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Conformation- and Fusion-Defective Mutations in the Hypothetical Phospholipid-Binding and Fusion Peptides of Viral Hemorrhagic Septicemia Salmonid Rhabdovirus Protein G

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Fourteen single and two double point mutants in the highly conserved region (positions 56 to 159) of the G gene of viral hemorrhagic septicaemia virus (VHSV), a salmonid rhabdovirus, were selected and obtained in plasmids by site-directed mutagenesis. Fish cell monolayers transfected with the mutant plasmids were then assayed for protein G (pG) expression, conformation-dependent monoclonal antibody (MAb) reactivity, and cell-cell fusion. Some mutations located in the phospholipid-binding p2 peptide (positions 82 to 110; mutants P86A, A96E, G98A, and R107A) abolished both MAb recognition and fusion activity, while others (P79A, L85S, and R103A) abolished MAb recognition but retained fusion at similar or lower pHs compared to those for the wild type. Phospholipid-binding assays of p2-derived synthetic peptides suggested that phosphatidylserine binding was not affected by the mutations studied. On the other hand, three (P79A, L85S, and T135E) of the four mutants retaining fusion activity mapped around two locations showing amino acid variation in 22 VHSV isolates and in neutralizing MAb-resistant mutants described previously. Mutations located in the hypothetical fusion peptide (positions 142 to 159; mutants F147K, P148K, and W154K) abolished both MAb recognition and fusion activity. The existence of mutants with altered conformation and defective fusion in both p2 and fusion peptides provides further evidence in favor of the participation of these and adjacent regions in some of the steps of the VHSV fusion processes, as suggested by previous studies. In addition, because the studied region induced strong immunological responses in trout, some of the mutants described here might be used to design attenuated VHSV vaccines.

Rhabdoviruses cause highly damaging diseases in fish reared by the worldwide salmon culture industry. Among fish rhabdoviruses (novirhabdoviruses), the viral hemorrhagic septicemia virus (VHSV), which originated in Europe but has recently been found in America (26, 41), affects not only salmonids but also cod, turbot (38), sea bass, eels, flatfish, and shrimp (8, 24, 27). Despite many efforts, including successful DNA vaccines at the laboratory level, a commercial vaccine against VHSV is not yet available (1, 18, 25, 30).

The whole genome of VHSV has been sequenced (23), and the disulfide structure of its protein G (pG) has been elucidated (12). Reverse genetic methods have been developed for the VHSV-related rhabdovirus infectious hematopoietic necrosis virus (5–7), which could allow the design of new vaccines. The study of rhabdovirus fusion in the VHSV model could be important in the design of alternative vaccines to fight these diseases.

The pG mutants of vesicular stomatitis virus (VSV), a well-studied mammalian rhabdovirus, with an altered extent of fusion and/or optimal fusion pH in vitro, had mutations located either in the fusion peptide or in carboxy-terminal regions affecting the low-pH conformational changes required for fusion (39, 40, 43). Alignment of the pG sequence of VSV with those of 14 other animal rhabdoviruses representing vesiculoviruses, lyssaviruses, ephemeroviruses, and novirhabdoviruses, made it possible to predict the locations of hypothetical fusion peptides in these other rhabdoviruses including VHSV (42). According to that model, the expected fusion peptide of VHSV could be located between positions 142 and 159.

On the other hand, evidence obtained by the use of synthetic and recombinant peptides of the pG of VHSV suggested that the sequence from positions 56 to 110 (frg11), containing non-canonical heptad repeats (11) and the phospholipid-binding peptide (p2), may also be involved in fusion (14, 15, 32). For instance, recombinant frg11 showed dramatic changes in both solubility and β-sheet conformation at low pH and induced low-pH-dependent cell-cell fusion by itself when added to cell monolayers (15). Furthermore, some VHSV pG mutants (mutated in the sequence from positions 118 to 161) obtained on the basis of resistance to neutralization by monoclonal antibody (MAb) C10, despite having alterations in its conformation, retained fusion activity (21). MAb C10 (37) or antipeptide antibodies anti-frg11, anti-p2(82–109), and anti-p4(123–144) (15) inhibited fusion, with all of the above data suggesting that these regions should be implicated in fusion. Because there is a disulfide bond between positions 110 and 152 (12), the p2 and fusion peptides must occupy nearby positions in the native pG of VHSV. However, there is no direct evidence for the participation of these two peptides in fusion.

In this study, G genes with mutations in the sequence encoding p2, as well as the peptide proposed by others to be the putative fusion peptide and in regions in between, have been obtained, expressed in the membrane of a fish cell line, and then assayed for reactivity with conformation-dependent MAb and cell-cell fusion at different pHs. The study has identified four mutations that, although eliminating MAb reactivity, re-

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sulted in mutants that retained some fusion activity similarly to the MAb C10-resistant mutants (21). Three of these mutations mapped around two pG locations, showing amino acid variations among VHSV isolates. Because 40% of the VHSV-immunized trout strongly recognized linear epitopes on these regions (15, 17, 35), the mutants described here might be useful in attempts to design attenuated VHSV strains to be used in DNA vaccines (2, 3, 18) or in vaccines obtained by reverse genetic methods (5, 6).

MATERIALS AND METHODS

In vitro site-directed mutagenesis. The in vitro site-directed mutagenesis Quick-Change technique (Stratagene, La Jolla, Calif.) was used to generate the mutated G genes in a supercoiled double-stranded pGEMTeasy plasmid containing the wild-type G gene (provided by M. Bremont, Institut National de la Recherche Agronomique, Jouy en Josas, France) (5). Two complementary primers of 15 nucleotides each containing the respective mutations were designed for each mutant. In all cases, primers were extended during thermal cycling with *Pfu* turbo DNA polymerase (Stratagene), generating a mutant plasmid in a nicked-circle form. Then DpnI endonuclease was added to digest the parental methylated DNA, and the nicked plasmid incorporating the desired mutation was introduced into XL1-Blue cells (Stratagene). The mutated pG gene mutants were subcloned into the EcoRI site of pMCV1.4 (ReadyVector, Madrid, Spain) (36,37) by using standard methods with *Escherichia coli* Top10 (16). Large amounts of plasmid were prepared by using the Wizard Megaprep DNA purification system (Promega, Madison, Wis.). Plasmid solutions were adjusted to 0.5 to 1 mg of total DNA per ml (absorbance at 260 nm). The mutated sequences were confirmed by sequencing plasmid DNA across the changed region in both directions.

Transfection of EPC cells with mutated plasmids. Epithelioma papulosum cyprini (EPC) cells (19) were grown at 28°C in 96-well plates containing RPMI Dutch medium, 20 mM HEPES, and 10% fetal calf serum (100 μl per well). The cells were transfected (~100,000 cells/well) with 0.3 μg of the different pMCV1.4-G mutants previously complexed with 0.5 μl of Fugene 6 (Roche, Barcelona, Spain) (28, 36) and incubated at 20°C under 5% CO_2 during 2 days.

Staining of the transfected EPC cell monolayers. After transfection, EPC cell monolayers were stained with anti-pG polyclonal Abs (PAbs) obtained in rabbits (provided by N. Lorenzen, Danish Institute for Food and Veterinary Research, Århus, Denmark) (29), in culture medium plus 2% rabbit serum, 2% goat serum, and 2% *E. coli* extract for 1 h after permeabilization with 2-perm (BD-Biosciences, Becton-Dickinson Madrid, Spain) (to estimate the cytoplasmic expression) or without permeabilization (to estimate the membrane expression). The cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit $F(ab)_2$ fragment (FITC-GAR) (Caltag, San Francisco, Calif.), washed, and either observed under an inverted fluorescence microscope (cytoplasm expression) or detached with fluorescence-activated cell sorter (FACS) buffer (Beckton-Dickinson) and analyzed by flow cytometry (FL1 region, 514 to 545 nm, green) in a Beckton-Dickinson (San Jose, Calif.) FACScan apparatus using the LYSYS II program (membrane expression). Background fluorescence profiles were obtained using nontransfected EPC cells and varied slightly from experiment to experiment. To calculate the percentage of fluorescent cells for each experiment, the following formula was used: [(area under the curve obtained with transfected cells − area under the curve obtained with transfected cells overlapping with the background curve)/total area under the curve obtained with transfected cells] × 100. To calculate fluorescence intensities for each experiment, the background fluorescence peak value was subtracted from the fluorescence peak value obtained with transfected EPC cells. The fluorescence intensity was expressed in fluorescence relative units (FRU).

Transfected EPC cell-cell fusion assays. To perform the cell-cell fusion assays, EPC cells plated in 24-well plates (~500,000 cells/well) were transfected with 0.6 μg of the different pMCV1.4-pG mutants complexed with 2 μl of Fugene (16, 28, 34) and incubated at 20°C. At 2 days later, the transfected cell monolayers were incubated for 15 min in RPMI Dutch culture medium plus 20 mM HEPES and 20 mM morpholineethanesulfonic acid (MES) (Sigma Chemical Co., St.Louis, Mo.) at different pHs (5.0, 5.3, 5.6, 6.0, 6.3, 7.0, and 7.3) at 20°C. Syncytium formation assays could not be performed at pHs lower than 5.0 because of detachment of the EPC cell monolayers. The monolayers were then incubated for 2 h at pH 7.6, fixed with cold methanol, washed, dried, and stained with Giemsa (37). The results were expressed as the percentage of nucleic in syncytia calculated by the formula: (number of nuclei in syncytia of three or more cells per syncytium/number of nuclei) × 100.

Phospholipid-binding assays. To assay for phospholipid binding, 100 μl of 0.1-mg/ml synthetic peptides (Chiron-Mimotopes, Victoria, Australia) per well (1 μg per well) were dried in 96-well plates as described previously (13, 14). Labeled L-3-phosphatidyl-[L-C-3–^{14}C]serine (PS) (55 mCi/mmol; Amersham, Little Chalfont, England) was dried under vacuum in glass tubes and sonicated in 0.1 M phosphate-citrate buffer (pH 7.6) (22). The labeled PS was then added at 100 μl per well to the solid-phase peptides (200 pmol per well). After 4 h of incubation at 20°C, the plates were washed and extracted with 100 μl of 2% sodium dodecyl sulfate per well in 50 mM ethylenediamine (pH 11.5) at 60°C for 30 min. The supernatants were then pipetted into 96-well polyethylene terephthalate plates containing 100 μl of Hiload scintillation liquid (LKB, Loughborough, England) per well and counted on a 1450-Microbeta scintillation counter (Wallac, Turku, Finland). The background binding obtained in the absence of peptides (1.25 pmol per well) was subtracted from all the data, and the counts were transformed into picomoles of PS.

RESULTS

Selection of point mutations. A comparison was performed among the pG sequences from 22 VHSV isolates to select which point mutations to introduce on the G gene. The amino acid sequences corresponding to the hypothetical phospholipid-binding and fusion peptides (positions 56 to 159) from the 22 isolates were obtained from the GenBank (accession numbers A10182, AB060725, AB060727, AF143862, AF345857, AF345858, AF345859, AJ233396, NC000855, U28799-1, U28799-2, U28747, U88056, U28800, U88050, U88051, U88052, U88053, U88054, U88055, X73873, and X66134). Amino acid translated sequences were highly conserved among the isolates. Amino acid variations among VHSV isolates were concentrated mainly at two locations around positions 80 and 140 (Fig. 1). Thus, most of the changes were found at position R81, which changed to Q or K, in 16 isolates and at position D136, which was changed to N, in 14 isolates. Less abundant amino acid variations, found in two to four isolates, were also observed at positions 71, 80, 97, 112, 118, 138, and 139. Positions at which amino acid variations were detected were excluded from the mutant design, because altered fusion activities have not been reported in any of these isolates.

The positions selected for mutation were changed to A when possible or to an amino acid with different physicochemical properties from those of the original position, depending on the possibilities for each nucleotide change. The positions selected in the hypothetical phospholipid-binding peptide (p2+frg11) included the helix-breaking, highly conserved P and G (P65, P79, P86, and G98) and the charged arginines located in its carboxy-terminal part (R103 and R107). All these were changed to an A. Other positions selected in amino acids, belonging to some of the noncanonical hydrophobic heptad repeats (I82, L85, and A96), were changed to hydrophilic (S) or charged (E) amino acids. Positions F115 and T135, located between the hypothetical phospholipid-binding and fusion peptides, were mutated to a charged amino acid (K and E, respectively). Because the hydrophobic amino acids F147, P148, and W154, located in the putative fusion peptide motif (F/Y)PXPXXCX(W/F), were conserved among 14 animal rhabdoviruses (42), these were also mutated in VHSV to a charged amino acid (either E or K).

Expression of the pG mutants in EPC transfected cells. All pG mutant-containing plasmids obtained for VHSV were expressed in the cytoplasm of permeabilized transfected EPC

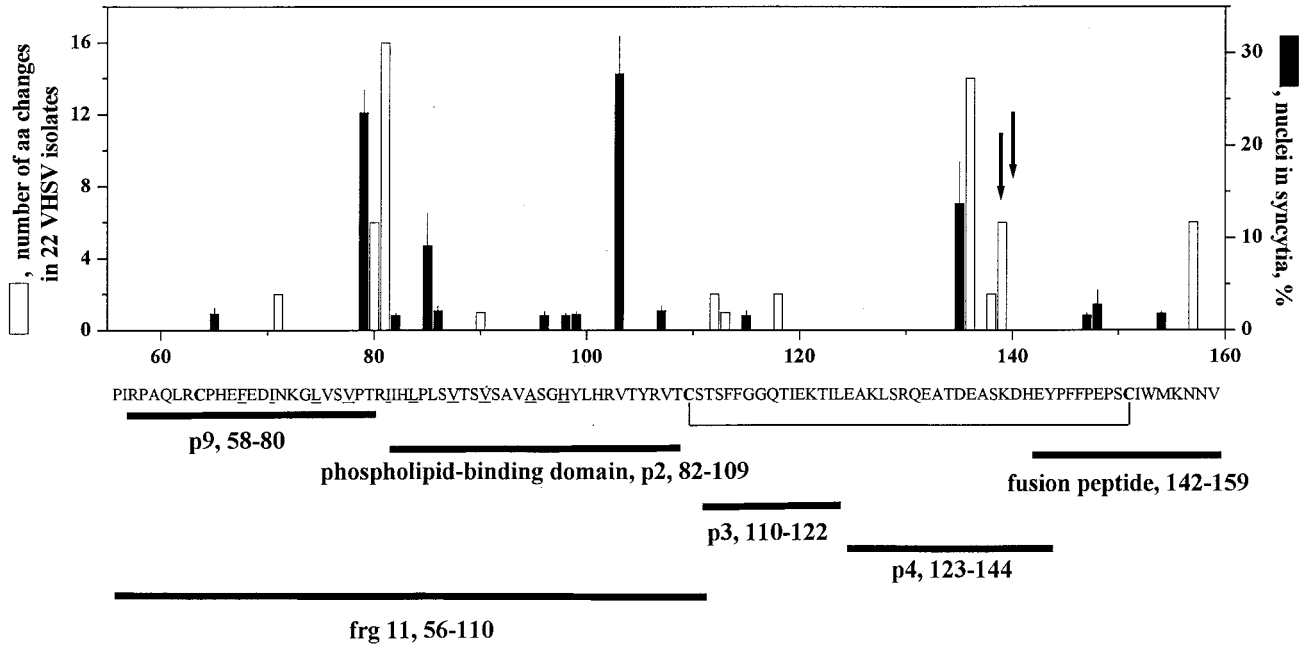

FIG. 1. Location of the amino acid changes in 22 isolates of VHSV and percentage of nuclei in syncytia of mutants in the region from amino acids 56 to 159 of the pG of VHSV. Cysteines are shown in bold. The disulfide bridge between C-110 and C-152 is represented as horizontal line connecting the two cysteines (12). The positions and locations of p9, p2 (phospholipid-binding domain), p3 (cold water fish rhabdovirus conserved sequence), p4 (hydrophilic loop), and frg11 (p9 + p2) sequences in pG, as defined in previous work (15), and of the putative fusion peptide (42) are indicated by thick horizontal lines. Vertical arrows indicate the locations of mutants resistant to MAb C10 at positions 139 and 140 that did not abolish fusion (21). Hydrophobic heptad repeat amino acids are underlined (11).

cells as verified by direct immunofluorescence with anti-G PAbs (Table 1).

Table 1 shows that the percentage of transfected EPC cells expressing pG in their membranes, estimated after averaging the results from the two to six replicates for each mutant, varied between 42.5 and 77.2% (except for I82S, which was not expressed). An estimated 53.5% ± 11% of the EPC cells transfected with the wild-type G gene expressed pG in their membranes. Similarly, pG was expressed in 50.2 to 77.3% of the EPC cells transfected with the mutants P65A, L85S, P86A, P86AG98A, G98A, G98AH99S, R103A, R107A, F115K, P148K, and W154K. Mutants P79A and A96E were not as efficiently transfected as the rest of the mutants (42.5 and 44.5%, respectively, of the transfected EPC cells), and the expression of mutant I82S in the membranes of transfected EPC cells was very low or not significantly different from the background level (1.3% ± 0.3% of the transfected cells).

For each mutant, Fig. 2 shows nontransfected and transfected FACS-stained cell profiles representative of the two to six replicates indicated in Table 1. Because the fusion efficiency depends strongly on the pG density at the cell surface, we estimated the relative level of expression per cell from the FACS profiles, assuming that the Ab recognizes all of the mutants equally. Since the background obtained with nontransfected EPC cells (grey curves in the graphs) varied slightly from one experiment to another, to compare the expression of the pG among the mutants, the area overlapping with the background was subtracted from the fluorescence for each experiment. The average intensity of the FACS fluorescence was then calculated from the replicates. The estimated intensity obtained for the wild-type pG was 18.7 ± 4.1 (n = 6) FRU, and the intensity for the rest of the mutants varied only from 10.8 to 22.5 FRU, except for the I82S mutant (Table 1).

Because, in contrast to what occurs in VSV, a proteolytic assay for low-pH-induced conformational changes in the pG of VHSV is not available, we used an assay of binding to conformation-dependent neutralizing MAbs as an estimation of possible conformational changes induced by mutation. Correct folding of pG was therefore analyzed by using conformation-dependent MAb C10, which binds simultaneously to positions 140 and 433 (4, 21), and MAb 2F1A12 which maps at position 253 (N. Lorenzen, personal communication). An estimated 21.6% ± 9% of the EPC cells transfected with the wild-type pG gene expressed the MAb C10 epitope in their membranes. In contrast, only 0.3 to 1.6% of the EPC cells transfected with any the mutants expressed the MAb C10 epitope (Table 1). Similar results were obtained with MAb 2F1A12 (data not shown).

EPC transfected cell-to-cell fusion assays. Figure 3 shows the typical appearance of syncytia and the fusion kinetics obtained in G-gene-transfected cell-cell assays for the wild-type G gene and its mutants. Under the present experimental conditions, wild-type fusion was maximal at pH 5.6 and decreased to about 70% at pH 6.0 and to 0% at pH 6.6. Only EPC cells transfected with the mutants P79A, L85S, R103A, and T135E showed fusion activity. Mutants R103A and T135E showed maximal fusion at pH 5.0, and the percentage of nuclei in syncytia were reduced to 27.7% ± 4.1% and 13.7% ± 4.5%, respectively. Mutants P79A and L85S showed maximal fusion at pH 5.3 to 5.6, and the percentage of nuclei in syncytia was

TABLE 1. Cytoplasmic and membrane expression of pG and induced nuclei in syncytia at the optimal pH in mutant pG-transfected EPC cells

Domain	Mutant	IF[a]	FACS of anti-pG PAb[b]			MAb C10[d]		Fusion		
			Stained cells (%)	Fluorescence intensity (FRU)	*n*	Stained cells (%)	*n*	pH	% of nuclei in syncytia[c]	*n*
	Wild type	+	53.5 ± 11	18.7 ± 4.1	6	21.6 ± 9	6	5.6	44.7 ± 8.1	3
Upstream of p2	P65A	+	54.1 ± 8	13.3 ± 3.1	3	0.9 ± 0.1	4		1.8 ± 0.6	3
	P79A	+	42.5 ± 10	13.1 ± 3.6	4	0.3 ± 0.1	3	5.3	23.5 ± 2.4	2
Phospholipid-binding peptide, p2 (82–110)	I82S	+	1.3 ± 0.3	0	3	1.6 ± 0.6	4		1.6 ± 0.2	2
	L85S	+	51.6 ± 29	13.6 ± 1.1	2	0.5 ± 0.1	3	5.0	9.2 ± 3.5	2
	P86A	+	56.8 ± 15	13.7 ± 5.8	4	1.1 ± 0.6	3		2.1 ± 0.5	3
	P86A G98A	+	50.6 ± 13	12.2 ± 3.5	4	1.4 ± 0.1	4		1.4 ± 0.3	3
	A96E	+	44.5 ± 19	13.3 ± 3.3	3	1.2 ± 0.6	3		1.6 ± 0.4	2
	G98A	+	77.2 ± 9	13.2 ± 4.7	2	0.6 ± 0.2	3		1.6 ± 0.2	2
	G98A H99S	+	71.5 ± 4	22.5 ± 2.5	2	0.5 ± 0.3	2		1.7 ± 0.3	2
	R103A	+	64.7 ± 14	11.7 ± 3.2	2	1.6 ± 0.8	2	5.0	27.7 ± 4.1	2
	R107A	+	63.5 ± 23	13.1 ± 2.1	2	0.9 ± 0.3	2		2.1 ± 0.5	2
Downstream of p2	F115K	+	52.0 ± 23	10.8 ± 3.4	3	0.4 ± 0.2	2		1.6 ± 0.5	3
	T135E	+	55.6 ± 20	11.6 ± 8.4	3	1.3 ± 0.8	2	5.0	13.7 ± 4.5	3
Fusion peptide (142–159)	F147K	+	69.0 ± 1	11.5 ± 0.5	2	1.3 ± 0.3	3		1.6 ± 0.2	2
	P148K	+	50.2 ± 2	22.5 ± 2.5	2	1.5 ± 0.3	2		2.8 ± 1.5	2
	W154K	+	76.3 ± 13	13.1 ± 1.8	2	1.0 ± 0.7	3		1.8 ± 0.2	2

[a] G-gene expression in the cytoplasm of the transfected EPC cells was estimated by immunofluorescence (IF) with anti-pG PAbs. The results are given as + when fluorescence was detected above the background obtained in nontransfected EPC cells.

[b] Results are given as means ± standard deviations of the percentages of cells stained with the indicated PAbs, determined by comparison with the numbers of cells stained with the same PAbs in nontransfected EPC cells (Fig. 2). The mean ± standard deviations of the fluorescence intensity of the transfected cells relative to the fluorescence intensity of the nontransfected cells was estimated in arbitrary FRU.

[c] Results of the fusion assay at the maximal fusion pH (Fig. 3) are given as percentage of nuclei in syncytia (syncytia of at least three cells per syncytium; $n \approx 1{,}300$, four fields per well at ×100). The percentage of nuclei in syncytia in a nontransfected EPC cell monolayer after transfer to pH 5.6 was 1.3% ($n = 1{,}300$); however, 95% of those detected syncytia were only of three nuclei per syncytium.

[d] MAb C10 maps simultaneously at positions 139 and 140 (4, 21).

also reduced to 23.5% ± 2.4% and 9.2% ± 3.5%, respectively. The P79A and L85A (amino-terminal) and R103A (carboxy-terminal) mutations flank the more internal sequences of the p2 phospholipid-binding domain.

On the other hand, the P86A, P86AG98A, A96E, G98A, G98AH99S, and R107A mutants (with most mutations located inside the hypothetical phospholipid-binding peptide) and the P65A and F115K mutants (with mutations around the phospholipid-binding peptide) were completely defective in fusion at all the pHs studied. Mutants F147A, P148A, and W154A, with mutations located in the highly conserved positions of the hypothetical fusion peptide, were also defective in fusion at all the pHs studied (pH ≥ 5.0).

The I82S mutant although expressed in the cytoplasm, was not detected in the membranes of transfected cells, and therefore no conclusions could be drawn from its defective fusion properties (Table 1).

Phospholipid binding of synthetic peptides corresponding to the p2 region. Since p2(82–109) was the main region of a 51 peptide pepscan of pG which showed PS binding (13,15), single-amino-acid changes were introduced into 15-mer synthetic peptides derived from the p2 sequence to study whether mutations in that region could affect PS binding. The sequence from amino acids 93 to 107 (including the two positively charged R103 and R107 residues) was selected to synthesize the peptides because it showed the maximal PS-binding activity of p2 (13). Each amino acid of this sequence was changed to A, and its effect in solid-phase PS binding was measured. The PS-binding activity of the wild-type sequence was 2.47 ± 0.34 pmol of PS per μg of peptide (Table 2). The PS-binding activity varied only from 2.1 ± 0.46 to 4.1 ± 0.53 pmol of PS per μg of peptide among the 15 synthetic peptides with single amino acid changes.

Because both ionic and hydrophobic interactions participate in PS binding by p2 (14), synthetic peptides were then obtained in which more drastic changes in the charged R positions were introduced. One of the positions 103 or 107 could be changed to K without significantly changing PS binding (2.5 ± 0.42 or 2.6 ± 0.33 pmol of PS per μg of peptide, respectively). PS binding could be lowered only when both R positions were simultaneously changed to either K or E (1.3 ± 0.19 or 0.75 ± 0.36 pmol of PS per μg of peptide, respectively).

The substitution of several amino acids by a series of A residues at positions 104 to 106, 95 plus 104 to 106, and 99 to 102 plus 104 to 106 also decreased PS binding to 2.1 ± 0.28, 1.69 ± 0.29, and 0.69 ± 0.21 pmol of PS per μg of peptide, respectively.

DISCUSSION

VHSV pG mutants with conformational changes and defective, reduced, or pH-altered fusion have been obtained in the hypothetical p2 phospholipid-binding and fusion disulfide-bonded peptides (12). Because the existence of similar VSV

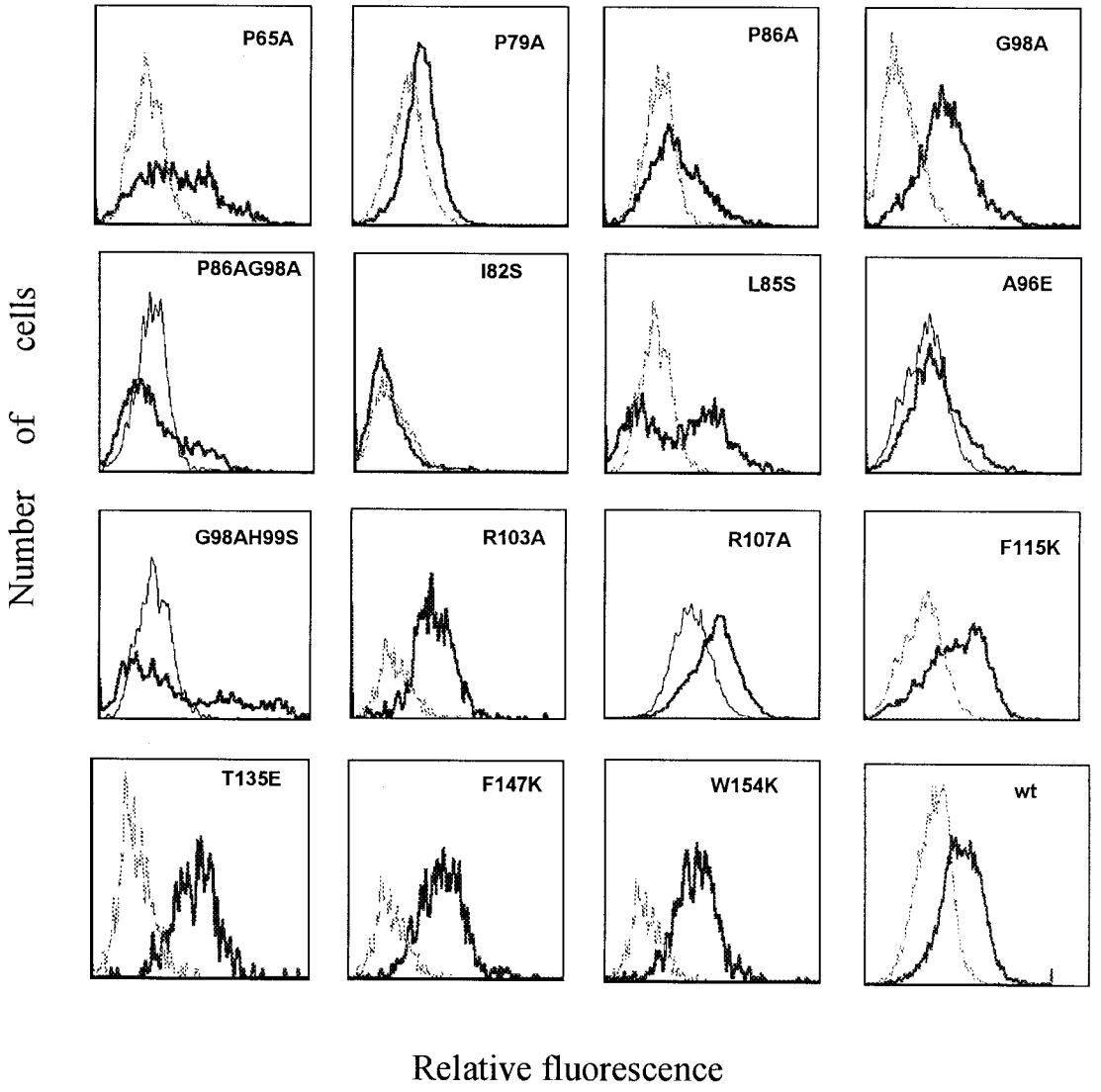

FIG. 2. Representative FACS profiles obtained by staining nontransfected and mutant pG-transfected EPC cells with anti-G PAbs. EPC cell monolayers were transfected with the pMCV1.4 plasmids coding for each of the pG mutants. Nontransfected EPC cell monolayers were prepared in parallel. Two days later, both the nontransfected and transfected EPC cell monolayers were stained with anti-G PAbs and FITC-GAR. The cells were then detached from the monolayers and analyzed by FACS. The experiments were repeated two to six times per mutant. The result of a representative experiment is shown in the figure, while the mean and standard deviations are shown in Table 1. The mutant P148K was omitted from the figure for best presentation. Relative fluorescence is in logarithmic units. Grey lines indicate nontransfected EPC cells; black lines indicate transfected EPC cells. wt, wild type.

fusion-defective or fusion-reduced mutants with an acid shift in the optimal pH has been previously interpreted as an indication of the role in fusion of these mutated positions (20, 33, 39, 40), we may also conclude that the regions mentioned above also participate in VHSV fusion processes. Previous results of studies of either penetration in membrane models by isolated p2 at the fusion pH (32) or inhibition of fusion obtained with antipeptide Abs corresponding to different parts (p2, frg11, and p4) of the region of the pG of VHSV from positions 56 to 144 (15), are also consistent with the participation of both peptides in some of the steps of VHSV fusion. However, since all the VHSV mutants studied have alterations in their pG reactivity with conformation-dependent MAbs, it is also possible that the mutations primarily affect the conformation of the pG and that this conformational difference is responsible for the observed alterations in fusion activity.

Despite the alteration in MAb C10 binding, the P79A, L85S, R103A, and T135E mutants were still able to undergo the low-pH conformational changes that must precede fusion, although P79A fused at only 50% of wild-type pG levels at 0.3 pH unit lower while the others required pH 5.0 (or lower) to achieve 25 to 50% fusion. In a similar way, VHSV mutants resistant to neutralization by MAb C10, which have lost their binding to MAb C10, were still able to perform fusion, and therefore, the mapped epitopes of MAb C10 may be related to VHSV fusion (21). The VHSV mutants in which some fusion

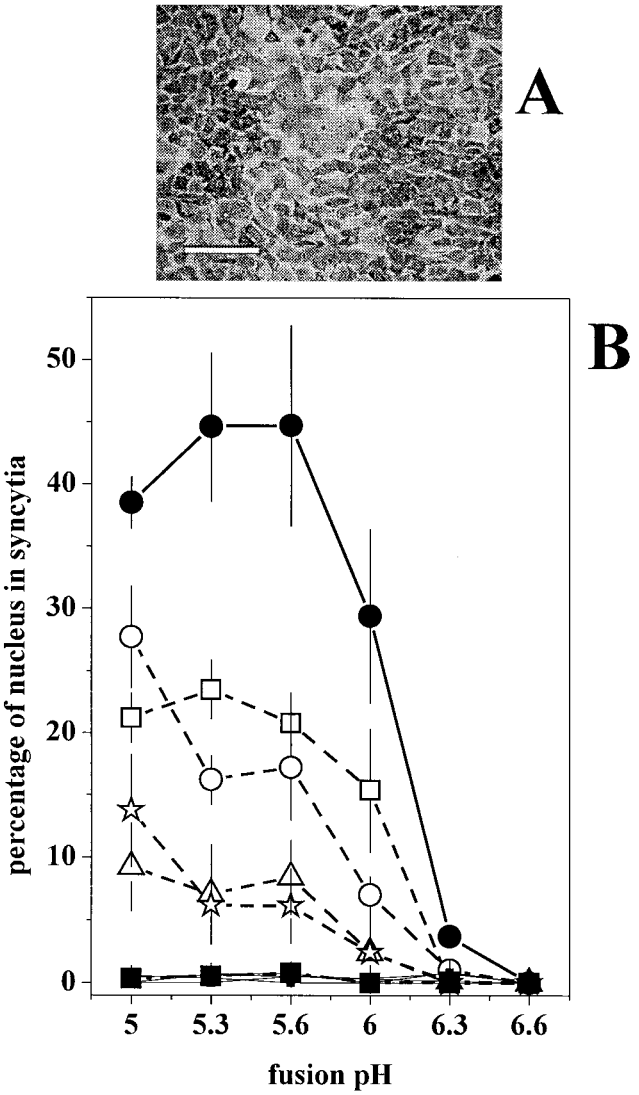

FIG. 3. Appearance of syncytia (A) and percentage of nuclei in syncytia induced by low pHs in EPC cells transfected with the pMCV1.4 mutant pG (B). EPC cell monolayers were transfected with the pMCV1.4 plasmids coding for each of the pG mutants. Two days later, the cell culture medium was replaced with medium at different pHs for 15 min and then with medium at pH 7.4 for 2 h. Monolayers were fixed and stained, and the number of nuclei in syncytia were counted (n = 1,300). Averages of two or three experiments per mutant are represented in the figure. ●, wild type; □, P79A; ○, R103A; △, L85S; ☆, T135E; ■, P86A, P65A, P86A G98A, R107A, F115K, F147K, W154K, P148K, I82S, and A96E.

was retained had mutations either flanking the innermost core of p2 (P79A, L85S, and R103A) or mutations in the hydrophilic loop (p4) between the p2 and the fusion peptides (T135E). In all these cases, the change in conformation at physiological pH at position 140 or 433 (as estimated by binding of MAb C10) and 235 (as estimated by binding of MAb 2F1A12) did not abolished fusion. The increase in the binding of MAbs C10 and 2F1A12 to wild-type pG at low pH (31) might indicate that the conformation at low pH required for fusion is less strongly affected by these mutations. Figure 1

TABLE 2. Labeled PS binding of solid-phase mutant p2 peptides (positions 93 to 107)

A position	15-mer sequence[b]	PS binding (pmol of PS/μg of peptide)[a]
93	**A**AVASGHYLHRVTYR	2.17 ± 0.47
94	SAVASGHYLHRVTYR	2.47 ± 0.34
95	SA**A**ASGHYLHRVTYR	2.10 ± 0.46
96	SAVASGHYLHRVTYR	2.47 ± 0.34
97	SAVA**A**GHYLHRVTYR	1.82 ± 0.50
98	SAVAS**A**HYLHRVTYR	2.66 ± 0.16
99	SAVASG**A**YLHRVTYR	2.41 ± 0.15
100	SAVASGH**A**LHRVTYR	2.99 ± 0.23
101	SAVASGHY**A**HRVTYR	3.24 ± 0.31
102	SAVASGHYL**A**RVTYR	4.10 ± 0.53
103	SAVASGHYLH**A**VTYR	2.72 ± 0.23
104	SAVASGHYLHR**A**TYR	2.80 ± 0.37
105	SAVASGHYLHRV**A**YR	2.64 ± 0.31
106	SAVASGHYLHRVT**A**R	2.84 ± 0.33
107	SAVASGHYLHRVTY**A**	2.40 ± 0.28
107	SAVASGHYLHRVTY**K**	2.50 ± 0.42
103	SAVASGHYLH**K**VTYR	2.60 ± 0.33
103,107	SAVASGHYLH**K**VTY**K**	1.30 ± 0.19
103,107	SAVASGHYLH**E**VTY**E**	0.75 ± 0.36
104–106	SAVASGHYLHR**AAA**R	2.10 ± 0.28
95,104–106	SA**A**ASGHYLHR**AAA**R	1.69 ± 0.29
99–102,104–106	SAVASG**AAAA**R**AAA**R	0.69 ± 0.21

[a] Radioactively labeled PS was estimated by binding to a solid phase coated with mutant synthetic peptides derived from the p2 partial sequence ^{93}SAVAS GHYLHRVTYR^{107}. Counts bound above background were then converted to picomoles of PS per milligrams of peptide, and averages from two different experiments, each done in triplicate, and the standard deviations are shown.

[b] The amino acid sequences are given in single-letter code. The amino acids mutated are in bold type.

shows that the mutants with fusion activity at position P79 or L85 (around position 80) and position T135 (around position 140) and the mutants resistant to neutralization by MAb C10 (21) were mapped at positions around either amino acid 80 or 140, the two locations around which the number of amino acid changes in 22 isolates of VHSV was the greatest. The location around the amino acid variations in natural isolates of MAb-resistant and site-directed point mutants retaining fusion activity (except position 103) suggests that in order to preserve fusion activity, most amino acid changes allowed in the region from positions 56 to 159 are those around positions 80 and 140.

The reduction in the binding capacity of conformation-dependent MAbs in the VHSV pG mutant might indicate that those mutants are misfolded. Therefore, the mutations would be affecting the conformation of pG, which would be the primary reason behind the observed alterations in fusion activity. It is not possible to determine whether the studied mutations have an indirect effect on fusion due to changes in the pG conformation, a direct effect on its fusion capacity, or both, since none of the VHSV mutants were recognized by the C10 or 2F1A12 MAb and there is not yet any other available VHSV neutralizing MAb (17) or any other assay for pG conformation. Furthermore, it is not yet possible to make a direct comparison with the properties of similar VSV fusion-defective mutants described above. Thus, alterations in the binding of neutralizing or conformation-dependent MAbs by VSV fusion-defective mutants has not been reported yet. On the other hand, no differences were found among VSV wild-type and fusion-de-

fective mutants in the increase of pG resistance to trypsin digestion at low pH (the biochemical assay used for conformational changes) (20, 39, 40). Recognition by conformation-dependent anti-VSV MAbs could also be altered in those VSV mutants, since it is known that conformation is altered extensively along pG during fusion (9, 10).

To study whether mutations in p2 could affect fusion by reducing its phospholipid-binding properties, a series of experiments were performed with mutated synthetic peptides corresponding to p2 sequences showing the highest PS-binding activity, as previously described (13, 14). To decrease the PS binding in this model, more than three amino acid substitutions had to be simultaneously introduced in the native p2 sequence, in agreement with previous indications in which both ionic and hydrophobic interactions were required for maximal PS binding (14). Although not all possible mutations in p2 have been studied, these results made it unlikely that the mutations in the single or double (P86A G98A or G98A H99S) mutants studied could cause a significant reduction in PS binding.

The pG proteins with mutations within the hypothetical fusion peptide (F147A, P148A, and W154A) were completely defective in fusion at all pHs studied, despite being expressed in the membranes of transfected cells at a similar level to those in the wild type or mutants with some fusion activity (Table 1). The F125Y and P126L mutants of VSV showed a 34 and 48% reduction in fusion compared with that obtained with wild-type pG, respectively (39), while in the equivalent F147K and P148K mutants of VHSV, the reduction of fusion was 100%. The lower fusion activity observed in VHSV could be due to the more drastic amino acid changes introduced into the VHSV mutants. Alternatively, it might be due to differences in the conditions of the cell-cell fusion assays (exposure at low pH for 2 or 15 min in VSV and VHSV, respectively).

Because serum from VHSV-immunized trout strongly reacted with solid-phase frg11 (15, 35) by recognizing its linear epitopes (17) and the mutants with mutations in frg11 were expressed in the cellular membrane independently of its conformation, most of the pG fusion-defective mutants described here are likely to induce immune responses in trout. Therefore, some of the mutations described in this work could be used to design attenuated VHSV vaccines, including DNA vaccination with the mutated G gene (2, 3, 18) or recombinant viruses obtained through reverse genetic methods (5, 6).

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ADDENDUM IN PROOF

Amino acid-translated sequences from other 67 G genes of new VHSV isolates (K. Einer-Jensen, P. Ahrens, R. Forsberg, and N. Lorenzen, J. Gen. Virol. **85:**1167–1179, 2004) confirm major amino acid variations to be around positions 80 and 140.

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