A Protein G Fragment from the Salmonid Viral Hemorrhagic Septicemia Rhabdovirus Induces Cell-to-Cell Fusion and Membrane Phosphatidylserine Translocation at Low pH*

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The fusion-related properties of segments p9, p3, p4, and p9 + p2 surrounding the p2 phospholipid-binding domain of the protein G (pG) of the salmonid rhabdovirus of viral hemorrhagic septicemia (VHSV) (Nunez, E., Fernandez, A. M., Estepa, A., Gonzalez-Ros, J. M., Gavilanes, F., and Coll, J. M. (1998) Virology 243, 322–330; Estepa, A., and Coll, J. M. (1996) Virology 216, 60–70) have been studied at neutral and fusion (low pH) values by using its derived peptides. Cell-to-cell fusion, translocation of phosphatidylserine, and inhibition of fusion of pG-transfected cells defined the p9 + p2 (fragment 11, sequence 56–110) as a fragment with higher specific activity for anionic phospholipid aggregation than the previously reported p2. While fragment 11, p2, and p3 showed interactions with anionic phospholipids, p9 and p4 showed no interactions with any phospholipids. When added to a cell monolayer model at low pH, fragment 11 induced pH-dependent cell-to-cell fusion and translocated phosphatidylserine from the inner to the outer leaflet of the membrane. At low pH and in the presence of anionic phospholipids, fragment 11 showed more than 80% β-sheet conformation (IR and CD spectroscopies). Finally, anti-fragment 11 antibodies inhibited low pH-dependent pG-transfected cell-to-cell fusion. All of the data support the conclusion that fragment 11 is a primary determinant of some of the viral cell fusion events in VHSV.

The protein G (pG)1 of mammalian and fish rhabdoviruses have (500 amino acids. The pG contains 2–6 glycosylation sites, 6–8 highly conserved disulfide bridges (3, 4), two or three noncanonical hydrophobic heptad repeats (5), a carboxyl-terminal cytoplasmic domain, and a removed signal peptide (6) (Fig. 1A). The pG forms homotrimers to bind to the host cell receptor(s) and after endocytosis fuses with the host membranes (7), following structural rearrangements triggered by the low pH of the endosome (8). Contrary to many other enveloped viruses, in rhabdoviruses there is no proteolytic processing of pG to expose a fusion domain (the so-called fusion peptide). Furthermore, the putative fusion domain is not hydrophobic but neutral, it contains an internal cysteine (except in rabies virus) (9, 10), and at least some of the conformational changes required for low pH fusion are reversible (11). Fusion-defective mutants in vesicular stomatitis virus (10, 12) and neutralizing monoclonal antibody (mAb)-resistant mutants at low pH in vesicles virus (13) identified fusion-related domains in these rhabdoviruses. An alignment model based in highly conserved cysteines identified homologous putative fusion domains in other 14 animal rhabdoviruses (3). However, there is not yet a mechanism or model to explain how fusion occurs when these viruses; nor has a complete fusion-defective mutant screening been performed to identify cell-to-cell fusion domains (14). All of the above mentioned data make rabbit viral fusion an intriguing subject to study viral entry in cells.

The major anionic phospholipid-binding domain of the pG (p2, sequence 82–109) of viral hemorrhagic septicemia virus (VHSV), a rhabdovirus of salmonids (15, 16), is related to fusion after VHSV binding to its cell receptors (17, 18). For instance, anti-p2 antibodies (Abs) inhibited phospholipid binding to VHSV (2) and VHSV-induced cell-to-cell fusion (19). In addition, p2 mediates phospholipid vesicle fusion, lipid mixing, and leakage of liposome contents and inserts itself into liposome membranes by adopting a β-sheet structure (1). Furthermore, phospholipid binding to VHSV (2) and fusion of pG gene-transfected cells (20) showed similar pH-dependent profiles. However, the location of the phospholipid-binding domain (sequence 82–109) (15) and the location of the putative fusion domain (sequence 142–156) (3) are separated in pG. On the other hand, based on mutants changing the fusion pH, it has been suggested that the complete fusion structure of VHSV could be constituted by two distant pG domains situated around sequences 110–118 and 144–154 (21). These sequences are maintained together by a disulfide bond between Cys78 and Cys102 (4). We selected four segments surrounding the p2 phospholipid-binding domain and located upstream of the putative fusion domain to study its possible relationship with fusion in the...
VHSV model. The selected segments were p9 (sequence 58–80), p3 (sequence 110–122), p4 (sequence 123–144), and fragment 11 (p9 + p2, fragment 11 from sequence 56–110) (Fig. 1, A and B). We then used synthetic or recombinant peptides derived from them to study their fusion-related properties at neutral and fusion (low pH). While fragment 11, p2, and p3 showed interactions with anionic phospholipids, p9 and p4 showed no interactions with any phospholipids. At the low pH of fusion, fragment 11 showed the highest anionic phospholipid binding specific activity and was unique in inducing noninfected cell-to-cell fusion and translocating phosphatidylserine (PS) from the inner to the outer leaflet of the membrane. Fragment 11 required low pH for most of its activities and at that pH showed a complete β-sheet structure. Finally, anti-fragment 11 inhibition of PG-transfected cell fusion confirmed the implication of fragment 11 in fusion of PG with cellular membranes. All the above mentioned data shows that fragment 11 participates in VHSV-cell fusion.

MATERIALS AND METHODS

Analysis of the PG of Rhabdoviruses—The alignment model proposed for cDNA-derived PG sequences of 14 animal rhabdoviruses belonging to family Rhabdoviridae was used to study the sequences related to the VHSV model. The selected segments were p9 (sequence 58–80) and three sequences of PG from three viruses, E. coli BL21 DE3 and E. coli BL21. The sequences were cloned into E. coli DH5α strain to select a positive clone, and then the construction was transferred to E. coli BL21 DE3 for expression as described before (27). The nucleotide sequences corresponding to p9 sequences 56–110 were present in the recombinant plasmid as demonstrated by DNA sequencing of the insert in both directions (not shown). For expression, isopropyl-1-thio-β-D-galactopyranoside was added following the manufacturer’s instructions, and the bacterial lysates in lysis buffer (20 mM NaHPO₄, 0.5 mM NaCl, 6 mM guanidine HCl, pH 7.8) were eluted from a nickel affinity column (ProBond™, Invitrogen) at pH 4.

Interaction of fragment 11 with itself was detected by its tendency to precipitate out of solution when dialyzed against 15 mM phosphate buffer at pH 7.6 (7). The highest concentration that could be obtained was around 50, 180, and 300 mg/ml for more than 1000 µg of dissolved fragment 11/ml were obtained by using pH values of 7.6, 6, 5.6, or 5.4, respectively. Only a small percentage of the aggregates were disulfide-bridged, as shown by densitometry of the Coomassie-stained bands of fragment 11 separated by 15% polyacrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol. The biotinylated peptide of 28 amino acids derived from fragment 11 pitropeptide for the VHSV (07.71) includes the signal peptide and is predicted to be transmembrane (EPC) cells and concentrated by polyethyleneglycol to about 70% purity. The biotinylated peptide of 28 amino acids derived from fragment 11 was chosen to decrease self-aggregation (biotin-phenol red in phosphate-buffered saline) were added to the plates and incubated for 1 h at pH 6 or 7.6 before performing polyacrylamide gel electrophoresis. On the contrary, only one band at about 10 kDa was obtained by polyacrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol, other higher molecular weight bands being undetectable at the highest concentrations electrophoresed (50 µg of fragment 11 well, n = 12). Since only one peak at A₂₈₀ and at the same position was obtained after fragment 11 was separated by Sephadex chromatography in buffers at pH 4 in the absence or in the presence of 0.14 M β-mercaptoethanol, Sephadex G-100 chromatography at pH 4 was used to partially separate other higher molecular weight fragments.

Induction of Anti-fragment 11, Anti-p2, Anti-p3, Anti-p4, and Anti-pG Abs in Mouse Ascites—Female BALB/c mice in groups of three were each immunized by injection with 20 µg of fragment 11, p2, p3, p4, or purified PG (29) in Freund’s complete adjuvant and then by four monthly similar peptide injections in Freund’s incomplete adjuvant. To obtain pooled diluted ascites containing the Abs, the immunized mice were intraperitoneally injected each with 0.5–2 × 10⁸ viable myeloma X63/Ag8.653 cells as described before (30).

Enzyme-linked Immunosorbent Assays—Confirmation of fragment 11 identity was performed by using polyclonal Abs (15) obtained in mice against concanavalin A affinity-purified PG from VHSV (29). Wells (Polygro, Nunc) were coated with serial dilutions of fragment 11 or other proteins in 100 µl of water and allowed to dry overnight at 37 °C. The coated plates were washed with dilution buffer before use. Then anti-G Abs (9 µg/ml) in dilution buffer (0.5% bovine serum albumin, 0.3% rabbit serum, 0.1% merthiolate, 0.005% phenol red in phosphate-buffered saline) were added to the plates and incubated for 1 h at room temperature. After the incubation of the corresponding Abs was carried out in plates coated with VHSV (0.5 or 1.5 µg of viral protein/ml) in 0.1 M citrate phosphate buffer at pH 7.6 and 6. Mice ascites containing Abs to p2, p3, p4, and fragment 11 were diluted 100–300-fold (to give a maximum absorbance of 1.5–2 units) in dilution buffer at different pH values and incubated for 1 h. Backgrounds, in wells coated with bovine serum albumin and incubated with the same antibodies at the same pH values as described above, were subtracted for all of the data. The relative absorbances were calculated according to the following formula: absorbance at pH 5.6/absorbance at pH 7.6 × 100.

Recognition of solid-phase VHSV plus fragment 11 mixtures by anti-G Abs was performed by coating the wells to dryness with serial dilutions of fragment 11 + VHSV in 100 µl of volume. Then anti-G Abs of known target epitope in dilution buffer at different pH values were added to the wells and incubated for 1 h at room temperature.

Recognition of lineal epitopes by fragment 11 was performed by using Pepscan peptides from the PG and a biotinylated peptide from fragment 11. The Pepscan peptides were then added to the plates coated with VHSV and incubated for 1 h. The color reaction was developed by the addition of 1 mg/ml o-phenylenediamine in 100 mM citrate buffer containing 3 mM H₂O₂. Optical density was measured at 492–620 nm (dual wave length) in an enzyme-linked immunosorbent assay reader as indicated before (31).
Solid-phase Phospholipid-binding Assays—Screening of phospholipid binding in the pG Pepscan peptides was made by using labeled anionic and nonionic phospholipids with the same carbon backbone to increase specificity. The labeled phospholipids used, 1 C/mmol, phosphatidyl-[2-3H]Linolenate 4,5-bisphosphate (PIPP) and phosphatidyl [2-3H]glycerol (PG), were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Annexin V-positive, PrIo-negative cells were determined by using basic extrusion apparatus with 100-nm polycarbonate filters (Avestin Inc.).

Vesicle Aggregation Assay—We used an assay based in the increase in the size of phospholipid vesicles quantified by optical density, since it has been used before as an indicator of vesicle fusion (32). The assay was performed as described before (1). Fragment 11 and p2 were added to a stock solution of 5 mg/ml in 20 mM MES at pH 5.6 to phospholipid vesicle suspensions in 1 ml of medium buffer at the desired pH.

Infrared Measurements—The measurements were performed as described before (1) in D2O buffer containing 10 mM HEPES, pH 7.0, 130 mM KCl, and 20 mM NaCl.

Circular Dichroism—The measurements were performed as described before (1). Aliquots of fragment 11 or p9 peptide from a solution at 5 mg/ml in trifluoroethanol were added to medium buffer (100 mM NaCl, 5 mM Tris, 5 mM citrate, 5 mM EDTA, 1 mM EDTA (medium buffer)) adjusted to the desired pH and incubated for 1 h at 37 °C and vortexed vigorously and adjusted to 0.14 mM final concentration. This suspension was extruded by 19 cycles in a Loposo- Fast™ Basic extrusion apparatus with 100-nm polycarbonate filters.

Results

Selection of pG Segments around the p2 Phospholipid-binding Domain of VHSV—A series of segments of the pG from the positions 56–144 that surround the phospholipid-binding domain (p2, sequence 82–109) was used to investigate which region(s) might be involved in membrane perturbation events (Fig. 1B). The segments were then synthesized as peptides p9 (sequence 58–80), p2 (sequence 82–109), p3 (sequence 110–122), and p4 (sequence 123–144). Peptide p9 contained 23 amino acids from position 56 to the beginning of p2. Following p2, p3 contained a sequence of 12 amino acids, 11 of which were totally conserved among cold water fish rhabdoviruses (VHSV, infectious hematopoietic necrosis virus, and hirame rhabdovirus) (3). Peptide p4 was from the end of p3 to the beginning of the putative fusion peptide (sequence 142–159). It contained many hydrophilic amino acids and one of the sites (K140) mutated in neutralization-resistant VHSV mutants of mAb C10 (21, 35).

Fragment 11 (sequence 56–110) containing the p9 + p2 sequences and five heptad repeats (sequence 68–99) (Fig. 1A) was obtained as a recombinant fragment. The segment corresponding to fragment 11 in the pG is flanked by disulfide bridges Cys84–Cys85, Cys101–Cys102, and Cys132–Cys133 (4), bringing together the phospholipid-binding and putative fusion domains (Fig. 1, A and B).

Binding of Labeled Phospholipid Vesicles to Solid-phase Peptides from a Pepscan of pG—To investigate p9, p2, p3, p4, or fragment 11 binding of anionic phospholipids, solid phases made of each of the five amino acids overlapping 15-mer peptides covering the whole sequence of pG (Pepscan) were used. Labeled phospholipids were PIPP as an anionic phospholipid and PI as the best control for PIPP, since it has less charge than and the same carbon backbone as its bisphosphate derivative. Maximal PIPP binding (14–15-fold the background level) was found by peptides covering the sequence corresponding to fragment 11 (p9 + p2). Lower PIPP binding (4–6-fold the background level) was found by peptides covering the sequence 109–133 (including p9) and by peptides covering the sequence 149–173 (including most of the putative fusion peptide) (Fig. 2A). However, Pepscan peptides covering the sequence of p9 and p4 did not show PIPP binding above background. Other peptides from the Pepscan also showed significant PIPP binding at other positions outside the studied region (e.g., those at around sequence 230). Very low binding was obtained for all peptides from the Pepscan when labeled PI was used (Fig. 2B).

Aggregation of Phospholipid Vesicles by p2 and Surrounding Segments—Concentrations from 1 to 100 µg/ml peptides were added in solution to 0.14 mM phospholipid vesicles of PS, phosphatidic acid, phosphatidyglycerol, or phosphatidylethanolamine at pH 7.6 and 5.6. The interactions of fragment 11 or p2 with plates (about 100,000 cells/well) were transfected with 0.6 µg of the plasmid G3-pcDNA/Amp complexed with 2 µl of Fugene in 100 µl of volume following the methods described before (33, 34). Abs were serially diluted in fusion medium at pH 6 (optimal fusion pHi) (19) and incubated overnight at 4 °C. The next day, the EPC plates were washed, cells were trypsinized from the well, and 100 µl of each treatment was added into the EPC wells. After 30 min of incubation, the cultures were washed with fusion medium at pH 7.6 and incubated for 2 h at 14 °C with fusion medium at pH 7.6.

In both assays mentioned above, after removal of the fusion medium, the EPC cell monolayers were fixed in 1% glutaraldehyde for 10 min, dried, and stained with Giemsa. The spread cells (cell surface of spread cells) were counted after 3-fold the control EPC cell surface) were counted among 400 cells. Results were expressed as the following formula: number of spread cells/number of total cells × 100. The number of nuclei in syncytia of three or more nuclei per syncytium were counted among 400 nuclei/well. Results were expressed as the percentage of nuclei in syncytia by the following formula: number of nuclei in syncytia/total number of nuclei × 100.

To assay for pG-induced fusion from within, EPC cells in 24-well plates were transfected with 0.6 µg of the plasmid G3-pcDNA/Amp complexed with 2 µl of Fugene in 100 µl of volume following the methods described before (33, 34). Abs were serially diluted in fusion medium at pH 6 (optimal fusion pHi) (19) and incubated overnight at 4 °C. The next day, the EPC plates were washed, cells were trypsinized from the well, and 100 µl of each treatment was added into the EPC wells. After 30 min of incubation, the cultures were washed with fusion medium at pH 7.6 and incubated for 2 h at 14 °C with fusion medium at pH 7.6.

In both assays mentioned above, after removal of the fusion medium, the EPC cell monolayers were fixed in 1% glutaraldehyde for 10 min, dried, and stained with Giemsa. The spread cells (cell surface of spread cells) were counted after 3-fold the control EPC cell surface) were counted among 400 cells. Results were expressed as the following formula: number of spread cells/number of total cells × 100. The number of nuclei in syncytia of three or more nuclei per syncytium were counted among 400 nuclei/well. Results were expressed as the percentage of nuclei in syncytia by the following formula: number of nuclei in syncytia/total number of nuclei × 100.
anionic phospholipid vesicles showed a 1.5-fold higher OD values at pH 5.6 than at pH 7.6 (n = 3), PS being the phospholipid showing the greatest anionic phospholipid vesicle aggregation (Fig. 2C and data not shown). Fragment 11 aggregated PS vesicles with 5-fold more efficacy when compared with p2 in a molar basis. In the presence of phosphatidylcholine, however, the increase in optical density was almost negligible even at pH 5.6. Peptide p9, p3, p4, or poly(H) did not induce any increase in the optical density at 360 nm in the presence of any of the phospholipids mentioned above (results not shown).

Induction of Cell Spreading and Syncytia by p2 and Surrounding Segments—When fragment 11 or p2 was added to EPC cell monolayers at pH 6, there was a 2–3-fold increase in the cell surface (cell spreading) in some of the cells (Fig. 3A). Thus, at pH 6 the addition of fusion medium, p2 (200 μg/ml) or fragment 11 (200 μg/ml) induced cell spreadings of 3.4 ± 0.2, 33.0 ± 1.0, or 36.7 ± 2.4% of the EPC cells, respectively (n = 2). The addition of p9, p3, p4, or poly(H) at pH 6 or fragment 11 at pH 7.6 to the EPC cell monolayers produced no detectable effects in cell spreading (not shown).

In the same experiments, syncytia of 3–6 nuclei also appeared but only in the presence of fragment 11 at pH 6 (Fig. 3C). The percentage of nuclei inside syncytia increased from 5 to 10% of the total number of nuclei when adding 50–200 μg of fragment 11/ml to EPC cell monolayers (Fig. 3E). The addition of p2, p3, p4, or poly(H) to the EPC cell monolayers did not cause numbers of syncytia higher than 2% (Fig. 3E and results not shown). Since ~10 μg of VHSV (containing about 1 μg of pG)/ml were required to obtain a similar highest number of nuclei in syncytia (not shown), fragment 11 was ~200-fold less active than pG in inducing fusion in cell monolayers. However, this fragment 11 concentration requirement for fusion is, most probably, an overestimation because of the tendency of fragment 11 to aggregate, as shown by the presence of visible microscopic aggregates covering the cell monolayers. Aggregates such as those are unlikely to cause the observable effects, that must be attributed to the fragment 11 molecules that remain in solution. Since only 15–30% of fragment 11 remains in solution in phosphate-citrate at pH 6, the concentration of the fusion-active soluble fragment 11 molecules that caused fusion at that pH are probably 3–6-fold lower than the concentration added. The percentage of cell to cell fusion was also dependent on the initial EPC cell concentrations. The higher the concentration was, the higher the percentage of nuclei in syncytia. However, concentrations of >300,000 cells/well resulted in such high numbers of nuclei in syncytia that they could not be quantitated.

Induction of PS Exposure in the Plasma Membrane by p2 and Surrounding Segments—Membrane fluorescence to FITC-labeled annexin was observed in some of the EPC cells exposed to 20 μg/ml fragment 11 at pH 6 (Fig. 4, A and B). To quantitate the translocation of PS to the other side of a biological membrane, we used the plasma membrane of EPC cells as a model and FITC-labeled annexin-binding to the cell surface of living cells as an estimation of PS exposure. An increase in the number of FITC-labeled annexin cells could be detected among the viable (PrIo-negative) cells after EPC cell monolayers were treated at pH 6 with increasing concentrations of fragment 11 (Fig. 4C) up to 400 μg/ml (not shown). The addition of p9, p2, p3, p4, or poly(H) was unable to induce such a significant number of annexin V-positive, PrIo-negative EPC cells in parallel experiments at those concentrations (Fig. 4C and data not shown).

Low pH- and Phospholipid-dependent Conformations of Fragment 11—Since fragment 11 appeared to be the most important segment in relation to fusion of those studied, the possible pH-dependent conformations suggested by the pH-dependent reversible aggregation of fragment 11 (see “Materials and Methods”) were measured by IR and CD spectra at different pH values.

From neutral to low pH, the band decomposition of the original amide spectra showed an increase in the band at 1633–1637 nm, corresponding to an increase in β-sheet content from 5.8 ± 1.2 to 25.4 ± 2.7% and other minor changes (not shown). The CD spectrum of fragment 11 at pH 5.6 shows the presence of 34.4% of β-sheet conformation, similar to the data obtained by the IR spectra. However, in the presence of PS vesicles, the β-sheet conformation of fragment 11 increased to
84.4% to become the major component (Fig. 5 and Table I), and at the same time the β turns decreased from 28.2 to 1.7%. Increasing the phospholipid/peptide molar ratio from 11 to 40:1 did not modify the CD spectrum any further, but at the intermediate phospholipid/fragment 11 molar ratio of 20:1, and despite the low pH, aggregates formed, which made it impossible to record any spectrum. Sonication was not capable of disrupting these aggregates, most probably formed as a result of neutralization of the electrostatic charges. The addition of PS to poly(H) did not induce any changes in its CD spectra (not shown), and the addition of phosphatidylcholine to fragment 11 produced only minor disturbances on the CD spectra of fragment 11 (not shown). At pH 7.6, the CD spectra could not be obtained because of aggregation of fragment 11 or of fragment 11 plus PS.

**Inhibition of Cell-to-Cell Fusion of pG Gene-transfected Cells by Antibodies to p2 and Surrounding Segments**—Because, in general, studies with peptides in isolation (such as those mentioned above) are difficult to interpret and sometimes the results bear little relation to what the corresponding sequences do in the context of the native protein, attempts were made to show the relevance of fragment 11 to VHSV fusion.

Because EPC cell monolayers transfected with a plasmid coding for the pG of VHSV caused about 25% of nuclei to be in syncytia when incubated at pH 6, an assay was designed to estimate the fusion in the presence of Abs to the selected segments around p2. To best interpret those experiments, binding of anti-peptide Abs to solid-phase VHSV pG were first studied at neutral (pH 7.6) and at fusion pH (pH 6). Thus, all of the anti-peptide Abs recognized each of their target pG segments in VHSV at both pH values. However, at low pH, Abs to fragment 11 plus PS.
most of its phospholipid-related activities. To make the microphotographs, EPC cell monolayers were treated with 20 μg of fragment 11/ml for 60 min at pH 6 and 14 °C and then stained with FITC-labeled annexin V and PrIo, detached with 10 mM EDTA in PBS, and analyzed by flow cytometry. Percentage of cells positive for PS and negative for PrIo was determined from the cells stained with FITC-labeled annexin V and PrIo, detached with 10 mM EDTA in PBS, and analyzed by flow cytometry. Percentage of FITC-labeled annexin V cells (% of total monolayer) was about 100%

**DISCUSSION**

**Participation of the Fragment 11/p2 in VHSV Fusion**—Of all of the selected segments p9, p2, p3, p4, and fragment 11 (p9 + p2), fragment 11 showed the highest anionic phospholipid aggregation specific activity and was the only one to induce non-infected cell-to-cell fusion and to translocate PS from the inner to the outer leaflet of the plasma membrane. Although PS translocation might be expected from a peptide interacting and destabilizing a membrane, to our knowledge it has not been described before for any viral derived peptide. Fragment 11 contained 84.4% β-sheet structure at the low pH required for most of its phospholipid-related activities.

Anti-fragment 11 inhibition of pG-transfected cell-to-cell fusion at low pH confirmed the implication of the fragment 11 domain in fusion. Fragment 11 showed different reversible pH-dependent conformations as shown by dramatic and reversible changes in its solubility and by changes in its IR or CD spectra (from 5.8 to 25.4–34.4% β-sheet content from neutral to low pH). It is likely that similar conformational changes in fragment 11 would contribute to its pH-dependent exposure at the surface of pG during fusion as suggested by its increased recognition and inhibition of fusion at low pH with anti-fragment 11 and anti-p2 Abs. Exposure of a previously buried region at the surface of pG at the low pH of fusion had been demonstrated before for rabies virus (8) and for vesicular stomatitis virus (36). Although the Abs mentioned earlier might also inhibit fusion by steric hindrance, the anti-fragment 11 and anti-p2 inhibition of fusion, together with all the other results of this work, strengthens the participation of fragment 11 and of p2 in fusion.

Although the addition of p2 at low pH to an EPC monolayer spreads the cells (most probably due to its insertion into the membrane) (1), only fragment 11 induced both spreading (Fig. 3A) and cell-to-cell fusion of the cells (Fig. 3C). However, fragment 11 was about 200-fold less efficient as pG in inducing cell-to-cell fusion. The estimated fragment 11 fusion-specific activity, however, can be at least 3–6-fold higher due to the absence of inert aggregates in the preparations of fragment 11 at the pH of fusion. Nevertheless, the results suggest that VHSV fusion requires not only the fragment 11 domain but also of some other domain(s) of pG, similar to what occurs in vesicular stomatitis virus, in which efficient viral cell fusion required not only fusion (12) but also carboxyl-terminal (10) and transmembrane (37) domains.

Since one molecule of p2 binds 15–17 molecules of anionic phospholipids (1) and a 1:20 molar ratio was required to form aggregates between fragment 11 and anionic phospholipids, we can speculate about the number of molecules participating in the fusion process involving pG trimers. Thus, assuming a similar situation in VHSV as in rabies virus fusion, where six pG trimers form the minimal fusion complex (8), the penetration of p2 in the membranes would cause a local increase of both p2 (6 × 3 p2 domains per fusion complex) and anionic phospholipids (270–360 anionic phospholipid molecules/fusion complex). The translocation of all of these molecules of anionic phospholipids from the inner to the outer leaflet of the membrane by the fragment 11 domains of a minimal fusion complex would cause a high local destabilization in most membranes, making them easier to fuse, as occurs in pH-sensitive liposomes (28).

**Participation of p9, p3, and p4 in VHSV Fusion**—Given the low affinity of fragment 11 for phosphatidylycholine, an external membrane phospholipid, the initial interaction of fragment 11 with the cellular membranes, contrary to what occurs with the anionic phospholipid vesicle models, must involve some protein-protein interactions. Most probably, those interactions are p9-dependent, since in fragment 11 only the p9 part does not bind phospholipids (Fig. 2 and CD spectra) and is free to interact with other peptide(s), at least after p2 has penetrated into the membrane. Also the covalent union between p9 and p2 must play an important part in the aggregation of fragment 11 at neutral pH, since there is no aggregation of the isolated p9, p2, or p9 + p2 parts of fragment 11 (not shown). Furthermore, aggregated PS vesicles were obtained with fragment 11 with 5-fold more molar specific activity than with p2, suggesting that some of the observed differences in PS aggregation by fragment 11 might be due to p9 interactions. On the other hand, it is difficult to explain how fragment 11 would undergo...
a reverse orientation in the membrane to cause PS translocation in the absence of additional pG sequences to aid in the process without the participation of p9. Therefore, the results suggest that p9 adds some peptide-peptide interactions to fragment 11, underline the importance of p9, and make it a good candidate to bring cellular membranes closer to each other before fusion.

The electrostatic attraction between the positively charged amino acids of p2 and the negatively charged phospholipids in the cytosol-facing monolayer of the endosome membrane could provoke the extension of the β-sheet structure of fragment 11 (CD of fragment 11 in the presence of anionic phospholipids). Because p9 (no changes in CD spectra and negative Pepscan PIPP binding) or p4 (high hydrophilicity and negative Pepscan PIPP binding) did not interact with anionic phospholipids, only p2 (1), p3 (noncharged amino acids and some Pepscan PIPP binding), and the putative fusion domain (some Pepscan PIPP binding) might be favored to get inside the hydrophobic part of the membranes. Inhibition of the penetration of the membrane by fragment 11/p2 could thus explain the inhibition of syncytia formation by anti-fragment 11/p2 Abs, whereas inhibition of p4 conformational changes required for fusion could explain the inhibition of syncytia formation by anti-p4 (Fig. 6).

In VHSV, as in other rhabdoviruses and contrary to other enveloped viruses, the putative fusion domain that inserts itself into the membrane remains uncleaved during fusion, is not hydrophobic, and contains a cysteine, and the low pH-induced conformational changes accompanying fusion are reversible. The reversible insertion of an internal fusion domain into a membrane bilayer would require at least one bending or a U structure. Since the low pH reversible conformational changes in fragment 11 described in this work correlate with the reversible low pH conformational changes of pG required for fusion in rhabdoviruses, the fragment 11 domain fulfills the theoretical expectations mentioned above for an internal fusion domain. On the other hand, if p2 penetrates the hydrophobic part of the membrane with a β-sheet extended structure (1), it has to carry with it both the p3 and the disulfide-bound putative fusion domain, while according to the results obtained, both p9 and p4 should remain outside the membrane.

Comparison of Fragment 11 Sequences in Other VHSV and
Rhabdoviruses—Only six amino acid positions in the fragment 11 sequences from eight VHSV isolates showed variation. Most of them were conservative (I71T, T80A, R81K, R81Q, T90N, A96V, and S97N), demonstrating the highly conserved nature of these sequences among different strains of VHSV. On the other hand, there were no amino acid identities in the fragment 11-like sequences from 14 animal rhabdoviruses except the highly conserved cysteine-proline Cys\(^\text{64}\) and Pro\(^\text{65}\) in VHSV, and a glycine (Gly\(^\text{98}\) in VHSV) situated at the end of the fragment 11-like sequence. All of the fragment 11-like sequences were found between two highly conserved cysteines, C\(_\text{I}\) (C\(_\text{I}\) bridged to C\(_\text{XII}\), which in VHSV corresponded to Cys\(^\text{64}–\text{Cys}^\text{315}\)) and C\(_\text{II}\) or C\(_\text{III}\) (C\(_\text{III}\) bridged to C\(_\text{V}\), which in VHSV corresponded to Cys\(^\text{110}–\text{Cys}^\text{152}\)). C\(_\text{II}\) was absent in cold water fish rhabdoviruses, and C\(_\text{III}\) was absent in rabies-like rhabdoviruses. The disulfide bridge between C\(_\text{III}\) and C\(_\text{V}\) (or between C\(_\text{II}\) and C\(_\text{IV}\) in rabies-like viruses) brings together both putative phospholipid-binding and fusion domains in all rhabdoviruses.

**Main Conclusions and Future Work**—In contrast to all of the pG segments studied, only fragment 11 when added at low pH was able to induce syncytia or to produce significant translocation of PS, suggesting a major participation of fragment 11 domain in VHSV fusion. Comparison of the effects of mutations in both fragment 11 and in the putative fusion domains (3, 21) of VHSV would assess its relative importance during fusion. On the other hand, the fragment 11 capacity to disrupt membranes only at low pH and the presence of its amino- and carboxy-terminal positive charges might serve particular purposes such as serving as a vehicle for DNA transfection (experiments in progress).

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**REFERENCES**


