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Salmonid viral haemorrhagic septicaemia virus: fusion-related enhancement of virus infectivity by peptides derived from viral glycoprotein G or a combinatorial library

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To search for enhancers and/or inhibitors of viral haemorrhagic septicaemia virus (VHSV, a salmonid rhabdovirus) infectivity, a total of 51 peptides from a pepscan of viral envelope protein G, a recombinant peptide from protein G (frg11) and 80 peptide mixtures from an α-helix-favoured combinatorial library were screened. However, contrary to what occurs in many other enveloped viruses, only peptides enhancing rather than inhibiting VHSV infectivity were found. Because some of the enhancer pepscan G peptides and frg11 were derived from phospholipid-binding or fusion-related regions identified previously, it was suggested that enhancement of virus infectivity might be related to virus–cell fusion. Furthermore, enhancement was significant only when the viral peptides were pre-incubated with VHSV at the optimal low pH of fusion, before being adjusted to physiological pH and assayed for infectivity. Enhancement of VHSV infectivity caused by the pre-incubation of VHSV with peptide p5 (SAAEASAKATAEATAKG), one of the individual enhancer peptides defined from the screening of the combinatorial library, was independent of the pre-incubation pH. However, it was also related to fusion because the binding of p5 to protein G induced VHSV to bypass the endosome pathway of infection and reduced the low-pH threshold of fusion, thus suggesting an alternative virus entry pathway for p5–VHSV complexes. Further investigations into VHSV enhancer peptides might shed some light on the mechanisms of VHSV fusion.

Introduction

The mechanisms of virus–cell fusion of some enveloped viruses have been characterized in detail at the molecular level. Thus, to fuse with cellular membranes, the envelope proteins of members of the families *Orthomyxo-*, *Paramyxo-*, *Retro-* and *Filoviridae* use a trimeric spike, require proteolytic cleavage of the envelope spike protein, have N-terminal fusion peptides, suffer conformational transitions involving trimeric α-helix, coiled-coil segments for fusion and contain viral envelope protein peptides that, when individually synthesized, are capable of inhibiting fusion (Bentz, 2000).

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Thus, synthetic peptides (6-mer to more than 30-mer) (Ferrer *et al.*, 1999; Owens *et al.*, 1990; Puras Lutzke *et al.*, 1995; Slepushkin *et al.*, 1993) derived from the segments forming trimeric α-helix, coiled-coils adjacent to either the fusion peptide (N peptides) (Owens *et al.*, 1990; Wild *et al.*, 1992) or the C-terminal region (C peptides) of the glycoprotein of human immunodeficiency virus (HIV) (Wild *et al.*, 1995) blocked *in vitro* HIV infection by inhibiting virus–cell fusion. Interactions among the N and C segments in the glycoprotein during fusion (Chan *et al.*, 1997) are inhibited by the synthetic peptides derived from them, thus explaining their antiviral activity (Chen *et al.*, 1995). Structural homologues to the inhibitory N and C peptides of HIV have been also found to be inhibitors of other enveloped viruses, such as paramyxo- (Rapaport *et al.*, 1995), parainfluenza- (Yao & Compans, 1996),

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respiratory syncytial (Lambert *et al.*, 1996) and measles (Wild & Buckland, 1997) viruses. In contrast to these results, no similar inhibitory peptides have been reported for rhabdoviruses.

While rhabdoviruses share some of the molecular features of fusion described above, their enveloped glycoproteins do not require proteolytic cleavage, are not predicted to form coiled coils and the evidence available suggests the presence of an internal fusion peptide (Fredericksen & Whitt, 1996, 1998; Li *et al.*, 1993; Shokralla *et al.*, 1998). For rhabdovirus fusion with cellular membranes, the G protein trimeric spikes find and bind to their target cells and, once the viruses are endocytosed, fuse with the internal cellular membranes at low pH. However, the molecular mechanisms involved in rhabdovirus fusion are not well understood (Coll, 1999; Durrer *et al.*, 1995; Gaudin *et al.*, 1999b).

To identify possible peptide inhibitors of rhabdovirus infection, we have followed, by analogy with other enveloped viruses, two different approaches. The first approach was to screen a pepscan dissection of a rhabdoviral G protein, including a recombinant fragment derived from it. The second approach was to screen an α-helix-restricted peptide library. As a model among the rhabdoviruses, viral haemorrhagic septicaemia virus (VHSV), a salmonid rhabdovirus, was chosen. We screened the viral peptides and the combinatorial library for both enhancers and/or inhibitors of VHSV infectivity. Of all the virus-derived or library peptides assayed, we did not find any single peptide or peptide mixture with inhibitory activity, which is in contrast to the inhibitory peptides found in most enveloped viruses. If the fusion process of rhabdoviruses cannot be inhibited by synthetic peptides, it might involve new principles yet to be discovered or else precise peptide sequences and/or conformations (that might be present only in whole proteins) are needed to inhibit rhabdovirus infectivity.

Methods

■ **Cells and rhabdoviruses.** Epithelioma papulosum cyprini (EPC) or rainbow trout gonad (RTG-2) cells were maintained as reported previously (Basurco & Coll, 1989). VHSV-07.71, which infects fish cells at 14 °C, was the virus strain used (LeBerre *et al.*, 1977). Supernatants from VHSV-07.71-infected EPC cells were clarified by centrifugation at 20000 ***g*** for 20 min and kept in aliquots at −70 °C. Viruses from EPC cell-infected clarified supernatants were concentrated to 10^{11} f.f.u./ml by ultracentrifugation at 100000 ***g*** for 45 min (Basurco & Coll, 1989).

■ **Virus infectivity assays.** To test the influence of pre-incubating VHSV with peptides on VHSV infectivity, we used a microneutralization test in 96-well plates (Lorenzo *et al.*, 1996). Briefly, VHSV and peptides were pre-incubated for 4 h in 25 μl RPMI-1640 supplemented with 20 mM HEPES and 20 mM MES cell culture medium (pH 5·6 or 7·6) at 14 °C, the optimal temperature for fusion and infectivity of VHSV. The pre-incubated mixtures (pH 5·6) were then adjusted to pH 7·6 with 0·2 N NaOH before the addition to EPC or RTG-2 cell monolayers, maintained in 75 μl cell culture medium at pH 7·6. After 1 day of incubation at 14 °C, pH 7·6, infected foci of individual cells were detected by the expression of the major VHSV protein (the nucleoprotein N) with an anti-VHSV N (2C9) monoclonal antibody (mAb), peroxidase stained and counted. Infectivity was expressed as f.f.u./ml. VHSV infectivity was unaffected by pre-incubation of the virus at pH 5·6, since the recovery of infectivity was 83·1 ± 25 % ($n = 6$) when VHSV was pre-incubated at pH 5·6 for 4 h at 14 °C, the pH adjusted with NaOH to pH 7·6 and then infectivity assayed at pH 7·6, as indicated above.

■ **Synthetic pepscans of VHSV G protein and assay of its effects on VHSV infectivity.** Synthesis of 15-mer peptides, overlapping by 5 aa, derived from the cDNA amino acid sequence of the G protein of VHSV-07.71 (Thiry *et al.*, 1991) was performed by Chiron (Victoria, Australia). The purity of the peptides was > 70 %, as determined by HPLC and mass spectrophotometry, other contaminants being incomplete peptides. The peptides diluted in 5 mM HEPES (pH 7) were added to 96-well plates (Dynatech) in volumes of 10 μl per well at a final concentration of 0·3 μmol per well. VHSV (50 μl per well, 10^8 f.f.u./ml) in RPMI-1640 supplemented with 20 mM HEPES and 20 mM MES was then added, incubated for 4 h at 14 °C, pH 5·6 or 7·6, adjusted to pH 7·6 and then added to the EPC cell culture wells in a total volume of 100 μl.

■ **Synthesis of a combinatorial peptide library with a high propensity to form α-helixes.** A 17-mer peptide combinatorial library with a high propensity to form α-helixes was synthesized in the so-called positional-scanning format. The combined and fixed positions on the helical scaffold were designed to preserve the tendency to fold into an α-helix conformation, to avoid alternative secondary structure conformations and to prevent aggregation processes, while allowing enough sequence diversity. Thus, residues Ser^1 and Gly^{17} have, respectively, N and C terminus α-helical, end-capping properties (Richardson & Richardson, 1988). Two residues, Glu and Lys (residues 4–8 and 12–16) were incorporated to favour solubilization and the formation of a salt-bridged, stabilized α-helix conformation (Esteve *et al.*, 2001). Ala residues were also included in the sequence framework due to their intrinsic α-helix stabilization properties (O'Neil & DeGrado, 1990). When only Ala residues were included, the scaffold peptide showed a 68 % tendency to form α-helical conformations by the AGADIR program (Muñoz & Serrano, 1997). Consequently, we chose to design new peptides by replacing the four positions from one face of the putative α-helix: residues 6, 9, 10 and 14. Hence, the scaffold sequence $S^1A^2A^3E^4A^5X^6A^7K^8X^9X^{10}E^{11}A^{12}X^{14}A^{15}K^{16}G^{17}$ or $O_5XO_2XXO_3XO_3$ (where O indicates fixed positions and X represents any of the 19 L-amino acids, except Cys, thereby avoiding the formation of disulphide bridges) was synthesized (Perez-Paya *et al.*, 1996). Each peptide sample thus contains 19^3 (6859) different peptides. The library comprises four sublibraries: SAAEAOAKXXAEAXAKG-NH_2, SAAEAXAKOXAEAXAKG-NH_2, SAAEAXAKXOAEAXAKG-NH_2 and SAAEAXAKXOAEAXAKG-NH_2 (Pastor *et al.*, 2002). After deconvolution of the library from the VHSV infectivity assay, peptides with the individual sequence SAAEASAK(O_1)TAEAT(O_2)AKG (O_2 = T, O_1 = A or O_1 = N in the p5 or p6 sequence, respectively; O_2 = E, O_1 = A or O_1 = N in the p3 or p4 sequence, respectively) were synthesized (Diver Drugs). The randomized amino acid sequence of peptide p5 (scrambled) was ASKASEAATAAETKGAA. The purity of the peptides was > 90 %, as determined by HPLC and mass spectrophotometry, other contaminants being incomplete peptides.

■ **Cloning and expression of G protein fragment 11 (frg11).** The DNA sequence corresponding to positions 56–110 of the VHSV G protein (Thiry *et al.*, 1991) involved in VHSV fusion was amplified, cloned and purified to 95 %, as described previously (Estepa *et al.*, 2001; Rocha *et al.*, 2002). The N-terminal poly(H) peptide added to frg11 by the pRSET plasmid was also synthesized as a control.

■ **Solid-phase enzyme immunoassays.** Wells from high-binding plates (Costar) were coated with 100 µl of either serial dilutions of peptides (to assay for G- or VHSV-binding) or VHSV + peptide mixtures (to assay for anti-G mAb-binding) in distilled water and allowed to dry overnight at 37 °C. All samples and reagents were diluted in buffer containing 0·5% BSA, 0·3% rabbit serum, 0·1% Tween-20, 0·01% merthiolate and 0·005% phenol red in PBS. To assay for VHSV-binding, purified VHSV was added to solid-phase peptides, incubated for 1 h and then incubated for 90 min with anti-N mAb 2C9. To assay for G-binding, purified G protein (Perez *et al*., 1998) was added to plates containing solid-phase peptides, incubated for 1 h and then incubated with anti-G polyclonal antibodies (pAbs). To assay for anti-G mAb-binding, anti-G mAbs of known target epitopes were added to the plates containing solid-phase VHSV + peptide mixtures and incubated for 1 h. Background binding of anti-G mAbs to solid-phase peptides was estimated in parallel experiments and subtracted from all data. In all assays, after washing, horseradish peroxidase-conjugated rabbit anti-mouse antibodies, diluted 1000-fold (Sigma), were added and the colour reaction developed by adding 1 mg/ml *o*-phenylenediamine in citrate buffer containing 3 mM H_2O_2. Absorbance readings at 492 and 620 nm for estimation of each individual well background readings were measured using an ELISA plate reader (Anthos).

■ **Fusion induced by addition of VHSV to uninfected cells.** VHSV (8×10^9 f.f.u./ml) was incubated with the peptides at 10 µg/ml in RPMI-1640 supplemented with 20 mM HEPES, 20 mM MES and 2% foetal calf serum at different pHs for 1 h at 14 °C. Then, the VHSV + peptide mixtures were added to EPC cell monolayers on 96-well plates in a final volume of 100 µl per well. After 1 h at 14 °C, the VHSV + peptide mixtures were removed, the cell monolayers were washed and incubated with fresh medium, pH 7·4, for 2 h at 14 °C. To measure the extent of fusion, the cells were fixed with cold methanol, dried and stained with Giemsa (5 mg/ml in PBS). The percentage of fusion was calculated using the following formula: (number of nuclei in syncytia/total number of nuclei) × 100 (Estepa & Coll, 1997).

Results

Enhancement of VHSV infectivity by pepscan peptides derived from the VHSV G protein

After some preliminary experiments to search for viral peptides with inhibitory activity of VHSV infectivity, each of the 15-mer peptides from a pepscan set of 51 peptides of the VHSV G protein was pre-incubated with VHSV at pH 5·6, adjusted to pH 7·6 and then assayed for infectivity on EPC cell monolayers using a focus-forming assay. No inhibitory peptides were found but some peptides significantly enhanced ($\sim$ 4- to 5-fold) the number of f.f.u. over the background number of f.f.u. (43 ± 12 f.f.u. in the absence of peptides). The most active peptides were derived from the regions located from aa 99 to 113, 199 to 213 and 369 to 383 (Fig. 1b). Peptides spanning aa 99–113 and 199–213 were reported before as the region of highest phosphatidylserine binding of the G pepscan (Estepa & Coll, 1996), whereas the peptide spanning aa 99–113 was part of frg11 (aa 56–110) (Fig. 1b, lower panel), which participates in VHSV fusion (Estepa *et al*., 2001).

In contrast, by pre-incubating the peptides and VHSV at pH 7·6 and then adding the mixtures to the EPC monolayers (Fig. 1a), peptides spanning aa 79–93, 99–113, 199–213, 219–223, 239–243 and 369–383 only increased 2- to 3-fold the number of f.f.u. over the background number of f.f.u. Again, no inhibitory peptides were found.

Peptides with enhancing or inhibitory effects were not found among the pepscan peptides by incubating EPC cells with VHSV before adding the peptides (Fig. 1c) or by incubating the peptides with the EPC cells before adding VHSV (Fig. 1d). Therefore, all these results suggested both the absence of peptides among the peptides derived from the G protein that are inhibitory to VHSV infection and the low-pH requirement for the peptides to interact with VHSV in order to significantly enhance virus infectivity.

Enhancement of VHSV infectivity by frg11

More than an 8-fold enhancement of VHSV infectivity was obtained when VHSV was pre-incubated at pH 5·6 with 200 µg/ml frg11 (frg11, aa 56–110, contained the sequences of the enhancer peptide spanning aa 99–113) and then the pH adjusted to 7·6 just prior to performing the infectivity assay (Fig. 2). Because of the tendency of frg11 to aggregate at pH 7·6 (Estepa *et al*., 2001), it is estimated that at that pH, a concentration of 200 µg/ml is about 30 µg/ml of soluble frg11. In parallel experiments, pre-incubation of frg11 with VHSV at pH 7·6 produced no enhancement of infectivity (Fig. 2b), confirming the requirement for a pre-incubation step at low pH before adjusting the pH back to physiological values to produce significant VHSV enhancement.

The results mentioned above suggested that the interactions between all VHSV-derived G peptides with VHSV required a pre-incubation step at low pH to produce enhancement of infectivity when added to cell monolayers at physiological pH. Because the low pH changes the conformation of the VHSV G protein, as shown by changes in the mAb-binding patterns (data not shown), and as it occurs in the G protein of all rhabdoviruses, the viral peptides must interact with the low-pH, fusion-active conformation of protein G to cause the observed enhancement of infectivity. Therefore, at low pH, G protein conformational changes and viral peptide enhancement of infectivity should be all related to VHSV–cell fusion.

Enhancement of VHSV infectivity by 17-mer peptide mixtures from a combinatorial library

To search further for peptides with inhibitory activity of VHSV infection, we designed a combinatorial library of synthetic peptides. Since most of the inhibitory viral peptides have a length between 18- (Ferrer *et al*., 1999) and 37-mer (Rapaport *et al*., 1995) and were derived from helical segments of its envelope glycoprotein sequences, a minimal length of 17-mer was chosen to synthesize a combinatorial library with a high propensity to produce α-helical peptides (see Methods). Then, each of the 80 peptide mixtures from the combinatorial

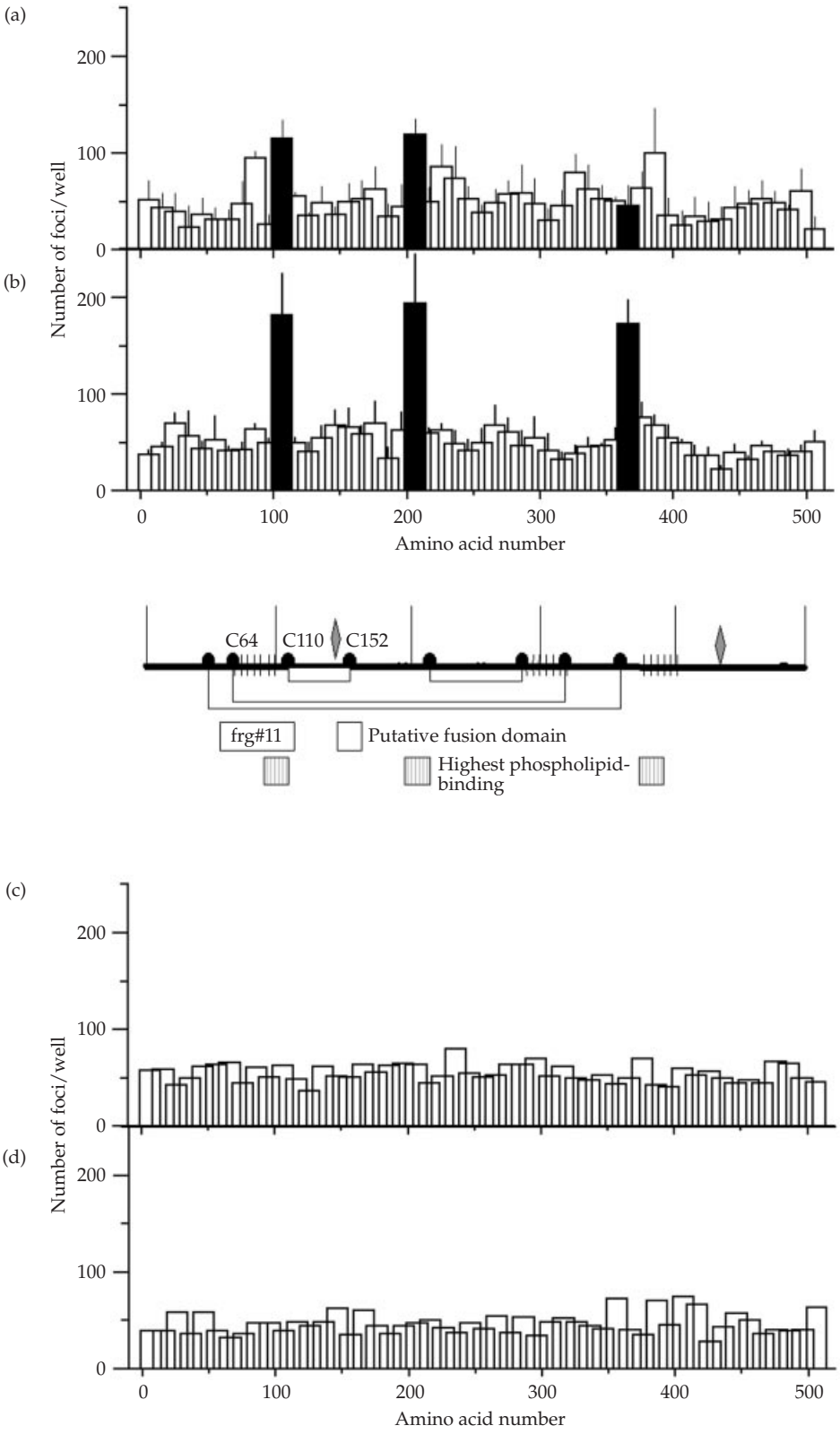

Fig. 1. VHSV infectivity after pre-incubation of VHSV with G pepscan peptides at pH 7·6 (a) or 5·6 (b) before adding them to cells, of cells with VHSV before adding the G pepscan peptides (c) or of cells with G pepscan peptides before adding VHSV (d). Peptides from a G protein 15-mer pepscan at ~ 3 μM were pre-incubated with 50 f.f.u. per well of VHSV for 4 h at 14 °C

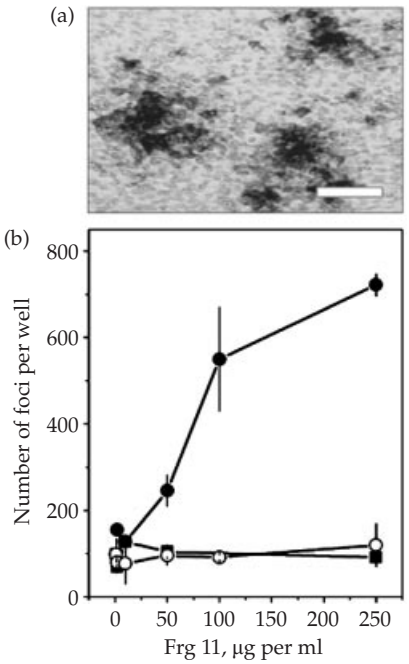

Fig. 2. Foci of VHSV-infected EPC cells (a) and VHSV infectivity after pre-incubation of VHSV with recombinant frg11 (b). VHSV was incubated for 2 h at 14 °C with several concentrations of frg11 at pH 7·6 or 5·6. VHSV + frg11 mixtures were then adjusted to pH 7·6 where appropriate, immediately added to EPC cell monolayers and infection allowed to proceed at pH 7·6. Mean ± SD of three experiments, each in duplicate, is represented. Frg11 pre-incubated at pH 7·6 with VHSV (■); frg11 pre-incubated with VHSV at pH 5·6 and then adjusted to pH 7·6 (●); poly(H) peptide (○). (a) Bar, 100 μm.

library was assayed after pre-incubation with VHSV at pH 7·6 for its effect on VHSV infectivity. Again, we found only peptide mixtures that enhanced the number of f.f.u. by 3- to 4-fold over the number of f.f.u. obtained in the absence of peptides (data not shown). No peptide mixtures were found that would inhibit VHSV infectivity.

Enhancement of VHSV infectivity by synthetic peptide sequences derived from the results of combinatorial library screening

From the deconvolution of the library, four peptide sequences that were expected to have the strongest enhancing properties were defined: SAAEASAKATAEATAKG (p5) and SAAEASAKNTAEATAKG (p6), differing at position 9 (underlined), and p3 and p4, differing from p5 and p6, respectively, at position 14 where they have Glu (E) instead of Thr (T). Peptides corresponding to all those sequences were synthesized, pre-incubated with VHSV and their effects on VHSV infectivity estimated. Only p5 and p6 significantly increased the enhancement seen in the peptide mixtures by 6- to 8-fold of the number of f.f.u. when VHSV was pre-incubated with at least 5 μM peptide at pH 7·6 in a volume of 25 μl (Fig. 3a). In contrast to the virus infectivity enhancing peptides, p5 and p6 enhanced VHSV infectivity whether pre-incubation with VHSV was performed at pH 5·6 or 7·6 before adjusting the pH to 7·6 and adding them to the EPC monolayers at pH 7·6 (data not shown). Therefore, the enhancement of p5 and p6 did not require the low-pH pre-incubation step, as with the VHSV-derived peptides.

Enhancement of virus infectivity occurred in both EPC (a carp cell line) and RTG-2 (a trout cell line) cells; both cell lines are susceptible to VHSV infection (Fig. 3a, b). Fig. 3(c) shows that there was no enhancing effect when the EPC monolayers were first incubated with p5 or p6 before infection with VHSV. The extent of enhancement increased by ~ 0·5-fold when the pre-incubation temperature was increased from 4 to 14 °C (data not shown) and by 2- to 3-fold when the total volume of the pre-incubation mixture was reduced from 100 to 25 μl. The extent of enhancement increased with time of pre-incubation of the VHSV + peptides mixture during the first 4 h of incubation (Fig. 3d) and then remained constant for up to 24 h (data not shown), allowing a reduction in peptide concentration [0·15 (p5 only) or 0·6 μM] (Fig. 3d) and confirming the concentration- and time-dependence of the VHSV + peptide reaction.

Since (i) there was no enhancement of virus infectivity when p5 or p6 were incubated with the cells rather than with VHSV, (ii) the extent of enhancement showed dependence of peptide and virus concentrations during pre-incubation, (iii) the effect was equally effective whether the cells were from carp (EPC) or from trout (RTG-2) and (iv) there was no enhancement of infectivity in other unrelated fish viruses, such as infectious pancreatic necrosis virus, it was concluded that p5 and p6 must interact with VHSV to enhance its infectivity.

either at pH 7·6 (a) or at pH 5·6 (b). After adjusting to pH 7·6, the peptide + VHSV mixtures were added to the EPC cell monolayers and incubated overnight at pH 7·6. (b, lower panel) Main disulphide bridges, heptad repeats and neutralizing mAb C10-resistant mutant positions of VHSV G protein: sequence of G from VHSV (____); Cys residues connected by horizontal lines indicate pairing by disulphide bridges (Einer-Jensen *et al.*, 1998) (●); Cys residues around the phospholipid-binding domain are C^{64} (C_{I}), C^{110} (C_{III}) and C^{152} (C_{IV}) [I, II and III, according to Walker & Kongsuwan (1999)]; hydrophobic heptad repeat regions, according to Coll (1995) (||||||); mutants resistant to mAb C10 mapping at positions 140 and 433 (◊). Location of phospholipid-binding domain p2 (aa 82–109), frg11 (aa 56–110) and putative fusion peptide (aa 142–159) (□). Cys residues 64, 110 and 152 are in bold and C^{110} is bridged to C^{152} (horizontal line). (c) EPC cell monolayers were incubated with each of the peptides from the pepscan for 4 h at 14 °C, pH 7·6, washed with cell culture medium and infected with 50 f.f.u. per well of VHSV. (d) EPC cell monolayers were infected with 50 f.f.u. per well of VHSV for 1 h at 14 °C, pH 7·6, washed with cell culture medium and then peptides added. In each case, the day after infection, VHSV foci were stained and counted. Black vertical bars show the peptides that demonstrated higher enhancing effects when pre-incubated with VHSV at pH 5·6. Mean ± SD of three experiments are represented in (a) and (b).

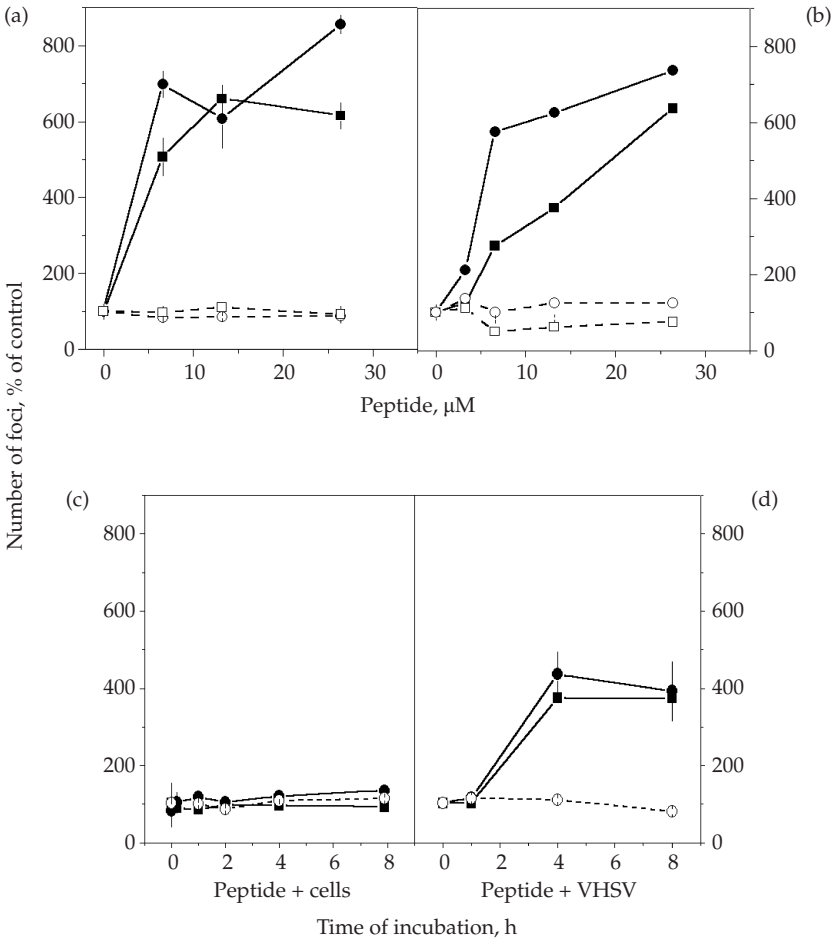

Fig. 3. Enhancement of VHSV infectivity on cell monolayers caused by pre-incubation of VHSV with library-selected peptides: concentration-dependence and time-course of pre-incubation. Peptides, at the indicated μM concentrations, were pre-incubated with 100 f.f.u. per well of VHSV (4 h at 14 °C, pH 7·6) in 25 μl of medium before infection of EPC (a) or RTG-2 (b) cell monolayers. (c) EPC cell monolayers were pre-incubated at different times with 25 μM peptides (14 °C, pH 7·6), then washed and infected with 500 f.f.u. per well of VHSV. (d) VHSV infectivity after VHSV was pre-incubated with peptides at 0·6 μM at different times before infection of the EPC cell monolayers (14 °C, pH 7·6). Similar enhancing effects were obtained with 0·15 μM p5 but not with p6. Results were expressed as the percentage of the number of foci in the control, calculated using the following formula: (number of foci in the presence of peptides/number of f.f.u. in the absence of peptides) × 100. Mean ± SD from duplicates are represented. ●, p5; ■, p6; ○, randomized p5 sequence peptide (scrambled p5); □, p3. Similar results were obtained with p4.

Confirming those observations, solid-phase p5 and p6 peptides bound to purified VHSV, as determined by recognition of bound VHSV with specific antibodies (data not shown). However, 1 μg per well of p5 (corresponding to 5 μM when the well was filled with 100 μl), bound 4-fold more VHSV than p6. To obtain a similar binding extent than p5, the amount of p6 had to be increased to 2 μg per well. In contrast, neither p3 nor p4 bound any VHSV at those concentrations (data not shown). The interaction of p5 and p6 with VHSV could be due to binding to the G protein or to the lipids of the viral membrane, the only types of molecules exposed on the VHSV surface. Because preliminary biophysical assays, such as lipid vesicle aggregation or circular dichroism spectra analyses, did not show any evidence for strong interactions between enhancer peptides with lipids (data not shown), we studied the possible interaction of p5 with the VHSV G protein.

Evidence for the interaction of p5 with purified G

Fig. 4(a) shows strong, specific and p5 concentration-dependent recognition of purified G bound to solid-phase p5 by specific anti-G pAbs and mixtures of mAbs. By using mAb C10, however, only a small increase in the absorbance readings could be obtained. Solid-phase scrambled p5 (randomized p5 sequence peptide) showed no G protein-binding (Fig. 4a) and p6 showed slightly lower G protein-binding than p5 (data not shown).

Binding of protein G to solid-phase p5 in the presence of an excess concentration of soluble p5 was reduced to background

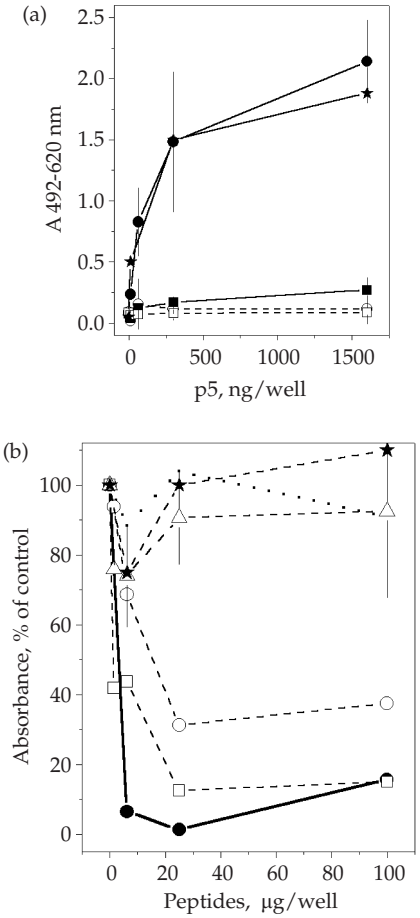

Fig. 4. Binding of purified G protein to solid-phase p5 (a) and competition of the binding with the addition of selected soluble peptides (b). (a) Different amounts of p5 or scrambled p5 were dried onto solid-phase 96-well plates. A sample of 3 µg per well of purified VHSV G protein was then added and incubated for 1 h. Anti-G antibodies were then added and the reaction developed with rabbit anti-mouse IgGs. Background binding of anti-G antibodies to solid-phase p5 in the absence of added G protein was subtracted from all data. Mean ± SD from duplicates are represented. ●, Mice polyclonal anti-G antibodies over solid-phase p5; ○, mice polyclonal anti-G antibodies over solid-phase scrambled p5; ★, mixture of mAbs (mAbs C10, 2F1A12, IP1H3 and I10) over solid-phase p5; ■, mAb C10 over solid-phase p5; □, mAb C10 over solid-phase scrambled p5 (similar results were obtained with the mixture of mAbs over scrambled p5). (b) A 1 µg sample of p5 was dried onto solid-phase 96-well plates. Purified VHSV G protein (3 µg) was pre-incubated with several concentrations of peptides in 100 µl volume at pH 5·6. After 1 h, the mixtures were adjusted to pH 7·6 with 0·2 N NaOH and then pipetted onto the p5 solid-phase. Anti-G antibodies were then added and the reaction developed with rabbit anti-mouse IgGs. Background binding of anti-G antibodies in the absence of protein G was subtracted from all data. Mean ± SD from duplicates are represented. Competition of binding of protein G to solid-phase p5 with soluble p5 was also obtained at pH 7·6. ●, p5; ---, scrambled p5; ○, viral peptide spanning aa 99–113; □, viral peptide spanning aa 199–213; △, viral peptide spanning aa 369–373; ★, frg11.

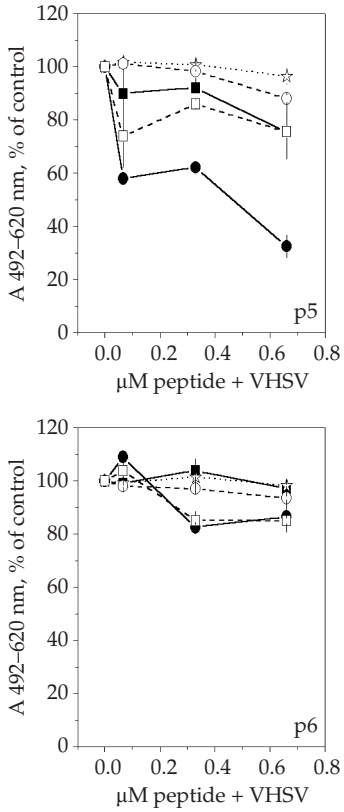

Fig. 5. Recognition of solid-phase VHSV + peptides by anti-VHSV G mAbs. A total of 1 µg of VHSV pre-incubated with different concentrations of peptides (p3, p4, p5, p6 or scrambled p5) for 4 h at 14 °C, pH 7·6, was dried over solid-phase 96-well plates. The anti-VHSV G antibodies were then added and the reaction developed with horseradish peroxidase-conjugated anti-mouse IgGs. Background binding of each of the mAbs to each of the solid-phase peptides was subtracted for each experimental point. Mean ± SD from duplicates are represented. Results are expressed using the following formula: (absorbance reading in the presence of peptide/absorbance reading in the absence of peptide) × 100. Results obtained with scrambled p5, p3 or p4 were similar to those obtained with p6. ●, mAb C10, neutralizing anti-VHSV (MAR mutants mapping at positions 140 and 433 of protein G) (Bearzotti *et al.*, 1995; Gaudin *et al.*, 1999a); ■, mAb 2F1A12, neutralizing anti-VHSV (MAR mutants mapping at position 253 of protein G) (N. Lorenzen, personal communication); ○, mAb IP1H3, anti-VHSV G (mapped by pepscan, aa 399–413 of protein G) (Fernandez-Alonso *et al.*, 1999); □, mAb I10, anti-VHSV G (mapped by pepscan, aa 139–153 of protein G); ☆, mAb 2C9, anti-VHSV N protein (Sanz & Coll, 1992).

levels obtained in the absence of G protein, thus confirming the interaction between p5 and protein G. Binding of G to solid-phase p5 was also competed by the enhancer peptides spanning aa 99–113 and 199–213 but not by the peptide spanning aa 369–383 and frg11 (Fig. 4b).

To confirm the binding of p5 to protein G and to map the positions on the G protein to which it binds, the influence of VHSV pre-incubation with p3, p4, p5, p6 or scrambled p5 on the subsequent binding of previously mapped anti-G mAbs to VHSV was studied by ELISA. Fig. 5 shows that only p5 inhibited the binding of mAb C10, one of the neutralizing mAbs tested. Neither 3F1A12-, IP1H3- nor I10-binding was inhibited by p5. Furthermore p3, p4, p6 or scrambled p5 did not inhibit the binding of any of the mAbs tested. Because the level of interaction of the mAbs with each of the peptides was subtracted from each data set, the results confirm that p5 interacts with the G protein. Furthermore, p5-binding could be

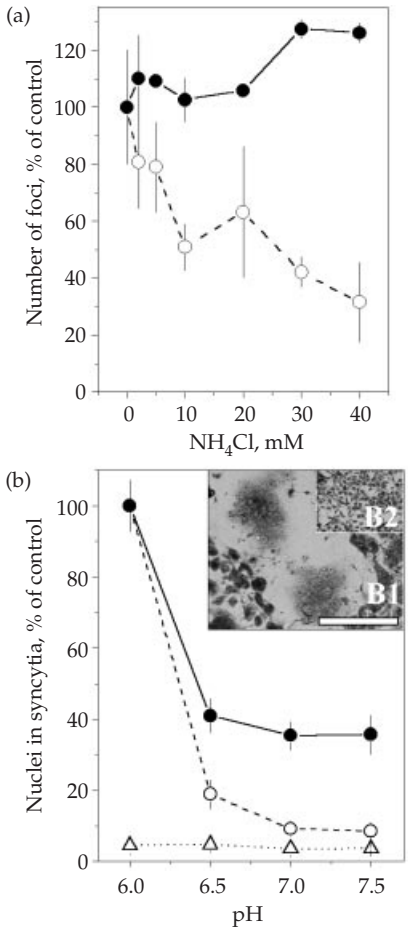

Fig. 6. Pre-incubation of VHSV with p5 prevents the inhibition of VHSV infectivity by NH_4^+ ions (a) and reduces the low-pH requirement for VHSV-induced cell–cell fusion (b). (a) EPC cell monolayers in 96-well plates were infected with VHSV or pre-incubated p5 + VHSV (10 μg p5 + 200 f.f.u. VHSV) in the presence of increasing concentrations of NH_4Cl. After 1 h at 14 °C, pH 7·6, the virus–peptide mixtures were removed, the cells were washed and 100 μl of fresh medium added. The numbers of f.f.u. per well obtained were $19 \pm 2{\cdot}1$ and $60{\cdot}5 \pm 8{\cdot}5$ per well for VHSV and for p5 + VHSV, respectively. Results are expressed as percentages of the number of f.f.u. obtained for each situation and by several concentrations of NH_4Cl, calculated using the following formula: (number of f.f.u. in the presence of NH_4Cl/number of f.f.u. in the absence of NH_4Cl) × 100. Results are expressed as mean ± SD of three experiments. VHSV pre-incubated with 10 μg scrambled p5 gave results similar to VHSV alone (data not shown). (b) Cell–cell fusion of uninfected EPC cell monolayers was induced by adding purified VHSV or p5 + VHSV. VHSV ($2{\cdot}5 \times 10^9$ f.f.u./ml) was pre-incubated with 40 μg/ml p5 for 4 h at 14 °C and then 100 μl per well of the mixtures were added to the EPC cell monolayers in 96-well plates. After VHSV or p5 + VHSV mixtures were removed, the monolayers were incubated with medium at pH 5·6 or 7·6 for 30 min at 14 °C, followed by incubation with medium at pH 7·6 for 2 h. The cells were then fixed with cold methanol and stained with Giemsa. The percentage of fusion was calculated using the following formula: (number of nuclei in syncytia/total number of nuclei) × 100. Results are expressed as the percentage of fusion at pH 6 using the following formula: (percentage of fusion at each pH/percentage of fusion at pH 6) × 100. Results are expressed as mean ± SD from duplicates. One of three experiments is represented. VHSV pre-incubated with 40 μg scrambled p5 gave results similar to VHSV alone. ●, p5 + VHSV; ○, VHSV; △, uninfected EPC cells. Inserts showing the aspect of VHSV-induced syncytia (B1) compared to a control EPC cell monolayer (B2) are shown. Bar, 100 μm.

located near the mapped epitope of mAb C10 MAR mutants (a conformational region mapping simultaneously to aa 140 and 433) (Gaudin *et al.*, 1999a). Alternatively, p5-binding could cause G protein conformational changes affecting mAb C10-binding.

Inhibition of the neutralization of VHSV by mAb C10 by pre-incubation of VHSV with p5 confirmed further the interaction of p5 with the epitope defined by neutralizing mAb C10 (data not shown).

All these data demonstrated that p5 and at least some of the enhancing viral peptides could bind to the G protein at the same region and that the binding interferes with binding to G and neutralization to VHSV by mAb C10 (aa 140 and 433). Further experiments were designed to explore the possibility of p5 interfering in VHSV fusion, as the possible implication of the region around aa 140 of the G protein in fusion (Gaudin *et al.*, 1999a; Walker & Kongsuwan, 1999) and all data mentioned above suggested that the enhancement of VHSV infectivity could be related to virus–cell fusion.

Evidence for interference of p5 with VHSV fusion

Rhabdoviral low-pH-dependent fusion and subsequent infection occur in the endosome after cellular binding and internalization. Therefore, agents that will inhibit the lowering of pH in the endosomes, such as NH_4^+, will inhibit the fusion and infectivity in rhabdoviruses. As expected, the addition of 40 mM NH_4^+ reduced VHSV infectivity from $19 \pm 2{\cdot}1$ to $4{\cdot}7 \pm 1{\cdot}5$ f.f.u. per well. However, when VHSV was pre-incubated with p5, enhancement of VHSV infectivity was unaffected by NH_4^+. Thus, VHSV infectivity after pre-incubation with 10 μg of p5 per well was enhanced from $19 \pm 2{\cdot}1$ to $60{\cdot}5 \pm 8{\cdot}5$ f.f.u. per well and it was maintained to about the same level in the presence of several concentrations of NH_4^+ (Fig. 6a). Similar results were obtained when 0·05 ($39{\cdot}3 \pm 2{\cdot}6$ f.f.u. per well) or 0·1 ($46{\cdot}3 \pm 5{\cdot}7$ f.f.u. per well) μg of p5 per well was used, thus showing that pre-incubation of VHSV with p5 causes VHSV to bypass the low-pH endosome pathway for VHSV fusion.

Furthermore, pre-incubation of VHSV with p5 allows some VHSV-dependent fusion between plasma membranes of uninfected cells to occur at physiological pH, as measured by syncytium formation in the presence of purified VHSV in uninfected EPC cell monolayers. Thus, by using uninfected EPC cell monolayers at pH 7·6, after the addition of VHSV pre-incubated with p5, 21 ± 4% (36·8% of control fusion with VHSV at pH 5·6) of nuclei in syncytia were found, whereas after adding VHSV, only 4% of nuclei in syncytia were found (Fig. 6b). In control parallel experiments carried out at the optimal conditions for fusion (pH 5·6) and after adding VHSV, 57 ± 5% of nuclei in syncytia were found. These results show that after pre-incubation of VHSV with p5, VHSV–p5 complexes bypass the low-pH requirement for VHSV-induced fusion and suggest that the enhancement of infectivity detected

by the focus-forming assay could be due to the penetration of the VHSV–p5 complexes directly throughout the plasma membrane to the cytoplasm.

Discussion

Contrary to what has been reported for many other enveloped viruses, peptides derived from envelope glycoprotein sequences were not strong inhibitors of VHSV infectivity. In contrast to the inhibitory peptides, we report here the enhancement of virus infectivity produced by the pre-incubation of VHSV at low pH with three of the 15-mer pepscan peptides derived from its own envelope protein, the G protein.

The mechanism of enhancement by VHSV peptides seems to be related to fusion because the virus enhancer peptide spanning aa 99–113 and enhancer frg11 belong to a region implicated previously in low-pH-dependent fusion (Estepa & Coll, 1996; Estepa *et al*., 2001). In addition, enhancer peptides spanning aa 99–113 and 199–213 were among the peptides from the G protein that show the highest phosphatidylserine-binding activity (Estepa & Coll, 1996). Furthermore, the enhancement effect was only significant when peptides and virus were pre-incubated at pH 5·6 (the optimal pH for VHSV fusion), even though subsequent infectivity assays were always performed at physiological pH, suggesting that interaction of virus infectivity enhancing peptides with VHSV requires the fusion-active, low-pH conformation of the VHSV G protein. Frg11, which induces syncytium formation when briefly incubated with uninfected cells at pH 5·6 (Estepa *et al*., 2001), also requires a pre-incubation step with VHSV at pH 5·6 before being added to the cell monolayers at pH 7·6 in order to induce enhancement of VHSV infectivity. Because the conformation of frg11 is also dependent on pH (Estepa *et al*., 2001), these results suggest that not only the G protein but also frg11 fusion-active conformations are required to induce the enhancement of VHSV infectivity.

Because no inhibitory peptides were found among the VHSV-derived peptides, we then searched among a larger number of peptides from an α-helix-restricted combinatorial library. The results again detected only peptides causing enhancement rather than inhibition of infectivity. Thus, p5 and p6, defined by the enhancement of virus infectivity results of the screening of 80 17-mer peptide mixtures of the library, were found [sequence SAAEASAK(A,N)TAEATAKG (p5 had an A and p6 had an N)]. The peptides synthesized according to the defined library sequences confirmed the increase in infectivity of VHSV on susceptible cells. The substitution of the T for E residues at position 14 in any of the two peptides (p5 → p3 or p6 → p4) or the scrambling of the amino acid sequence of p5 (randomizing p5 sequence) abolished the effect of enhancement, thus showing the specificity of the interaction and its amino acid sequence dependence. Enhancement of virus infectivity was described before for 2- to 3-mer peptides in bovine leukaemia virus (Voneche *et al*., 1993) and for 4-mer peptides in xenotropic murine retrovirus (Suk & Long, 1983) but not for larger peptides. To our knowledge, only a peptide sequence with antiviral activity against grass carp haemorrhage reovirus has been selected before from a nona-peptide library displayed on phages (Wang *et al*., 2000). Therefore, this should be the first report on the enhancing effects of rhabdovirus infectivity by peptides derived from the viral envelope protein or from a synthetic library.

The reason why we did not find any rhabdovirus inhibitory peptides might be simply because of the restricted nature of the virus pepscan and/or the peptide library. We have not tested every possible 15- or 17-mer peptide or other possible peptides of different sizes. Although unlikely, it cannot be completely ruled out that inhibitory rhabdoviral peptides could be found among shorter or longer peptide sequences or that some 15-mer peptides from the pepscan of the G protein could be inhibitory if used at much higher concentrations. However, both length and concentration are related in the antiviral peptides reported in other enveloped viruses. Thus, previous studies have shown than synthetic peptides derived from similarly located viral sequences (Owens *et al*., 1990) or from combinatorial libraries (Puras Lutzke *et al*., 1995) were effective to inhibit HIV infectivity at mM concentrations for 6-mer peptides, μM concentrations for 11-mer peptides (Ferrer *et al*., 1999; Slepushkin *et al*., 1990) or nM concentrations for 22-mer peptides (Slepushkin *et al*., 1993). Similar concentration ranges were found in inhibitory peptides from paramyxoviruses (Lambert *et al*., 1996; Rapaport *et al*., 1995). Since 15-mer peptides were tested for the VHSV G pepscan at ∼ 3 μM concentrations and p5 (17-mer) could be active at ∼ 0·15 μM concentrations, it is unlikely that longer peptides will show different qualitative effects.

Peptide p5 must interact with VHSV G protein rather than with the cells or the viral membranes to induce the enhancement effect because no enhancement was obtained when p5 was pre-incubated with the cells rather than with VHSV. Before infection, the extent of enhancement depended on both peptide and virus concentration, time and temperature, it occurred whether the cells were from carp (EPC) or from trout (RTG-2), it was virus specific and no interactions of p5 with phospholipid liposome models could be demonstrated. Confirming the above-mentioned expectation, solid-phase p5 bound purified G protein, which could be out-competed by the addition of soluble p5 and p5-inhibiting, neutralizing mAb C10 binding to solid-phase VHSV and mAb C10 neutralization of VHSV. Furthermore, bypassing the low-pH requirement for fusion by the p5–VHSV complex and the p5 reduction of the low-pH requirement for triggering VHSV-dependent uninfected cell–cell fusion demonstrated that the enhancement effect of p5 after binding to G is related to VHSV–cell fusion. All the results obtained thus suggest that binding of p5 to VHSV at physiological pH produces similar conformational changes in the G protein than those produced by low pH and

are required for regular VHSV–cell fusion. An increase of virus–cell fusion at physiological pH could explain the observed enhancement of VHSV infectivity by allowing virus entry throughout the plasma membrane, independently of virus entry throughout the endosomal pathway. Similar mechanisms of enhancement could operate with the viral peptides that competed with the G protein binding to solid-phase p5, although those would require protein G to be in the low-pH conformation. Thus, in the absence of p5, only VHSV particles that are internalized and survive the enzymes of the endosome lysosome pathway are infectious when the pH lowers and are detected by the focus-forming assay. In the presence of p5, some conformational change occurs in the G protein, which allows VHSV to fuse with the plasma membrane at physiological pH, when endosomal lysosome-inactivating enzymes and the low pH are avoided and thus many more viral particles are infectious and detected by the focus-forming assay.

The existence of enhancement peptides and the absence of inhibitory peptides in VHSV, if extended to other rhabdoviruses, might be due to the different mechanisms involved in the infectivity/fusion steps in rhabdoviruses compared to other enveloped viruses. In rhabdoviruses, including VHSV, the so-called fusion peptide remains internal to the G protein during fusion and the low-pH-induced conformational changes in protein G are reversible. On the contrary, in most enveloped viruses, protease cleavage of envelope proteins exposes the N-terminal part of the fusion peptide just before fusion and the subsequent conformational changes are usually not reversible.

Besides helping the understanding of the underlying mechanisms of rhabdovirus fusion or shedding light on the processes in the early steps of rhabdovirus infections, the present findings might also serve to improve the sensitivity of rhabdovirus detection tests based on VHSV infectivity (Perez *et al*., 2002).

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