give approximately equivalent levels of viral particles in each assay. The luciferase activity of wild-type was normalized to 100%, and the infectivity of viral particles containing mutant VSV G was compared with wild type (Fig. 5, A–D). As with cell-cell fusion assays, we found that Trp-72 was a critical residue; mutation to phenylalanine (W72F) resulted in only 12% of the wild-type infection, and all other mutants tested (W72V, W72A, and Trp-72Y) were below the level of detection. Mutation of the tyrosine residues in the VSV G fusion loops also markedly affected infection, although in this case substitution of other aromatic residues was well tolerated. Infection of mutants Y73F, Y73W, Y73V, and Y73A was not significantly different from wild type. In contrast, substitution of tyrosine for residues with nonpolar, aliphatic R groups (Y73V, W73A, Y116V, and Y116A) could not be tolerated. Mutations at position 117 were more readily accepted. In this case, the alanine residue could be replaced with either an aromatic or uncharged group (A117F and A117N); such substitutions seem to be better tolerated in the context of a virus particle, than in cell-cell fusion assays (see Fig. 4). Alanine 117 could not be replaced with an amino acid having a positively charged R group (Lys, Arg, or His), whereas substitution with a negatively charged residue (A117D) resulted in residual infectivity.

DISCUSSION

Using quantitative membrane fusion and virus entry assays, we show here that the bipartite fusion loops predicted from the crystal structure of VSV G play a fundamental role in membrane fusion mediated by the protein. In particular, we show a critical role of the single tryptophan in loop I (Trp-72), the most proximal residue of the fusion loops. Substitution of Trp-72 with any other residue had a profound effect on membrane fusion. The only substituted residue in this position that allowed some degree of fusion activity during virus entry was
fusion form. gB contains an analogous fusion domain to VSV domain IV (domain I for gB) that comprises an extended β-hairpin with hydrophobic tips. However, VSV G and HSV-1 gB differ in the detailed architecture of the hydrophobic tips; whereas the single tryptophan (Trp-72) is the most distal residue of the VSV G fusion loops (and shown here to be the most critical residue for fusion), the single tryptophan residue (Trp-174) in the fusion loops of HSV-1 gB (comprising residues 173VWFGHRY179–258VEAFHRY265) projects more to the side of fusion loop 1, with Phe-262 being the more distal hydrophobic residue. In contrast, VSV G appears to present a more optimal fusion loop with a single distal tryptophan, in addition to two tyrosine residues. The coalescence of these polar aromatic residues in the phenylalanine, although tyrosine allowed residual activity in cell-cell fusion assays, especially at lower pH values. We also show important roles for the two tyrosine residues in the fusion loops (Tyr-73 and Tyr-116). In both these cases, although the tyrosine residues could be substituted with either tryptophan or phenylalanine without major effects, other substitutions could not be tolerated. The fourth amino acid comprising the fusion loops (Ala-117) was less critical. Ala-117 could be substituted with a variety of amino acids, but not with positively charged residues. The important role of tryptophan and tyrosine in the VSV G fusion loops is completely consistent with the known preference of these amino acids for the interfacial lipid head groups: the planar indole ring of tryptophan is thought to favor interfacial localization due to its compatibility with the liquid-crystalline order of the lipid bilayer, which varies depending on the depth within the bilayer (40, 41), with its interfacial location due to the balance of several factors: a hydrophobic effect that would drive it out of the aqueous solvent, complex electrostatic interactions that favor its location within the hydrated head group region, and repulsive forces that keep it out of the hydrocarbon core (42).

When the structure of VSV G was first solved, it was observed that the glycoprotein B (gB) of herpes simplex virus 1 (HSV-1) showed significant structural homology (14, 43, 44). In the case of gB, the structure is believed to be that of the post-fusion form, however it could also represent a pre-fusion-active form of a VSV G trimer would be predicted to facilitate an interfacial interaction between the fatty acid chains and polar head groups of the lipid to initiate membrane destabilization during the fusion reaction.

The fusion domain of VSV G shows a high degree of similarity to the fusion domain of class II fusion proteins (14), with the notable difference that the fusion loops of VSV G are bipartite and comprise two separate loops, whereas for class II fusion proteins the fusion loops are continuous and comprise a more conventional “fusion peptide” (13, 17, 45). In general, the fusion loops of class II fusion proteins have not been the subject of intensive mutagenesis studies. The Semliki Forest virus fusion loop has undergone the most extensive mutagenesis, with a variety of phenotypes observed following mutation of the hydrophobic residues in the fusion loop (comprising residues DYQCKVYTGVYPFMWGGAYC) (46), however mutation of the single tryptophan was not performed. For the tick-borne encephalitis virus envelope protein, leucine 109 was shown to be a critical component of the fusion loop (20), although other bulky hydrophobic residues (e.g. the single tryptophan, Trp-101) were not mutated in this study due to predicted problems in protein transport or assembly. In the case of LaCrosse virus, an alanine substitution of the single tryptophan (Trp-1066) in the predicted fusion loop of the glycoprotein (Gc) did not affect cell surface expression yet completely abrogated fusion (47). Substitutions other than alanine were not tested in this study.

In terms of the optimal number of tryptophan residues that might constitute a fusion domain, the addition of a second tryp-
Mutational Analysis of the VSV G Fusion Loops

tophan to the VSV G fusion loops (loop II, Y116W), resulted in no significant change in fusion activity. A quite different result was found with HSV-1 gB, where the presence of tryptophans in both fusion loops resulted in a large decrease in fusion activity (48). Both VSV (Rhabdoviridae) and HSV-1 (Herpesviridae) belong to an extended family of viruses with equivalent fusion domains. In the case of the herpesviruses, the Epstein-Barr virus and human herpesvirus 6, 7, and 8 gB homologs all appear to have more exposed tryptophan residues (49), with in one case (Epstein-Barr virus) three tryptophan residues present (50). Animal rhabdoviruses in general have a single tryptophan residue in their fusion loops. This can be in loop I, as is the case for VSV, or in loop II, e.g. with rabies virus (see Fig. 1). Exceptions to this include Flanders virus of birds and bovine ephemeral fever virus, which have a tryptophan residue in both fusion loops. However, for both Flanders virus of birds and bovine ephemeral fever virus the second amino acid in fusion loop II is polar, unlike other rhabdoviruses where the residue at this position is non-polar or aromatic. Overall, it seems, that increasing the number of tryptophan residues offers little or no obvious advantage to the virus in terms of its infectivity in vivo, or fusion activity in vitro. Interestingly, the importance of tryptophan residues in the membrane-interacting loops structures also seems to be shared with certain bacterial toxins, e.g. perfringolysin O. In the case of perfringolysin O a more complex loop structure containing six tryptophan residues is present in domain 4 of the protein (51). Domain 4 of perfringolysin O confers the initial interaction of the soluble toxin molecule with cholesterol-containing membranes, prior to conformational changes in the toxin structure and the formation of an oligomeric β-barrel-lined pore in the membrane (52). Of these six tryptophan residues Trp-436, Trp-438, and Trp-439 appear to be important for membrane insertion, with Trp-438 being most critical (53, 54). Thus the interfacial interaction of one or more tryptophan residues with the membrane appears to be a common property of membrane-interacting loops from quite evolutionarily divergent proteins.

One notable feature of VSV G is that in its pre-fusion state the fusion loops are set wide apart in a tripod arrangement, with the tips pointing toward the viral membrane (15). In contrast, the fusion loops of class II fusion proteins and herpesvirus gB are buried at an oligomeric interface. Interestingly, mutation of the hydrophobic residues of the gB fusion loop did not cause any generalized problem in protein trafficking to the cell surface (48, 49). In contrast, we found that even conservative changes in the hydrophobic residues of the VSV G fusion loops caused significant problems in protein transport to the cell surface. To date, we have not carried out an analysis of the transport defect(s) of the mutant proteins. In the structure of the pre-fusion form of VSV G, the membrane proximal region is missing, and so the positioning of the fusion loops relative to the viral membrane cannot be determined. To account for the transport defect in the fusion loop mutants, we consider one possibility, that the hydrophobic tips of the fusion loops may be inserted into the membrane during G protein transport to the cell surface, thus stabilizing the oligomeric state of the protein and facilitating transport. Upon fusion activation the fusion loops would be withdrawn from the viral membrane and, following low pH-induced conformational changes, inserted into the host cell membrane. Such a model is consistent with the ability of the VSV fusion domain to reversibly interact with membranes (55). In this regard, VSV G may show substantial difference to both herpesvirus gB and the class II fusion proteins.

VSV entry into host cells is known to occur from acidic endosomes, and some early reports showed a pH maximum for VSV fusion at approximately pH 6.1–6.2 (31, 56), leading to the suggestion that fusion in vivo occurred from early endosomes. However, it has now become established that VSV G-mediated fusion has a relatively broad pH optimum below pH 6.0 (57), with fusion occurring from endosomal carrier vesicles that traffic to the late endosome (2, 58). The luciferase-based VSV G cell-cell fusion assay employed here indicates that the minimum pH at which fusion becomes maximal is 5.7–5.8. We did not observe a peak in the pH profile of fusion as reported by others (22); in other words fusion remains fully active between pH 5.7 and 5.1. In this report, we used three distinct assays of VSV-mediated membrane fusion (visual scoring of syncytia, a luciferase reporter gene, and pseudovirions), and in general the mutants behaved in a very similar manner between these assays. Principal exceptions to this were the W72F, Y73W, A117D, and A117F mutants. W72F showed some degree of fusion in cell-cell fusion assays but with very limited activity in virus infection assays. A117D was able to mediate fusion in cell-cell fusion assays but required very low pH. Y73W and A117F both consistently gave near normal fusion in virus entry assays yet were significantly compromised in cell-cell fusion assays at pH 5.7. These discrepancies may be explained by possible differences in the pH of fusion in the endosome of cells, different curvature or lipid composition of the membrane, or density of glycoprotein in the membrane. As such, the W72F, Y73W, A117D, and A117F mutants may be particularly illuminating in future studies examining VSV G-mediated fusion.

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Mutational Analysis of the VSV G Fusion Loops
