Characterization of the Putative Fusogenic Domain in Vesicular Stomatitis Virus Glycoprotein G

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The envelope glycoprotein G of vesicular stomatitis virus induces membrane fusion at low pH. Site-directed mutagenesis of specific amino acids within a segment spanning amino acids 123 to 137 of G protein, which is highly conserved in vesiculoviruses and was previously shown by us to be involved in fusogenic activity (Y. Li, C. Drone, E. Sat, and H. P. Ghosh, *J. Virol.* 67:4070–4077, 1993), was used to determine the role of this region in low-pH-induced membrane fusion. The mutant glycoproteins expressed in COS cells were assayed for acid-pH-induced cell-cell fusion. Substitution of the variant Pro-123 with Leu had no effect on the fusogenic activity, while substitution of conserved Phe-125 and Asp-137 with Tyr and Asn, respectively, shifted the pH optimum of membrane fusion to a more acidic pH value and decreased the fusion efficiency. The deletion of amino acid residues 124 to 127, 131 to 137, or 124 to 137 produced mutants defective in transport. Mutation of the conserved residues Gly-124 and Pro-127 to Ala and to Gly or Leu, respectively, inhibited cell-cell fusion activity by about 90% without affecting transport of the mutant proteins to the cell surface, suggesting that these two residues may be present within the fusion peptide and thus may be directly involved in fusion. This highly conserved domain containing neutral amino acids of G protein may therefore represent the putative fusion domain of vesicular stomatitis virus G protein.

The entry of enveloped viruses involves fusion of the viral envelope and cell membrane induced by the viral spike glycoprotein. Virus-cell membrane fusion occurs either at neutral pH or under conditions of acidic pH (27, 31, 32, 37, 38, 40). In the case of viruses requiring neutral pH, virus-cell fusion occurs at the plasma membrane, while viruses that require a low-pH trigger for fusion are endocytosed and in this case virus-cell fusion occurs in the acidic compartments of endosomes (1, 31, 32, 37, 38). Cell-cell membrane fusion also takes place when spike glycoprotein genes are expressed in the absence of other viral gene products (9–11, 18, 29, 39). In addition to the hydrophobic signal peptide and the membrane anchoring domain, viral envelope glycoproteins are believed to contain a hydrophobic peptide segment which is involved in membrane fusion (32, 37, 38). Viruses such as influenza virus, Newcastle disease virus, and human immunodeficiency virus contain this fusion peptide at the NH₂ terminus, while viruses such as Semliki Forest virus (SFV) and Rous sarcoma virus contain the fusion peptide in internal regions of the glycoprotein (32, 37, 38). The fusion domains of these viral glycoproteins were identified and characterized by mutational analyses of the cloned spike protein genes (4, 10, 11, 13–16, 18, 24, 25, 37, 38, 41, 43).

Vesicular stomatitis virus (VSV) has been studied extensively as a prototype of the negative-stranded rhabdovirus family (23). The single transmembrane glycoprotein G is involved in virus entry and cell fusion. VSV G protein can induce virus-cell or cell-cell fusion under conditions of acidic pH (3, 9, 28, 29, 40). A reversible conformational change induced in the acidic condition is believed to activate the fusion domain (3, 28). However, the fusion domain has not yet been definitely identified. Examination of the primary sequence of

G protein of VSV Indiana (30), New Jersey (12), Chandipura (26), or Cocal (2) did not show the presence of any obvious hydrophobic sequence other than the signal sequence and the carboxy-terminal membrane anchoring domain. A glycosylation mutant of G protein containing an extra oligosaccharide chain in the proximity of a sequence of uncharged amino acids was defective in low-pH fusogenic activity of wild-type G protein, suggesting that this sequence of uncharged amino acids may be required for fusogenic activity (41). To characterize the domain(s) of G protein that is involved in acid-induced membrane fusion as well as to test the requirement for a hydrophobic sequence in the fusogenic domain, we studied the fusogenic activities of a number of mutants of G protein constructed by introducing two or three amino acids in the ectodomain of G protein by using linker-insertion mutagenesis (25). The results show that of 14 mutants isolated, only 4 (H2, H5, H10, and A4) were fusion defective. The mutation in one of these mutants, H2, is within a region of conserved uncharged amino acids that had been proposed as a possible fusogenic domain (27, 41).

To determine whether the sequence adjacent to the site of the insertion mutation of mutant H2 was involved in fusogenic activity, we used site-directed mutagenesis to change specific amino acids within this conserved region. The mutant G proteins were expressed in COS cell and assayed for membrane fusion. The results show that mutagenesis of specific amino acids within this conserved uncharged sequence can either block the fusion activity or shift the pH optimum for fusion to a more acidic value, which suggests that this region (amino acids 123 to 137) may represent a putative internal fusion peptide of VSV G.

MATERIALS AND METHODS

Construction of mutant G proteins. The gene encoding G protein of VSV Indiana serotype was cloned into the eukaryotic expression vector pXM (44) to produce the plasmid pXM-G as described earlier (25). A unique *Apa*I site was

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created in the G gene by substitution of the bases A-299 and G-302 with G and C, respectively, by using the primer 5'-TCCGCTGGTATGGGCCCAAGTATATAACA-3'. Mutagenesis was done as described previously (28a) by the procedure of Kunkel et al. (21). The 737-bp fragment generated by digesting a modified G clone containing an *Apa*I site [pG(+A)] with restriction enzymes *Apa*I and *Kpn*I was cloned into M13 mp18. The oligomers used for mutagenesis were GGCTGAATCTAGGCTTCCCT, TGAATCCAGCCTTCCCTCCT, ATCCAGGCTACCTCCTCA, TCCAGGCTTCCCTGGTC AAAGTTGTGGAT, CTCCCTCTTCAAAGTT, CTGTGACGAATGCCGAAGCA, CTTGGCTGAATCCAGCCGAA GCAGTGA, CTTGGCTGAATCCACAAAGTTGTGGATA, and CTCCTCAAAGTTGTGCCGAAGCAGTGAT for constructing mutants P123→L (carrying the P-123→L mutation), G124→A, F125→Y, P127→G, P127→L, D137→N, ΔA, ΔB, and ΔC, respectively. The deletion mutants ΔA, ΔB, and ΔC lacked residues G-124 to D-137, G-124 to P-127, and G-131 to D-137, respectively. The mutagenized fragments were screened by nucleotide sequence analysis and ligated to the 6-kb fragment obtained from *Apa*I and *Kpn*I digestion of pXM-G(+A) (pXM-G containing the *Apa*I site as described above), regenerating pXM-G harboring the desired mutation in the G gene.

Transfection, labeling, and immunoprecipitation. Subconfluent monolayers of COS-1 cells were transfected by the $\text{Ca}_3(\text{PO}_4)_2$ method as described earlier (25, 28a). The transfected cells were labeled with [^{35}S]methionine at 24 h post-transfection and processed for immunoprecipitation and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described before (25).

Cell-cell fusion assay. The fusogenic activities of the expressed wild-type and mutant G proteins were determined by exposing cells to pH 4.8 to 6.4 for a very short time and observing polykaryon formation as described previously (25). Polykaryons containing more than five nuclei were counted.

Localization and intracellular transport. The cell surface localization of the expressed glycoproteins was examined by indirect immunofluorescence of transfected cells fixed with paraformaldehyde and reacted successively with rabbit anti-G antibody and goat anti-rabbit immunoglobulin G conjugated to fluorescein as described previously (25). For quantitation of G protein expressed in the cell surface, labeling using lactoperoxidase-catalyzed iodination was carried out as described earlier (25). Transport of the mutant G proteins to the Golgi apparatus was determined by acquisition of endoglycosidase H (endo H) resistance by the procedure described previously (19, 25).

Folding and oligomerization. Proper folding of expressed proteins was determined by the recognition of mutant proteins by the conformation-specific monoclonal antibody I14 as described previously (8, 22, 34). The oligomeric state of the expressed G protein was determined by sucrose gradient centrifugation as described before (7, 8, 25).

RESULTS

Mutagenesis of the conserved region adjacent to mutant H2. Previously we have shown that insertion of a tripeptide between Asn-122 and Pro-123 of G protein of VSV Indiana produced a mutant, H2, which was blocked in low-pH-induced cell-cell fusion (25). The mutant H2, however, was transported to the cell surface, oligomerized properly, and was recognized by conformation-specific antibodies (25). This block in fusogenic activity of G protein suggests that the insertion could be occurring within a sequence directly involved in fusion or very

¹²³ Pro	Gly	Phe	Pro	Pro	Gln	Ser	Cys	Gly	Tyr	Ala	Thr	Val	Thr	Asp ¹³⁷
CCA	GGC	TTC	CCT	CCT	CAA	AGT	TGT	GGA	TAT	GCA	ACT	GTA	ACG	GAT
↓	↓	↓		↓										↓
Leu	Ala	Tyr		Gly										Asn
CtA	GcC	TaC		ggT										aAT
				Leu										
				CtT										

FIG. 1. Mutations in the H2 region of VSV G glycoprotein. The amino acid and corresponding nucleotide sequences of the peptide encompassing residues 123 to 137 of G protein of VSV Indiana serotype are shown. Conserved amino acids are underlined. The amino acids under the arrows are the replacement amino acids, and the oligonucleotide-directed base changes are shown in lowercase letters. The sequences of G proteins of VSV Indiana, New Jersey, Chandipura, Cocal, and Piry are given in references 30, 12, 26, 2, and 27a, respectively.

close to the fusion peptide. Since the sequences of fusion peptides of viral glycoproteins show high degrees of identity within the same subgroup of virus, we examined the sequences of G proteins of other vesiculoviruses. A comparison of the sequences of G proteins of VSV Indiana (30), New Jersey (12), Chandipura (26), Cocal (4), and Piry (27a) showed a conserved region between amino acids 123 and 137, containing 15 uncharged amino acids (Fig. 1). Of these 15 residues, 12 were identical in the G proteins of five vesiculoviruses. To determine whether this conserved region may be the fusion peptide of VSV G protein, six amino acid substitution mutants were constructed by site-directed mutation of the G gene of VSV Indiana (Fig. 1). In addition, three deletion mutants, ΔA, ΔB, and ΔC, encompassing residues 124 to 137, 124 to 127, and 131 to 137, respectively, were also constructed.

Synthesis and glycosylation of mutant G proteins. The mutant G genes were expressed in COS cells, and proteins labeled with [^{35}S]methionine were analyzed by immunoprecipitation with a polyclonal anti-G antibody followed by SDS-PAGE. Wild-type G protein contains two N glycosylation sites (30), which were also retained in the mutant G proteins. To test whether the two sites were glycosylated in the mutant G proteins, we expressed the proteins in the absence and in the presence of the N-linked glycosylation inhibitor tunicamycin (19) and analyzed them by SDS-PAGE. The results presented in Fig. 2 show that all six mutant G constructs express proteins which comigrate with wild-type G protein. The intensities of the bands corresponding to wild-type and mutant proteins were similar, suggesting that the substitution of amino acids did not affect the expression or stability of the protein. In the presence of tunicamycin, all of the expressed mutant proteins showed a decreased molecular weight corresponding to that of the nonglycosylated wild-type G protein (Fig. 2A). The three deletion mutants, ΔA, ΔB, and ΔC, also expressed proteins that were recognized by anti-G antibody and showed expected changes in molecular weights compared with the wild-type G protein (Fig. 2B). However, the amounts of mutant proteins expressed were reduced, possibly because of decreased stability of the mutants.

Cell-cell fusion activities of mutant G proteins. The mutant G proteins were next analyzed for low-pH-dependent fusion activity in a cell-cell fusion assay. COS cells transfected with wild-type and mutant G genes were treated at 37°C for 1 min with the fusion medium at pH 5.6, and polykaryons formed were fixed and viewed under a phase-contrast microscope. Cells transfected with wild-type G showed extensive polykaryon formation, and the polykaryons contained 20 to 50 nuclei (Fig. 3). The mutant P123→L also induced extensive fusion at pH

FIG. 2. Expression of the mutant G genes and glycosylation of mutant proteins. (A) COS cells were transfected and labeled with [^{35} S]methionine in the absence (–) and in the presence (+) of 2 μ g of tunicamycin per ml; the cell lysates were immunoprecipitated with anti-G antibody and analyzed by SDS-PAGE. WT, wild type. (B) The deletion mutants Δ A, Δ B, and Δ C, lacking amino acids 123 to 137, 123 to 127, and 131 to 137, respectively, were labeled in the absence of tunicamycin and analyzed as for panel A. Numbers at left are molecular weights (k, thousands).

5.6. The mutants F125 $\rightarrow$ Y and D137 $\rightarrow$ N could induce fusion at pH 5.6, but the extent of fusion was considerably less than that of the wild type. Also, there were fewer nuclei in the polykaryons produced by these two mutants than in those produced by wild-type G protein. In contrast, the three mutants G124 $\rightarrow$ A, P127 $\rightarrow$ G, and P127 $\rightarrow$ L showed very little fusion activity. Repeating the low-pH treatment for additional time periods did not increase the fusogenic activity.

Previous studies have shown that the pH optimum for membrane fusion by viral glycoproteins can be shifted by mutation within the fusion peptide (13, 24, 42). The pH dependence of each of the six mutant G proteins defective in fusion at pH 5.6 was therefore examined over the pH range of 4.8 to 6.4. The results presented in Fig. 4 show that, similar to the case with wild-type G protein, the mutant P123 $\rightarrow$ L had a broad pH range of fusion, with maximum fusogenic activity observed at pH 5.6. The mutants F125 $\rightarrow$ Y and D137 $\rightarrow$ N showed maximum fusogenic activities at pH 5.2 and 5, respectively, with efficiencies of about 30 and 50%, respectively, of the wild-type fusogenic activity. The mutant P127 $\rightarrow$ G also showed a shift in the pH optimum of cell fusion to pH 5.1; however, the fusogenic activity was about 20% of that observed in the case of wild-type G. The two mutants G124 $\rightarrow$ A and P127 $\rightarrow$ L had little fusogenic activity in the pH range of 4.9 to

FIG. 3. Polykaryon formation by mutant G proteins. COS cells transfected with wild-type (WT) and mutant G plasmids were exposed to fusion medium at pH 5.6 for 60 s, incubated in regular medium for 2.5 h, fixed, stained, and photographed.

FIG. 4. pH dependence of cell fusion induced by expression of mutant G proteins. COS cells transfected with wild-type (WT) or mutant G plasmids were exposed to fusion medium of varying acidity in the pH range 4.7 to 6.3 for 60 s. The cells were processed as described in the legend to Fig. 3. The total number of polykaryons containing more than five nuclei was counted in 16 random fields. Fusion activity was calculated by using the number of polykaryons produced by wild-type G protein at pH 5.6 as 100%. The graph represents data obtained from two separate sets of experiments. COS cells were transfected with plasmids encoding wild-type G (●) and mutants P123→L (○), D137→N (◇), F125→Y (□), P127→G (×), G124→A (△), and P127→L (—).

5.2 and had no fusion activity at pH 5.6 (Fig. 4). The average polykaryon sizes were 20 to 50 nuclei for wild-type G protein and the mutant P123→L and 20 to 30 nuclei for both mutants F125→Y and D137→N. In contrast, the polykaryons produced by mutants G124→A, P127→G, and P127→L were much smaller in size and contained 5 to 10 nuclei. The three deletion mutants ΔA , ΔB , and ΔC failed to show any polykaryon formation (data not shown).

Taken together, these results show that mutations of the four conserved amino acids G-124, F-125, P-127, and D-137 affected the fusogenic activity of the mutant G proteins by shifting the pH optimum for cell fusion to more acidic pHs as well as by reducing the efficiency of fusion. The substitution of the nonvariant G-124 with a conservative amino acid, alanine, inhibited the extent of fusion by 90%. The replacement of P-127 with leucine also caused a similar blocking of cell fusion even at the low pH of 4.8. Substitution of F-125 and D-137 with tyrosine and asparagine, respectively, caused both inhibition of fusion activity and a shift of the pH optima for fusion to more acidic values. Replacement of the nonconserved P-123 with leucine did not affect the fusogenic activity. These results, therefore, suggest that this region of uncharged amino acids, which is conserved in five of the vesiculovirus G proteins sequenced, may be the putative internal fusogenic domain.

Intracellular localization and transport of mutant G proteins. The observed defect in cell-cell fusion activities of the mutant G proteins could be due to defects in intracellular transport and localization in the cell surface. Indirect immunofluorescence was therefore used to examine the cell surface localization of the mutant proteins. Six mutants, P123→L, G124→A, F125→Y, P127→G, P127→L, and D137→N, showed strong staining at the cell surface (Fig. 5). The three deletion mutants ΔA , ΔB , and ΔC failed to show any surface immunofluorescence and thus were defective in transport. The

FIG. 5. Cell surface localization of mutant G proteins by immunofluorescence. Transfected COS cells were fixed with paraformaldehyde and reacted with rabbit anti-G antiserum and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G. Like mutant ΔA , mutants ΔB and ΔC did not show any surface labeling.

distribution of the glycoproteins was determined more quantitatively by using cell surface iodination of proteins catalyzed by lactoperoxidase. As shown in Fig. 6A and B, the mutant glycoproteins showed strong surface labeling with iodine; however, no surface iodination was observed with the mutant ΔA (Fig. 6C).

The relative amounts of mutant and wild-type G proteins on the cell surface were quantitated by densitometric scanning of the fluorogram exposed for a time period chosen to produce band intensities within the linear range of the densitometer. The relative amounts of the mutants P123→L, G124→A, F125→Y, P127→G, P127→L, and D137→N present on the cell surface were 110, 115, 91, 166, 110, and 126%, respectively.

The transport of the mutant proteins from the endoplasmic reticulum to the Golgi complex was evaluated by the acquisition of endo H resistance (19). The results presented in Fig. 7 show that following a 15-min pulse, all six mutant proteins were completely digested with endo H, indicating that they were glycosylated with N-linked oligosaccharides. After 1 h of

FIG. 6. Localization of the mutant G proteins on the cell surface by iodination. COS cells were transfected with wild-type (WT) or mutant G constructs and radioiodinated with ^{125}I in a lactoperoxidase-catalyzed reaction. Cell lysates were immunoprecipitated and analyzed by SDS-PAGE. Panels A, B, and C are from three separate experiments.

FIG. 7. Acquisition of endo H resistance by wild-type and mutant G proteins. COS cells transfected with wild-type (WT) or mutant constructs of G protein (residues 357 to 367) (22, 34), were labeled with [^{35} S]methionine for 15 min, chased with an excess of nonradioactive methionine for 0 min (A) or 60 min (B), and immunoprecipitated with anti-G antibody. One half of the sample was treated with endo H (+), and the other half was not (-). Samples were analyzed by SDS-PAGE. Numbers at left are molecular weights (k, thousands).

chase, all six mutant proteins became completely resistant to endo H digestion, indicating that they were transported from the endoplasmic reticulum to the medial Golgi complex.

Folding and oligomerization of mutant G proteins. Conformation-specific monoclonal antibody I14, which reacts with the B2 epitope of VSV G protein (residues 357 to 367) (22, 34), has been extensively used to distinguish between correctly and incorrectly folded mutants of G protein (6, 8). As shown in Fig. 8, all six mutants of G protein studied were immunoprecipitated with monoclonal I14 anti-G antibody, indicating that the B2 epitope was properly expressed. The decreased amounts of the immunoprecipitates observed in the cases of mutants F125→Y and D137→N were due to reduced amounts of proteins present in the extracts.

The fusion proteins HA1 of influenza virus and E1 of SFV were shown to require a homotrimeric structure for low-pH-induced membrane fusion (35). G protein also forms a trimer which is essential for its intracellular transport (7, 8, 20). We therefore determined the oligomeric structure of mutant G protein by sucrose density gradient centrifugation at acidic pH (8, 25). The results presented in Fig. 9 show that all of the mutants formed trimers similar to that formed by the wild-type G proteins, suggesting that the observed alterations in fusogenic properties were not due to incorrect oligomerization.

A summary of the results obtained in this study is shown in Table 1.

DISCUSSION

In this communication we have identified a sequence of conserved amino acids (residues 123 to 137) of the G protein

FIG. 8. Recognition of mutant G proteins by conformation-specific monoclonal antibody I14. [^{35} S]methionine-labeled cell extracts were made from COS cells transfected with plasmids containing wild-type (WT) or mutant G protein constructs. The samples were immunoprecipitated with monoclonal antibody I14. Numbers at left are molecular weights (k, thousands).

of VSV as a putative fusogenic peptide involved in low-pH-induced membrane fusion. This conclusion is based on the results of mutagenesis of specific amino acids present within this region, which shows that replacement of G-124 and P-127, which are also conserved, with alanine and with glycine or leucine, respectively, resulted in almost complete abolition of fusion activity without affecting the transport of G proteins to the cell surface. Substitution of two conserved residues, namely, F-125 and D-137, with tyrosine and asparagine, respectively, shifted the pH optimum for fusion to a more acidic value and caused decreased fusogenic activity. These two criteria, inhibition of cell fusion and shift of the pH optimum of fusion as a result of mutagenesis, have previously been used to establish the fusion peptides of a number of viral glycoproteins, including HA2 of influenza virus (13), gp41 of human immunodeficiency virus (4, 10, 11), F protein of simian virus 5 (15), and E1 of SFV (18, 24). Results of earlier mutagenesis experiments which inserted the tripeptide Leu-Glu-Asn between N-122 and P-123 showed a complete block of the fusogenic activity, suggesting that the mutation was either within or in the immediate proximity of the fusion peptide (25). The introduction of an additional oligosaccharide moiety at residue 117 as a result of mutagenesis of Q-117 to asparagine also abolished fusion activity, suggesting that this region may be involved in membrane fusion (41). Ohnishi (27) had earlier proposed this region of G protein as a potential fusogenic domain on the basis of the high conservation of sequence in the Indiana (30) and New Jersey (12) serotypes. A comparison of the amino acid sequences in this region of the G proteins of three other vesiculoviruses, namely, Chandipura (26), Cocal (2), and Piry (27a), has further shown a very high degree of sequence conservation, suggesting an important biological function(s).

Our current understanding of the mechanism of protein-mediated membrane fusion is based primarily on the studies of the fusogenic activity of influenza virus hemagglutinin (HA)

FIG. 9. Analysis of oligomer formation of mutant and wild-type G proteins by sucrose gradient sedimentation. COS cells transfected with wild-type (WT) or mutant G constructs were labeled with [³⁵S]methionine, and cells were lysed in Triton X-100 at pH 5.6. The cell lysates were loaded onto a 5 to 20% sucrose gradient at pH 5.6 and centrifuged. Fractions were collected from the bottom, immunoprecipitated with anti-G antibody, and analyzed by SDS-PAGE. The bottom fraction is to the left. 8S (aldolase) and 4S (bovine serum albumin) markers sedimented in fractions 12 and 16, respectively.

glycoprotein (5, 32, 33, 37, 38, 42). The fusion peptide of HA contains 24 hydrophobic amino acids located at the NH₂ terminus (13, 14, 17, 42). Exposure to acidic pH induces an irreversible conformational change in the HA molecule to make it fusion competent. In the fusion-competent conformation the previously buried hydrophobic fusion peptide of HA is exposed and relocated so that it can interact with the target membrane (3, 28, 36, 42). Recently, Carr and Kim (5) have proposed a spring-loaded mechanism for conformational change of influenza virus HA protein. A sequence with a high propensity for forming a coiled coil was identified adjacent to the hydrophobic fusion peptide. At acidic pH this sequence forms an extended coiled coil structure that exposes the highly hydrophobic NH₂-terminal peptide and induces fusion by moving the fusion peptide closer to the target membrane (5). Amino-terminal hydrophobic sequences have also been identified as fusion peptides for a number of viral glycoproteins, namely, F proteins of paramyxoviruses and gp41 of human immunodeficiency virus (4, 10, 11, 27, 32, 37, 45). Membrane fusion induced by these viral glycoproteins, however, does not require an acidic pH to trigger the fusion process (32, 37). Recently, E1 glycoprotein of SFV, which requires an acidic pH for fusion, was shown to contain a fusion peptide located not at the NH₂ terminus but in an internal position (residues 75 to 97). The SFV E1 fusion peptide is also hydrophobic and

TABLE 1. Summary of properties of site-directed mutants of VSV G protein^a

Protein ^b	Cell surface expression		Cell fusion (%) ^c	pH optimum of fusion ^d
	Immuno-fluorescence	Iodination (%)		
Wild type	+	100	100	5.6
P-123→L	+	110	100	5.6
G-124→A	+	115	~10	
F-125→Y	+	91	~30	5.2
P-127→G	+	166	~20	5.1
P-127→L	+	110	~5	
D-137→N	+	126	~50	5.0

^a Mutants ΔA, ΔB, and ΔC were not expressed on the cell surface and lacked fusogenic activity.

^b Mutants are designated by the original residue of G protein of the Indiana serotype of VSV (30), its position from the NH₂-terminal methionine, and the mutated residue.

^c Cell fusion was determined by counting the total number of polykaryons containing more than five nuclei in 16 random fields. Values expressed are percentage of the fusion observed at the pH optimum as derived from the data in Fig. 4.

^d The pH optimum was determined as described in the legend to Fig. 4.

conserved in a number of alphaviruses (16–18, 24). In contrast, the putative fusion peptide of VSV G protein suggested in this report is not hydrophobic but contains a stretch of uncharged amino acids which are conserved in G proteins of five vesiculoviruses (2, 12, 26, 27a, 30). Also, unlike with influenza virus HA protein, the acid-induced conformational change observed in the case of VSV G protein is reversible (3, 23, 28). Analyses of the VSV G sequence for the presence of a coiled coil structure (5a, 25a) failed to identify any predicted coiled coil domain. This suggests that the mechanism of acid-induced membrane fusion by G protein may be different from the mechanism involved in HA-induced membrane fusion.

A comparison of the sequence of the putative internal fusion peptide of E1 protein of SFV (16, 24) with that of the putative fusion peptide of VSV G protein (Table 2) shows an intriguing similarity in terms of the spatial locations of glycine, proline, and aspartic acid residues, and a common motif containing glycine, proline, and aspartic acid can be suggested. The two glycine residues and the proline and aspartic acid residues are conserved in the E1 proteins of a number of alphaviruses (16, 24) and in the G proteins of five vesiculoviruses (2, 12, 26, 27a, 30). Mutagenesis of these conserved residues affects the fusogenic properties of E1 and G proteins in a similar manner, either by abolishing the fusion activity or by shifting the pH optimum to more acidic values. Thus, mutation of G-83 to A and G-91 to A in E1 protein produced a shift in the pH threshold of fusion to more acidic values. The substitution of G-91 with the charged residue aspartic acid, however, blocked

TABLE 2. Comparison of sequences of VSV G putative internal fusion peptide and SFV E1 fusion peptide

Fusion protein	Sequence of fusion peptide ^a															
SFV E1 ^b	G -83	V	Y	P	F	M	W	G	G	A	Y	C	F	C	D -97	
VSV G ^c	G -124	F	P	P	Q	S	C	G		Y	A	T	V	T	D -137	
Common motif	G	-	-	P	-	-	-	G		-	-	-	-	-	D	

^a Conserved residues are shown in boldface. Glycine, proline, and aspartic acid residues present in similar positions are shown in boldface italics.

^b The sequence of the fusion peptide of SFV E1 protein is conserved in a number of alphaviruses (16).

^c The sequence shown is present in VSV Indiana G protein and is conserved in G proteins for four other vesiculoviruses (2, 12, 26, 27a, 30).

the fusion activity of E1 protein. Replacement of the conserved P-86 with the charged residue aspartic acid or the mutation of G-91 to P caused transport defects in E1 protein (24). In comparison, mutation of G-124 and P-127 to glycine and to glycine or leucine, respectively, in G protein abolished the fusion activity almost completely. Replacement of D-137 of G protein with asparagine caused a shift in the pH optimum of fusion. In the case of E1 protein, the corresponding residue D-97 was not studied, but mutation of D-75 to A resulted in a downward shift of the pH threshold for fusion. The observed shift in pH threshold or pH optimum for fusion of the mutant fusion peptides could be due to changes in the structure which require a higher proton concentration for the induced conformational change essential for fusion. Thus, the mutations D-75→A, G-83→A, and G-91→A of E1 protein and F-125→Y and D-137→N of G protein could cause an increased stabilization of the fusion peptide structure and therefore a requirement for increased acidity to destabilize and expose the fusion peptide for membrane fusion. The substitution of G-3, G-7, and G-12 with alanine in the conserved fusion domain of F protein of simian virus 5, however, resulted in increased fusogenic activity (15). It was suggested that a G→A change could stabilize the α -helical structure of the fusion peptide, thereby facilitating insertion into lipid bilayer (15). The presence of acidic residues in the fusion peptides of viral proteins that require a low pH for fusion has been observed in the cases of HA protein of influenza virus, E1 protein of alphaviruses, and G protein of vesiculoviruses (2, 12, 16, 24, 25, 26, 32, 37, 38). Protonation of acidic side chains at low pH results in a diminished electrostatic repulsion that destabilizes the folded conformation at neutral pH and thereby facilitates interactions with the lipid bilayer (27). Substitution of aspartic acid with asparagine would therefore cause a requirement for a lower pH for any conformational change required for membrane fusion. It may also be noted that proline residues are present near the centers of internal fusion peptides of both E1 protein of alphaviruses and G protein of vesiculoviruses (2, 12, 16, 25, 26–27a, 30, 37, 38). Similarly, the presence of proline in the middle of a potential internal fusion peptide of a candidate sperm fusion protein was reported (38). Replacement of P-127 of G protein with either the helix breaker glycine or the hydrophobic leucine caused inhibition of the fusion activity. Mutation of P-86 of E1 protein to a charged amino acid, aspartic acid, however, produced a transport defect, and the mutant E1 protein was retained in the endoplasmic reticulum (24). The presence of the strong helix-breaking residues proline and glycine within the internal fusion peptides would restrict the formation of helical structures by these peptides.

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