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Structure, binding and neutralization of VHSV with synthetic peptides

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Abstract

The phosphatidylserine binding region p2 of VHSV was characterized and was shown to be involved with fusion. Synthetic peptides corresponding to this region interact with phospholipids by penetrating into the membrane and changing to a beta sheet configuration. Computer modeling of this region shows the possible ways by which the interaction with the membranes can succeed. Inhibitory peptides are presently being sought by studying possible interactions within heptad repeats located in other regions of the G protein of VHSV. The heptad repeat region that includes the phosphatidylserine binding domain p2 has been cloned and preliminary experiments show that under certain conditions, peptides from this region can inhibit VHSV infectivity.

Keywords: *Salmonid rhabdovirus*; Inhibitory peptides; Viral peptides

1. Introduction

The glycoprotein G of mammalian and fish rhabdoviruses has been shown to bind to cellular membranes including phospholipids and fuse at low-pH (Gaudin et al., 1993; Lecocq-Xhonneux et al., 1994; Estepa and Coll, 1996a). Inhibition of vesicular stomatitis virus (VSV) infection by phosphatidylserine (PS) (Schlegel et al., 1982) and PS binding by viral haemorrhagic septicemia virus (VHSV) (Estepa and Coll, 1996b) have also been demonstrated. PS-binding to solid-phase 15-mer peptides (pepscan) of the glycoprotein G of VHSV was highest on the region from aa 82 to 109 (p2 peptide) (Coll, 1996, 1997; Estepa and Coll, 1996a,b). At the low pH required for fusion, anti-p2 polyclonal antibodies recognized p2 and VHSV (Estepa and Coll, 1996b) and inhibited PS-binding to VHSV in solution (Estepa and Coll, 1996a) as well as VHSV-induced cell to cell fusion (Estepa and Coll, 1997). Additionally, similar pH dependence profiles were shown by PS-binding to VHSV (Estepa and Coll, 1996a) and by glycoprotein G mediated cell to cell fusion (Lecocq-Xhonneux et al., 1994).

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The p2 peptide contained an a–d hydrophobic heptad-repeat (Coll, 1995a,b), that was completely conserved for other VHSV G published sequences (Thiry et al., 1991a; Lorenzen et al., 1993; Benmansour et al., 1997) and p2-like regions were present in all rhabdoviruses examined (Coll, 1995b) and bound PS (Coll, 1997). Mapping of fusion defective mutants and hydrophobic photolabeled regions of VSV and/or of rabies (Gaudin et al., 1995) showed those to be situated adjacent to the p2-like regions, thus suggesting that p2-like regions and fusion might be functionally related in all rhabdoviruses.

Synthetic peptides from similar N-terminal heptad repeats (N-peptides) adjacent to the fusion peptide (Owens et al., 1990; Wild et al., 1992) and from the C-terminal region (C-peptides) of the glycoprotein of HIV (Wild, et al., 1993; Wild et al., 1995) blocked in vitro viral infection and syncytia formation. The antiviral activity of these peptides seemed to be due to the ability to associate with each other in the glycoprotein sequence and to inhibit the conformational changes needed for fusion (Chen et al., 1995). Three N-peptides form an interior coiled-coil trimer while three C-peptides pack outside as deduced by X-ray crystallography, thus adding some evidence to the hypothesis mentioned above (Chan et al., 1997).

Structurally similar homologues to the N and C peptides of HIV have also been found to be potent inhibitors of paramyxoviruses (Rapaport et al., 1995), parainfluenza (Lambert et al., 1996; Yao and Compans, 1996), respiratory syncytial virus (Lambert et al., 1996) and measles virus (Lambert et al., 1996; Wild and Buckland, 1997) but not for rhabdoviruses.

Because hydrophobic heptad repeat structures have been detected in rhabdoviruses including VHSV, the synthetic peptide p2 from the VHSV glycoprotein G heptad repeat A, has been used to study the interaction with phospholipids by employing a variety of biophysical assays (Nuñez et al., 1998) and to investigate its ability to interact with other parts of the glycoprotein and to inhibit VHSV infectivity. The report herein describes preliminary results obtained using this approach which may offer a new therapeutic method to minimize disease.

2. Materials and methods

2.1. Peptide synthesis

The peptides covering the major amino-terminal PS-binding region A (Fig. 1) of the glycoprotein G of VHSV were synthesized (Clontech, Palo Alto, CA), with the sequences: p2 ^{82}IIPHLPLSVTSVSAVASGHYLHRVTYRVT^{109}, p9 ^{58}RPAQLRCPHEFEDINKGLVSVPT^{80}, and p11 ^{68}FEDINKGLVSVPTRIIHLPLSVTSV-SAV^{95} (Coll, 1997).

2.2. Cloning and expression of the heptad-repeat region A (fragment no. 11)

The glycoprotein G (Thiry et al., 1991a) cDNA sequence was amplified by the polymerase chain reaction (PCR) with specific amplimers. The amplimer 1 $^{5'}$GTCGGATCCATGCCGATTCGACCA hybridized with the cDNA region corresponding to the N-terminal part (amino acid [aa] 56) of the glycoprotein G contained a Bam HI restriction site and added an initiating methionine codon (ATG). The nucleotide sequence corresponding to the C-terminal part (aa 110) of the glycoprotein G contained an EcoRI restriction site and added a stop codon (TGA) in the amplimer 2 $^{5'}$CGAGAATTCTCAACAGGTGACTCG. The PCR generated DNA was recovered from the agarose after gel electrophoresis, digested with BamHI and EcoRI, and introduced in the linearized pRSET-A (Invitrogen, San Diego. CA) (a poliHis containing plasmid) and cloned in the *E. coli* DH5α strain. The expression of fragment no. 11 was at 28°C by IPTG induction in the BL21 *E. coli* strain. Bacterial pellets were lysed and the expressed protein was purified by a Ni affinity column (Probond resin, Invitrogen). Confirmation of the presence of the cloned sequence was performed by sequencing the recombinant plasmid and confirmation of the protein expression was done by immunoblotting of the purified expressed protein with polyclonal antibodies anti-G. After dialysis against 20 mM Tris pH 7.6 the fragment no. 11 was frozen until used.

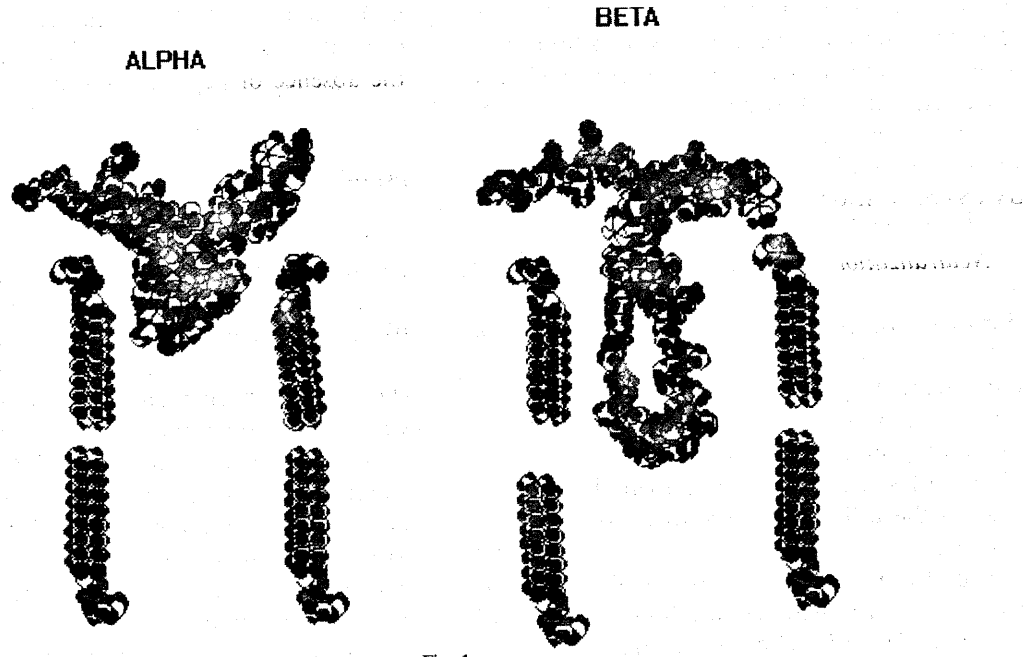

Fig. 1

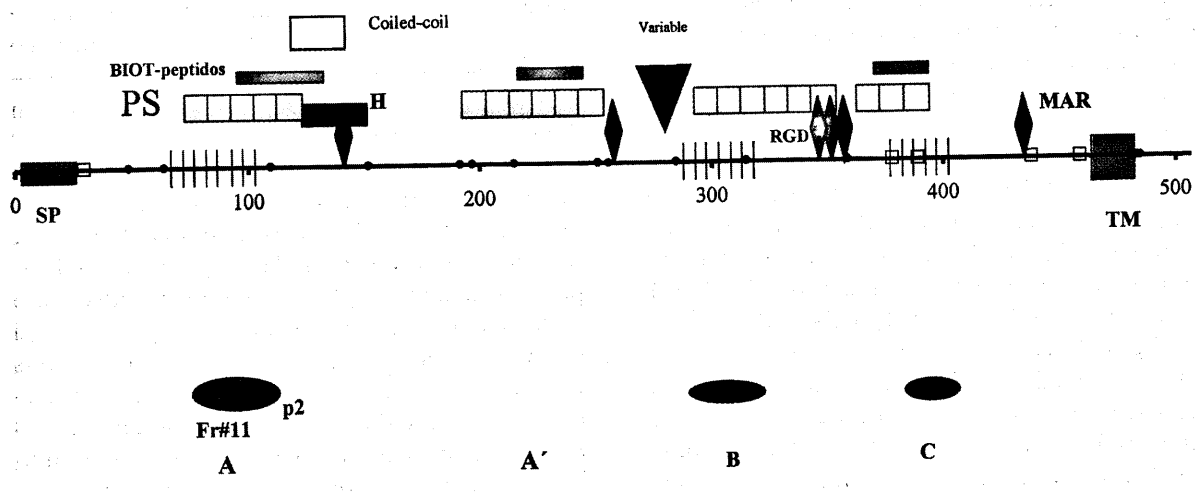

Fig. 2

Fig. 1. Models obtained with the fragment no. 11 (aa 56–110) by a computer simulator. The program used was the Desk Top Molecular Modeler version 3.0 Oxford University Press, 1994. The aa sequence of the fragment no. 11 obtained by recombinant *E. coli* without the poli-His tail is ^{56}PI RPAQLRCPHEFEDINKGLVSVPTRIIHLPLSVTSVSAVASGHYLHRVTYRVTC^{110} (In bold face are the aa that make the bending points of the all-β-sheet structure P65, 79 and 86 and G98). In the all-β-sheet structure, the distance between P65 and P79 is about 46 Å and between P86 and G98 is about 41 Å. Whereas the distance from P79 to P86 is about 22 Å. Eight phospholipid molecules arranged as in a phospholipid bilayer have been drawn to scale for comparison.

Fig. 2. The mapping of some motifs in the glycoprotein G of VHSV. SP, signal peptide. TM, transmembrane region. ●, cysteins; □, carbohydrates; ||||||, hydrophobic heptad repeat regions A, B and C (Coll, 1995b); ◆, MAR, aa changes in the monoclonal antibody resistant mutants (Bearzotti et al., 1995); □□□, PS phosphatidylserine binding region (Estepa and Coll, 1996a); — regions binding biotinylated peptides corresponding to the heptad-repeat B; □, high probability of a coiled coil forming region predicted by the Lupas algorithm (PCgene package) (Lupas et al. 1991); ▼, most variable region (Benmansour et al., 1997). H, most hydrophilic region (predicted with the program ANTIGEN from the PCGene package).

2.3. Molecular modelling of fragment no. 11

The program used was the Desk Top Molecular Modeler v 3.0 Oxford University Press, 1994. The aa sequence of the fragment no. 11 obtained by recombinant *E. coli* without the poli-His tail was ^{56}PIRPAQLRCPHEFEDINKGLVSVPTRIIHLP LSVTSVSAVASGHYLHRVTYRVTC^{110}

2.4. Neutralization microassay

Synthetic peptides covering different parts of the heptad repeat A (p2, p9 and p11) and a recombinant fragment (fragment no. 11) were used to study inhibition of VHSV infectivity. Virus and peptides were first mixed together at different pHs, neutralized and then the mixture added to the cell monolayers to measure VHSV infectivity.

About 10^3 $TCID_{50}$ per ml of VHSV 07.71 was incubated at 14°C with serial dilutions of G peptides at pH 7.6 or 5.6 (Lorenzo et al., 1996). After all samples were adjusted to pH 7.6, cultures of EPC cells in 96-well plates were infected with 100 μl of the different virus/peptide mixtures, adsorbed for 1 h at 14°C, rinsed with cell culture medium and filled with 100 μl/well. The VHSV infected EPC were incubated 18 h at 14°C. The VHSV infected EPC monolayers were fixed for 10 min in cold methanol and air dried. To detect the N antigen, the MAb 2C9 diluted 1000-fold in dilution buffer (130 mM NaCl, 2 mM KCl, 8 mM Na_2 HPO_4, 1.4 mM KH_2 PO_4, 0.24 mM merthiolate, 5 g of Tween 20/l, 50 mg of phenol red/l, pH 6.8) was added to the wells (100 μl/well) and incubated for 1 h. After washing with distilled water, 100 μl of peroxidase-labelled anti-mouse IgG (Sigma Che Co. M) was added to each well and incubation continued for 30 min. After three washings by immersion in distilled water, 50 μl of a 1 mg/ml solution of diaminobenzidine (DAB) (Sigma, Che. Co) in the appropriate buffer was added to each well, and the reaction allowed to proceed until brown foci were detected by inspection with an inverted microscope. After washing with water and air drying, brown foci (DAB positive foci) were counted with an inverted microscope provided with a 10 × ocular eye grid. The results were expressed by the following formula: number of DAB positive foci in the presence of peptides/number of DAB positive foci in the absence of peptides × 100.

2.5. Binding of biotinylated heptad-B to pepscan peptides

A series of 15-mer peptides that overlapped 5 aa and spanned the cDNA derived aa sequence of the glycoprotein G (Thiry et al., 1991b) of VHSV 07.71 were chemically synthesized (Chiron Mimotopes, Victoria, Australia). Polystyrene plates (Dynatech, Plochingen, Germany) were coated with 300–1000 pmol of peptides per well in 100 μl of distilled water, incubated at 37°C until dry, and stored sealed with blue silica gel at 4°C. The biotinilated heptad repeat B (aa 288–319) (Chiron Mimotopes) was diluted in dilution buffer (see above). The plates were blocked by incubation for 15 min with dilution buffer. After blocking, the plates were incubated for 60 min at room temperature with 100 μl/well of the diluted biotinylated peptide and washed once with distilled water. To detect the bound biotinylated peptide, streptavidin-peroxidase (Nordic, The Netherlands), in dilution buffer (200-fold dilution) was added (100 μl/well), incubated for 30 min and then washed three times with distilled water. For color development, 50 μl of substrate buffer (150 mM sodium citrate, 3 mM H_2O_2 and 1 mg/l *o*-phenylenediamine, pH 4.8) were pipetted into each well and the reaction was stopped with 50 μl per well of 4 mM H_2SO_4 after 30 min. The results were read in a Titertek multiskan RC at wavelengths of 492 and 620 nm. The absorbance at 620 nm was used to correct for individual-non significant differences between wells.

3. Results

3.1. Modeled structure of the heptad repeat region containing p2

According to the data obtained before by biophysical measurements (Nuñez et al., 1998) and by using a computer modeling program, some of

Table 1
Binding of biotinylated heptad-repeat B (aa 288-314) to G pepscan peptides[a]

aa	pH 7.6	pH 5.6
99–113	1.47 ± 0.03	1.49 ± 0.19
119–133	1.03 ± 0.23	1.18 ± 0.1
219–233	0.87 ± 0.22	0.87 ± 0.1
229–243	0.41 ± 0.12	0.57 ± 0.33
369–386	1.34 ± 0.26	1.28 ± 0.18
379–386	1.26 ± 0.37	1.19 ± 0.03

[a] Absorbance values obtained by ELISA binding assays from averages from two different experiments and standard deviations are shown. Average background absorbance were 0.36 ± 0.14.

the possible conformations of the complete heptad repeat region A (aa 56–110) containing the p2 peptide (aa 82–109) was studied. Fig. 1 shows the all conformation and the all β conformation. The all conformation, although interrupted by Prolines, showed a highly compact structure. However bendings were also found in the all β conformation because of the presence of Proline at positions 65, 79 and 86 and of Glycine at position 98. The all β conformation showed in the amino terminal extreme two Arginines in positions 58 and 63 followed by a first bend of 90° with Proline 65 and 14 aa of β extended conformation (~46 Å) until Proline 79. A new bending of about 90° occurs followed by a short length of 7 aa ends at Proline 86. The structure bends again another 90° followed by a β extended conformation of 12 aa (~41 Å) which ends with a Glycine at position 98 where it occurs another bending and a short stretch were the two arginines at carboxy-terminal positions 103 and 107 are situated (Fig. 1).

3.2. Binding of biotinylated heptad-repeat B to pepscan G

The biotinylated heptad repeat B (aa 288–319) (Fig. 2) reacted mostly with the peptides aa 99–113 (that contains the carboxy-terminal part of the p2), 119–133, 219–233, 229–243, 369–376 and 379–386 (heptad-repeat B is contained between the two former peptides) from the pepscan (Table 1). The rest of the 51 peptides in the pepscan of glycoprotein G did not give absorbance values above the background. The binding of biotinylated heptad B was independent of pH (pH 5.6 and 7.6). Similar, preliminary results were obtained with biotinylated heptad-repeat A.

3.3. Preliminary data on inhibition of VHSV infectivity

At pH 7.6 only the fragment no. 11 showed some inhibition of infectivity (68.5 ± 2.5% of the initial number of focus) respect to the control. However, when the mixing between VHSV and the peptides was performed at pH 5.6, p2, p11 and fragment no. 11 showed a reduction to 27.5 ± 5.2, 50.6 ± 7.2 and 36.1 ± 9.8% of the initial number of focus whereas p9 was still unable to inhibit VHSV infection (Table 2). The inhibition was dependent of the dosage of the peptide used between 1 and 50 μg/ml (not shown).

No significant inhibition was detected with p9, p11 or fragment no. 11 whether the assay was

Table 2
In vitro inhibition of VHSV infectivity by VHSV synthetic peptides corresponding to the p2 region[a]

Peptide	aa	% foci relative to control at 50 μg/ml of peptide pH 7.6	pH 5.6
p2	82–109	114.5 ± 10.5	27.5 ± 5.2
p9	58–80	107.0 ± 12.0	99.5 ± 3.5
p11	68–95	90.2 ± 0.2	50.6 ± 7.2
fragment no. 11	56–113	68.5 ± 2.5	36.1 ± 9.8

[a] VHSV virions were incubated with the peptides 1 h at pH 7.6 or 15 min at pH 5.6 and then neutralized to pH 7.6. Viral infectivity was then assayed at different peptide concentrations by a microneutralization method as described before (Lorenzo et al., 1996). Briefly, mixtures were added to EPC monolayers in 96-well plates, adsorbed during 1 h at 4°C, washed and filled with 100 μl/well of cell culture medium. After overnight incubation at 14°C, the cultures were fixed with cold methanol during 10 min and dried. Staining of the VHSV foci was with MAb 2C9, peroxidase labelled rabbit anti-mouse IgG and diamino-benzidine (DAB). Results are expressed by the following formula, number of VHSV foci in the presence of peptides/number of VHSV foci in control × 100. Averages and standard deviations of duplicate cultures are shown in the Table.

performed at pH 7.6 by binding the VHSV to the cells, washing and adding the peptides or by adding the peptides to the cells, washing and binding the VHSV (not shown). However p2 showed small but measurable inhibition in both of the above mentioned assays (to 60–80% of the initial number of focus) but only at the highest p2 concentration tested (50 μg/ml).

4. Discussion

Because a peptide (p2, aa 82–109) contained in the amino-terminal heptad repeat A (Fig. 1) of the glycoprotein G of VHS rhabdovirus binds anionic phospholipids (Estepa and Coll, 1996a,b) and anti-p2 antibodies inhibited VHSV-induced fusion (Estepa and Coll, 1997) and PS-binding to VHSV at low pH (Estepa and Coll, 1996a), we have further studied the possible structure of p2, the interactions with other peptides from G and the ability of p2 to inhibit VHSV infectivity.

The p2-phospholipid interaction forms peptide–phospholipid complexes in solution which can be isolated in sucrose gradients (Coll, 1997) and the addition of the p2 peptide to phospholipid vesicles was able to induce phospholipid vesicle aggregation, mixing and rapid leakage of vesicle-trapped fluorescent probes in a peptide concentration-dependent manner (Nuñez et al., 1998), most of the essential steps required for fusion.

Deconvolution of the CD spectra of p2 showed that at pH 7.0, the β-sheet is the major component (66%) and at pH 5.0 the percentage of this conformation is lower (28%). However, the presence of PS vesicles increases the proportion of β-sheet conformation on the p2 peptide to 71 and 91%, respectively (Nuñez et al., 1998). Fluorescence depolarization studies, demonstrated that the p2 peptide not only interacts but also inserts into the hydrophobic core of the anionic phospholipid bilayer maintaining or favoring the β-sheet conformation (CD studies) observed for this peptide in solution (Nuñez et al., 1998). There is increasing evidence that a β-sheet conformation of fusion-related peptides could also be involved in the fusion events mediated by other viruses (Epand et al., 1992; Nieva et al., 1994). Since computer programs predict a putative α-helix inside the p2 peptide (Coll, 1997) the p2 peptide could be in an α-helix conformation in the native glycoprotein G. The p2 peptide would acquire the β-sheet structure after a change in the conformation of the glycoprotein G mediated by the low pH thus enabling it to interact with the anionic phospholipids. A conformational change in the glycoprotein G of VHSV when exposed to low-pH seems to be occurring because the reaction of anti-p2 antibodies with VHSV only occurs at low-pH (Estepa and Coll, 1996a). Moreover, similar low pH induced conformational changes have also been reported in rabies and in VSV (Gaudin et al., 1993). A β extended conformation of p2, if present in all the heptad-repeat containing region (fragment no. 11) would theoretically be able to penetrate into the cellular membrane (Fig. 1). The unexpected bending of the β extended conformation (Fig. 1) of the whole region A due to the presence of prolines could explain how an internal fusion peptide would penetrate the membrane without previous cleavage, as it occurs in other enveloped viruses.

All the above mentioned findings and the fact that anti-p2 antibodies inhibited fusion (Estepa and Coll, 1997) and PS-binding to VHSV (Estepa and Coll, 1996a) support the idea that this region of the G protein might play an active role in the viral fusogenic processes. Therefore this region can be considered as an important target in the design of strategies for the prevention of VHSV infection in vivo by viral peptides (Chan et al., 1997).

Preliminarly results initiated to explore this hypothesis showed that incubation of VHSV with synthetic peptides mimicking the p2 region inhibited VHSV infectivity but only at the pH of fusion. All the results mentioned suggest that the region of the heptad repeat A of the glycoprotein G that causes the highest inhibition is found between aa 68 and 113. The highest inhibitory region contains, therefore, the p2 peptide, which inserts into the membrane, according to the biophysical data. While still preliminary, these results allow formulation of additional hypothesis including: (a) Because of complete inhibition of VHSV

infectivity was not observed, either more viral peptide sequences have to be tested (for instance, pepscan of the whole glycoprotein G, longer or shorter peptides) or other conditions of the experiment have to be examined (pH, time of exposure of peptides to VHSV, etc.); (b) Because VHSV infectivity is preserved even after a 15 min exposure to pH 5.6, it is concluded that the conformational change induced by the pH is reversible or it does not affect infectivity; (c) Because only when VHSV is exposed to the peptides at pH 5.6, inhibition of the peptides is observed, it can be concluded that only at the low-pH conformation, the viral peptide is able to interact with some of the viral G structures implicated in the exposition of the p2 peptide (Estepa and Coll, 1996a,b). All these hypothesis are based on analogies with the HIV viral peptide induced inhibition, in which the N-peptides might inhibit infectivity by binding to the C-peptide regions or the C-peptides might inhibit infectivity by binding to the N-peptide regions (Chan et al., 1997). However other mechanisms might be responsible for these type of inhibitions in rhabdoviruses. The mechanism(s) by which these peptides inhibit VHSV infectivity may have some relation to the conformational change of glycoprotein G, to the fusion mechanism or to some interaction of p2 with other parts of the glycoprotein G (heptad-repeats B, C, etc). More experimental evidence has to be obtained to decide among these and/or other possibilities.

Because other regions contiguous to the heptad repeats B or C have been recently implicated in fusion in the vesicular stomatitis rhabdovirus (Schnell et al., 1998; Shokralla et al., 1998) and we have shown by using biotinylated peptides that, at least, some heptad repeats of VHSV interact with each other, peptides belonging to those new regions should be also tested for possible inhibition of VHSV infectivity. It is noteworthy that MAR mutants (Bearzotti et al., 1995) and the highest variable region of the glycoprotein G (Benmansour et al., 1997) map outside the identified regions potentially involved in fusion. On the other hand, until now we have restricted our studies to inhibition of VHSV infectivity but both inhibition of infectivity and inhibition of fusion (Estepa and Coll, 1997) assays should be used to try to define a suitable test to search for all the viral regions potentially implicated in VHSV inhibition. Furthermore, the fusion inhibition assays could use not only VHSV-infected cells as a model but G-transfected cells or permanent cell lines expressing G or fragment no. 11. Those former assays could be much better models to study both fusion and fusion inhibition by the peptides.

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