Influence of Membrane Anchoring and Cytoplasmic Domains on the Fusogenic Activity of Vesicular Stomatitis Virus Glycoprotein G

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Chimeric proteins in which the transmembrane anchoring sequence (TM) or both the TM and the cytoplasmic tail (CT) of vesicular stomatitis virus glycoprotein G were replaced with corresponding domains of viral or cellular integral membrane proteins were used to examine the influence of these domains on acidic-pH-induced membrane fusion by G protein. The TM and CT of G were also replaced with the lipid anchor glycosylphosphatidylinositol. Hybrids containing foreign TM or TM and CT sequences were fusogenic at acidic pH but glycosylphosphatidylinositol-anchored G was nonfusogenic at acidic pH. The results suggest that the fusogenic activity of G protein requires membrane anchoring by a hydrophobic peptide sequence and the specific amino acid sequence of the TM has no influence on fusogenic activity.

The spike glycoprotein G of vesicular stomatitis virus (VSV) is a transmembrane (TM) glycoprotein which can induce membrane fusion at acidic pH in the absence of other viral gene products (3, 7, 26). Studies from our (18, 39) and other laboratories (6, 8, 37) have identified within the ectodomain an internal peptide (amino acids 117 to 137) as the fusion peptide. Unlike the other fusion peptides, the fusion domain of VSV G protein is not hydrophobic and contains neutral amino acids (8, 22, 39). Studies with influenza virus HA2 (35, 38), VSV G (7), and Semliki Forest virus E1 (17) proteins lacking the membrane anchor showed that the ectodomain alone cannot induce fusion, suggesting the requirement of membrane anchoring for fusogenic activity of the proteins.

Studies with the fusogenic TM glycoproteins of human immunodeficiency virus (HIV) (10, 12, 23) and Moloney murine leukemia virus (25) suggested that a specific amino acid sequence in the transmembrane domain plays a crucial role in membrane fusion. Studies with a glycosylphosphatidylinositol (GPI)-linked form of influenza virus hemagglutinin protein (HA) which lacks the membrane anchoring and cytoplasmic domains have shown that GPI-linked HA anchored to the membrane via GPI can induce hemifusion but not full fusion (16). It was proposed that the ectodomain of HA can induce hemifusion when anchored to the membrane via GPI but complete fusion of the membrane requires the transmembrane domain and possibly the cytoplasmic domain to induce the formation of fusion pores (13, 21). Studies with HIV or simian immunodeficiency virus TM fusion proteins (10) and fusion protein F of Newcastle disease virus (30) suggested that the cytoplasmic tail can affect fusion in a positive or a negative fashion. To understand the roles of the different domains of the envelope glycoprotein G of VSV in membrane fusion as well as to examine the requirement of sequence specificity within these regions for fusogenic activity, we have constructed a number of chimeric proteins in which the membrane anchoring region, singly or in conjunction with the cytoplasmic tail of VSV G protein, was replaced with corresponding domains

from a number of integral membrane glycoproteins and their fusogenic activities were determined. The results show that replacement of the transmembrane domain alone or both the transmembrane and cytoplasmic domains permitted membrane fusion at acidic pH by the chimeric proteins. However, the GPI-anchored ectodomain of VSV G protein failed to show low-pH-induced membrane fusion. Our results suggest that membrane fusion by VSV G protein requires not only the fusion peptide in the ectodomain but also membrane anchoring of the G protein by a hydrophobic peptide sequence and that the specific amino acid sequence of the membrane anchoring domain has no influence on fusion.

Construction and expression of chimeric G proteins. Chimeric proteins consisting of the ectodomain of VSV G protein joined to the membrane anchoring domain alone or to the membrane anchoring and cytoplasmic domains from foreign integral membrane glycoproteins such as herpes simplex virus type 1 (HSV-1) glycoproteins gB (1, 31) or gD (32), nonenveloped adenovirus type 5 glycoprotein E3-11.6 K (29), and the cellular receptor glycoprotein CD4 (20, 34) were constructed by using PCR mutagenesis (Fig. 1). To examine the effect of membrane anchoring via a lipid moiety, we constructed the GPI-anchored form of VSV G (G-GPI) by substituting the transmembrane and cytoplasmic domains of VSV G protein with the carboxy-terminal 37 amino acids of the GPI-anchored decay-accelerating factor (2, 15, 25, 28, 33) (Fig. 1). The amino acid sequences of the membrane-spanning and cytoplasmic domains of the G protein and of the chimeras are shown in Table 1. All of the chimeras contain the wild-type VSV G ectodomain (amino acids 1 to 462).

COS-1 cells transfected with the high-efficiency vector pXM (39) encoding the chimeras expressed all of the hybrids. The chimeras GAdG, GgDG, GCD4G, GgB3G, GgDgD, GCD4CD4, and G-GPI all show cell surface labeling as analyzed by indirect immunofluorescence. The distribution of the chimeric glycoproteins was quantitated by cell surface iodination of proteins catalyzed by lactoperoxidase (39). The chimeras were present on the cell surface in amounts of 110 to 190% relative to the wild-type G protein (Table 1). Acquisition of endo H resistance of the glycoproteins (39) showed that the rate of transport from the endoplasmic reticulum to the Golgi apparatus for the hybrids was similar to that of the wild-type G

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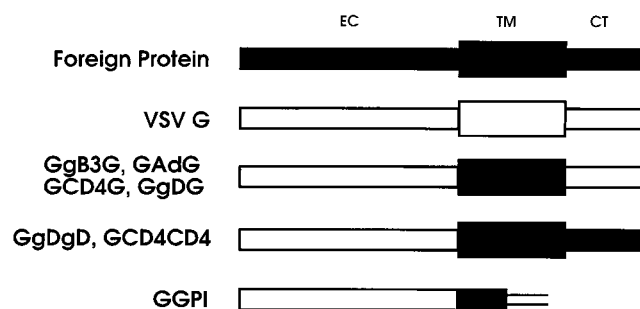
FIG. 1. Schematic diagram of wild-type and chimeric G proteins. Open boxes represent VSV G amino acid sequences, and closed boxes represent amino acids from foreign proteins HSV-1 gB (1) and gD (32), adenovirus type 5 E3-11.6 K (Ad) (29), human CD4 (CD4) (20), or decay-accelerating factor (2). Double black line represents the addition of a GPI lipid anchor (2, 15, 16). Amino acid sequences and construction of chimeric G constructs are described in Table 1. EC, ectodomain; TM, transmembrane anchor; CT, cytoplasmic tail.

protein except for GgDgD and G-GPI, which moved at a slower rate. Proper folding and acquisition of correct tertiary and quaternary structure of the chimeras were examined by determining their oligomeric structure at acidic pH (4, 5). All of the hybrids formed trimers at acidic pH.

Cell-cell fusion activity of chimeric G proteins. To determine the low-pH-induced fusogenic activity of the chimeric G proteins a syncytium-forming assay was used (39). COS cells transfected with wild-type and chimeric G genes were exposed twice at 37°C for 1 min with the fusion medium at pH 5.6, and the polykaryons that were formed were fixed and viewed under a phase-contrast microscope. We have previously observed that the pH optimum for membrane fusion by VSV G mutants can be shifted to a more acidic pH (39). We, therefore, also determined the fusogenic activity of the hybrid G proteins at pH 5.2 (Fig. 2). The results show that cells expressing GAdG, GgB3G, GCD4G, and GgDG showed polykaryon formation that was comparable to that of wild-type G. Hybrids GCD4 CD4 and GgDgD were fusion competent although at reduced

levels compared to wild-type G protein. In contrast, the chimera G-GPI, which lacks the transmembrane and cytoplasmic domains and is anchored to the cell surface via a GPI linkage, showed no polykaryon formation at pH 5.6 or 5.2. Comparison of the relative cell surface expression of hybrid G proteins with their fusogenic activities showed that the reduced fusogenic activity of GCD4CD4 and GgDgD as well as the lack of fusogenic activity for G-GPI was not due to reduced cell surface expression of the hybrids (Table 1). Earlier studies indicated that mutations in the fusion domain of VSV G protein can shift the pH threshold as well as the pH optimum for fusion to more acidic regions (9, 39). The pH dependence for fusion of the mutants GCD4CD4, GgDgD, and G-GPI was therefore examined over a wider range of pH conditions. Results presented in Fig. 3 show that both GCD4CD4 and GgDgD mutants have an altered pH threshold for fusion. Wild-type G protein shows initial fusion activity at about pH 6.3. In contrast, initial fusion for the chimeras GCD4CD4 and GgDgD occurred at the more acidic pH of 5.6. Maximum fusogenic activity for these two mutants was observed at pH 5.0, in comparison to pH 5.6 for the wild-type G as well as chimeras containing only the foreign transmembrane regions. Mutant G-GPI did not show any fusogenic activity over the entire range of pH conditions tested. Treatment of the cells expressing GCD4CD4, GgDgD, or G-GPI at low pH for longer periods of time, or at a higher temperature of 39°C, did not increase the fusogenic activity. It appears, therefore, that the cytoplasmic tail can influence the fusogenic activity. The fact that both the threshold pH as well as the pH optimum for fusion by mutants containing foreign cytoplasmic tails are shifted to more acidic-pH conditions suggests that kinetic components of fusion may be modulated by this region.

The hybrid G-GPI was anchored to membranes via a lipid moiety. Studies with a cell line expressing GPI-linked HA glycoprotein of influenza virus showed that the lipid-linked HA failed to induce cell fusion but could induce hemifusion of the outer membranes resulting in mixing of the lipids but not of the contents of the cells (16). We tested for lipid mixing by cells expressing G-GPI by using the octadecylrhodamine (R18) flu-

TABLE 1. Amino acid sequences of the membrane-spanning and the cytoplasmic domains of VSV G protein and the chimeras constructed, and quantitation of the cell surface expression and fusion activity

Construct[a]	Amino acid sequence			Surface expression (%)[b]	Fusion activity (%)[c]	
	Ectodomain	Transmembrane anchor	Cytoplasmic tail		pH 5.6	pH 5.2
G-wt	FSSWK	SSIASFFFIIGLIIGLFLVL	RVGIHLC...RLGK	100	100	90
G(AXB)	FSSWK	SSSSIASFFFIIGLIIGLFLVA	RVGIHLC...RLGK	100	100	84
GgB3G	FSSWK	FGALAVGLLVLAGLAAAFFAF	RVGIHLC...RLGK	170	100	100
GCD4G	FSSWK	SSSALIVLGGVAGLLLFIGLGIFFA	RVGIHLC...RLGK	169	97	77
GAdG	FSSWK	SSLGMWWFSIALMFVCLIIMWLICCL	RVGIHLC...RLGK	118	96	100
GgDG	FSSWK	NMGLIAGAVGGSLLAALVICGIVYWM	RRVGIHLC...RLGK	110	71	90
GgDgD	FSSWK	NMGLIAGAVGGSLLAALVICGIVYWM	RRRTRKAP...PLFY	110	10	30
GCD4CD4	FSSWK	SSSALIVLGGVAGLLLFIGLGIFFCV	RCRHRRRQ...CSPI	132	15	42
G-GPI	FSSWKSSSPNKGSGTTS	GTTRLLSGHTCFTLTGLLGTLVTMGLLT[d]		192	0	0

[a] Compared to the wild-type VSV G protein (G-wt) the construct G(AXB) contained an *Apa*I restriction enzyme site at nucleotide 299 (39), a *Xho*I site at nucleotide 1416 (37), and a *Bss*HII site at nucleotide 1473, all of which were created by site-directed mutagenesis. G(AXB) also contained two additional serines inserted between amino acids 463 and 464 and the leucine at residue 482 was replaced by alanine. The G protein coded by the G(AXB) gene showed wild-type properties with respect to transport to cell surface, oligomerization at low pH and low-pH-induced polykaryon formation. The construct GgB3G contained amino acids 775 to 795 of HSV-1 gB (1) between residues 462 and 482 of VSV G protein (27). Construct GCD4G was made by inserting amino acids 375 to 395 of CD4 (20) between residues 465 and 484 of G(AXB), while GCD4CD4 was made by joining residues 375 to 435 of CD4 after amino acid 485 of G(AXB). GAdG were made by insertion of residues 32 to 55 of adenovirus type 5 E3-11.6 K protein (29) between amino acids 464 and 483 of VSV G protein. GgDG was made by inserting residues 339 to 365 of HSV-1 gD (32) between amino acids 462 and 483 of VSV G, while GgDgD and G-GPI were made by joining residues 339 to 394 of HSV-1 gD and residues 311 to 347 of decay-accelerating factor (2, 15, 16), respectively, after amino acid 465 of G(AXB).
[b] Surface expression was quantitated as previously described (39). Values obtained for mutants are expressed as percentages of the wild-type value set at 100%.
[c] Fusion activity was determined as previously described (39). Values obtained for mutants are expressed as percentages of wild-type value set at 100%.
[d] Sequence for GPI lipid anchor begins with predicated point of attachment (2, 5).
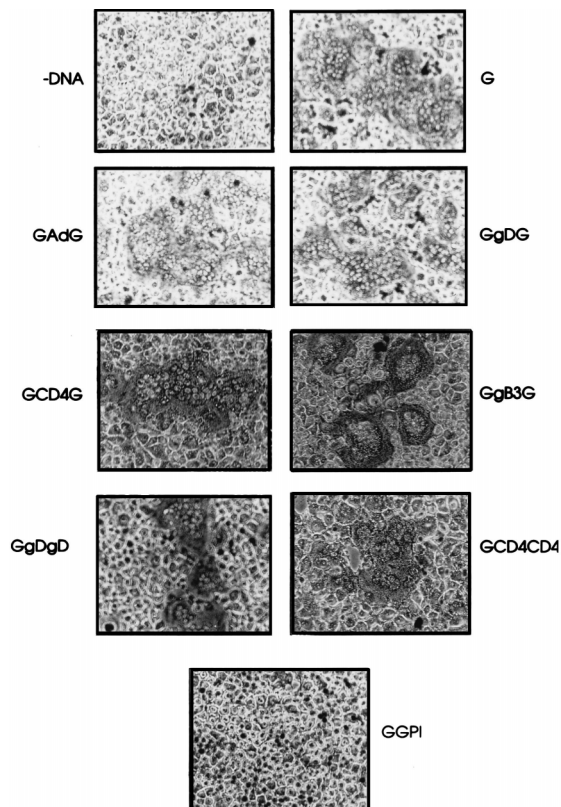
FIG. 2. Polykaryon formation induced by wild-type and chimeric G proteins. COS cells transfected with plasmids encoding wild-type or chimeric G constructs were exposed to fusion medium at pH 5.2 for 60 s and then incubated for 2.5 h with regular medium at 37°C. The fusion shock was repeated, and then cells were incubated in regular medium for 2 h, fixed, stained, and photographed (39).

FIG. 4. Lipid-mixing activity of cells expressing wild-type G and chimera G-GPI determined by R18 fluorescence-dequenching assay. BHK cells infected with a recombinant vaccinia virus (vTF7-3) expressing T7 polymerase were transfected with pBluescript (Stratagene) plasmids containing wild-type G or the mutant G-GPI gene cloned between the T7 promoter and T7 terminator by using liposomes composed of dimethyldioctadecyl ammonium bromide and L-α-dioleylphosphatidylethanolamine (9, 37). Plasma membrane vesicles prepared from Vero cells were labeled with octadecylrhodamine (R18) as described by Puri et al. (24). BHK cells transfected with wild-type G (G), hybrid G-GPI (GPI), or with no DNA (−DNA) were prebound to R18-labeled plasma membrane vesicles at 4°C. The vesicle-cell suspension was added to preequilibriated buffer (pH 7.2), the pH was lowered by addition of 0.5 M 2-(N-morpholine) ethanesulfuric acid (pH 5.0), and fluorescence was measured. Complete dequenching (100%) was observed after adding Triton X-100 to a final concentration of 0.05% (24).

orescence-dequenching assay developed by Puri et al. (24). Results shown in Fig. 4 indicate that cells expressing wild-type G protein mediated lipid mixing at pH 5.5 as shown by dequenching of fluorescence. Cells expressing G-GPI, however,
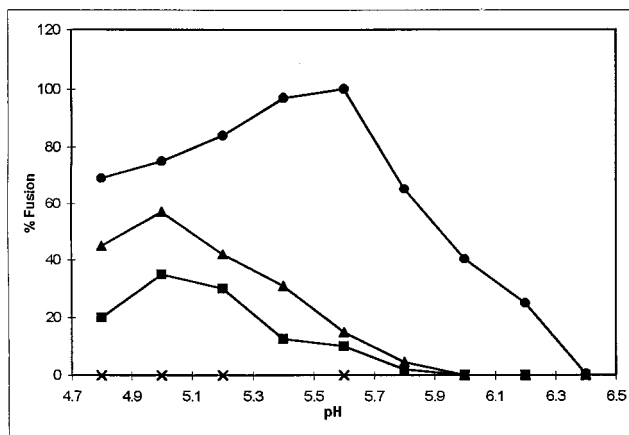

FIG. 3. pH dependence of cell fusion induced by expression of chimeric G proteins. COS cells transfected with plasmids encoding wild-type or chimeric G proteins were exposed to fusion medium of various acidities in the pH range of 4.8 to 6.4. The cells were processed as described previously (39). The total number of polykaryons containing more than five nuclei were counted in 25 random fields. Fusion activity was calculated by using the number of polykaryons produced by wild-type G at pH 5.6 as 100% (39). COS cells were transfected with plasmids encoding wild-type G (●) or mutants GgDgD (■), GCD4CD4 (▲), or G-GPI (X).
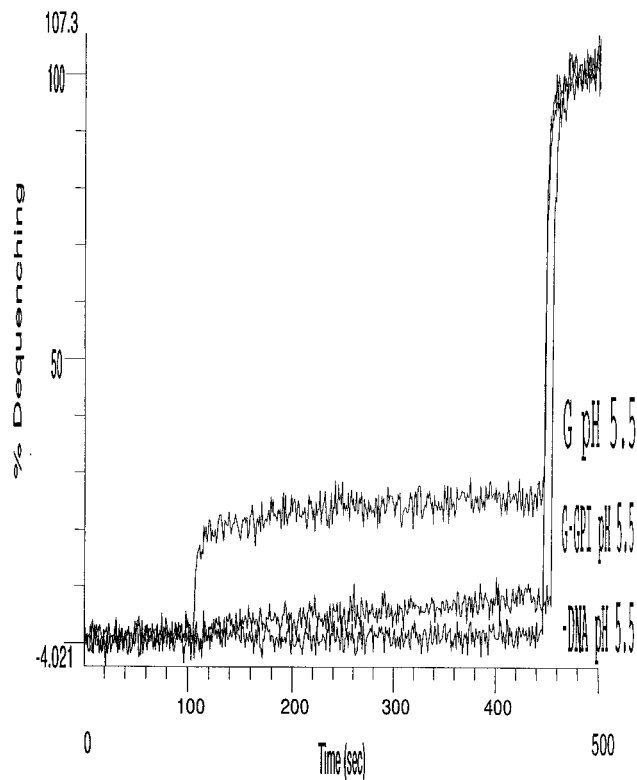
showed a small amount of dequenching of fluorescence at pH 5.5. Lowering the pH to 5.2 did not increase the level of fluorescence dequenching. The results suggest that G protein anchored to membranes via lipid may not induce hemifusion as observed for GPI-linked HA. It may be possible that the nine amino acids of the decay-accelerating factor present in the G-GPI hybrid (Table 1) may affect conformational changes involving hemifusion. GPI-linked viral fusion proteins, such as envelope TM proteins of HIV (28, 33) and Moloney murine leukemia virus (25), and bovine herpes virus type 1 glycoprotein gB (19) also failed to show fusion. However, the hemifusion activity of these GPI-linked proteins was not tested (19, 25, 28, 33).

pH-dependent trypsin sensitivity of chimeric G proteins. Viral fusion proteins that are activated at low pH show a fusion-inducing conformational change at acid pH (11, 13, 14, 36, 38). The sensitivity of influenza virus HA (11, 38) or rabies virus G (11) proteins to trypsin digestion at acid pH has been used to monitor the low-pH-induced conformational change. Recently, it was shown that incubation of VSV G protein at low pH renders them more resistant to trypsin digestion, pre-
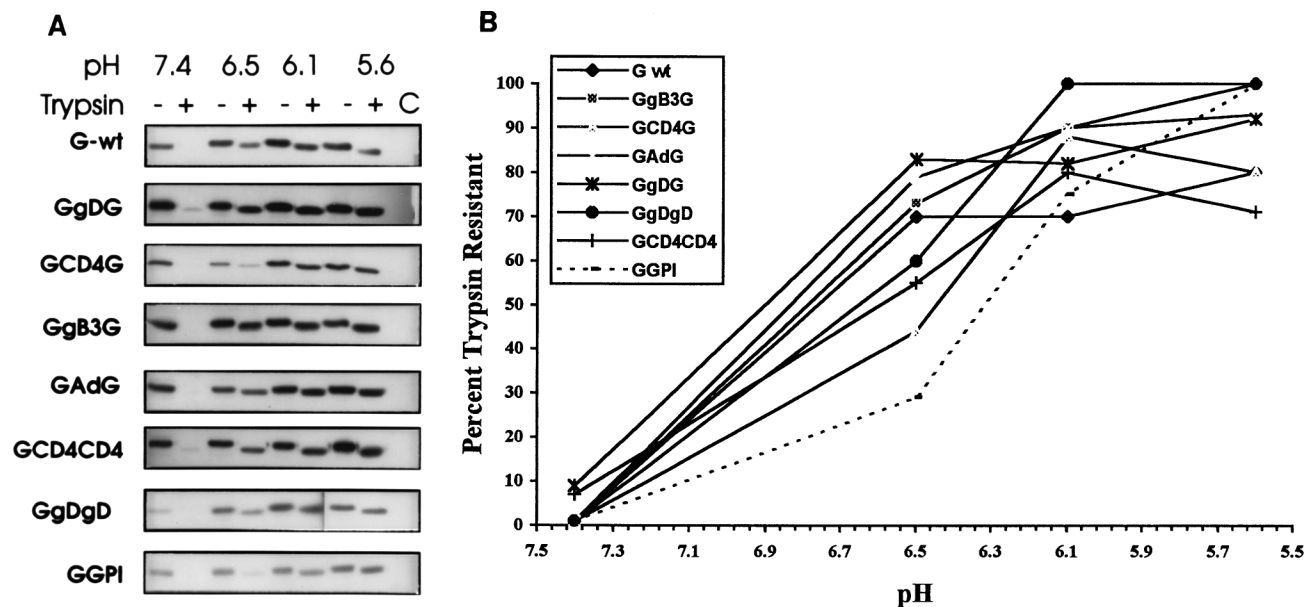
FIG. 5. pH-dependent resistance of wild-type and chimeric G proteins to trypsin digestion. (A) COS cells transfected with wild-type or chimeric G genes were labeled with [^{35}S]methionine for 30 min and chased with nonradioactive methionine for 60 min. Labeled cells were lysed in the presence of 1% Triton X-100 in a buffer containing 2× MNT [40 mM 2-(N-morpholine)ethanesulfonic acid, 60 mM Tris, 200 mM NaCl, 2 mM EDTA] at the indicated pH. Cell lysates were incubated with or without 10 µg of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-trypsin for 30 min at 37°C (9). In lane C samples digested at pH 5.6 were incubated with 0.3% sodium dodecyl sulfate. Lysates were immunoprecipitated with anti-G antibody and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9, 39). (B) Bands from the experiment described for panel A were quantitated by scanning densitometry. Percent trypsin resistance was calculated by quantitating the band intensity of the trypsin-digested samples relative to nontrypsin-digested samples at the same pH (9, 39).

sumably due to conformational changes induced by acid pH (9). We have therefore used the pH-dependent resistance to trypsin digestion of VSV G protein to examine acidic-pH-induced conformational change of wild-type and chimeric G proteins. Results presented in Fig. 5 show that the wild-type as well as the chimeric G proteins were completely digested by trypsin at neutral pH but showed increased resistance to digestion by trypsin at more acidic pH. All of the chimeric G proteins except G-GPI showed a digestion profile fairly similar to that of the wild type. The hybrid G-GPI was, however, more sensitive to trypsin digestion than wild-type G at pH 6.5.

Taken together, our results indicate that substitution of the transmembrane domain of VSV G with the membrane anchoring domain of a foreign glycoprotein does not affect the fusogenic ability of the hybrid protein. However, replacement of both the transmembrane and cytoplasmic domains of VSV G with the corresponding domains of a foreign protein reduces the fusogenic activity of the hybrid. In contrast, the anchoring of the ectodomain of VSV G to the cell surface via a glycolipid anchor abolished membrane fusion. Thus, our results suggest that the low-pH-induced fusogenic activity of VSV G protein requires membrane anchoring by a hydrophobic peptide sequence but the specific amino acid sequence of the transmembrane domain has no influence on membrane fusion and the cytoplasmic tail may have a modulating role. The membrane anchor and the cytoplasmic tail together may form an entity which can regulate the fusogenic behavior of a protein.

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