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Synthetic peptides reveal a phospholipid binding domain in the glycoprotein of VHSV, a salmonid rhabdovirus

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Summary — Using phosphatidylserine (PS) binding to solid-phase synthetic 15-aa peptides, which covered the full length glycoprotein G of a salmonid rhabdovirus, viral haemorrhagic septicaemia virus (VHSV), evidence is presented showing the mapping of its major phospholipid-binding region. Three overlapping peptides were the dominant but not exclusive, reactive peptides that defined the phospholipid-binding main region. A 28-aa synthetic peptide (p2, aa 82-109), defined by the sequences of the 3 above-mentioned peptides, contained a putative α-helix domain with 3 consecutive hydrophobic amino acid a-d heptad-repeats (amphipathic α-helix), and 2 arginines at its carboxy terminal part. This peptide showed a higher apparent specific activity of PS-binding than the 15-aa peptides. Only native, denatured or recombinant fragment G4 viral glycoprotein G showed PS-binding. This did not occur for any of the other VHSV proteins tested. The highest specific activity of PS-binding, however, was found for purified VHSV. PS-binding to purified VHSV was abolished by any VHSV treatment that removed the glycoprotein G from the virions and was partially inhibited by anti-p2 mouse antibodies. It was higher at pH 5.6 than at pH 7.6. The identification of the fish rhabdovirus main phospholipid binding domain allowed some preliminary comparative sequence studies that showed that p2-like sequences exist in all rhabdoviruses.

phospholipid / glycoprotein / rhabdovirus / VHSV

Résumé — Des peptides synthétiques révèlent un domaine de fixation des phospholipides dans la glycoprotéine G de VHSV, un rhabdovirus des salmonidés. *L'utilisation d'un test de fixation de la phosphatidylsérine sur des peptides synthétiques de 15 aa, couvrant toute la longueur de la glycoprotéine G du virus de la septicémie hémorrhagique virale (VHSV), un rhabdovirus des salmonidés, a permis la localisation de la région principale de fixation des phospholipides. Trois peptides chevauchant se sont révélés dominants, mais non exclusifs, et donc définissaient la région principale fixant les phospholipides. Un peptide synthétique de 28 aa (p2, aa 82-109), défini par les séquences des 3 peptides mentionnés ci-dessus, contenait probablement un domaine en hélice α, comprenant un motif répété de 3 acides aminés hydrophobes consécutifs et 2 arginines à son extrémité C-terminale (α hélice amphipathique). Ce peptide montrait une activité spécifique apparente de fixation des phospholipides plus grande que les peptides de 15 aa. De toutes les protéines virales testées, seuls les fragments G4 naturels, dénaturés, ou recombinants, de la glycoprotéine G, montraient une fixation des phospholipides. Cependant, l'activité spécifique de fixation la plus importante a été observée dans le virus purifié. La fixation des phospholipides au virus purifié était supprimée par tous les traitements du virus qui enlèvent la protéine G des virions. Elle était partiellement inhibée par des anticorps de souris anti-p2, et était plus forte à pH 5,6 qu'à pH 7,6. L'identification de ce domaine principal de fixation des*

phospholipides des rhabdovirus de poissons a permis une étude comparée des séquences, qui a montré que des séquences équivalentes à p2 existent également chez tous les rhabdovirus.

rhabdovirus / VHSV / glycoprotéine / phospholipide

INTRODUCTION

Mammalian rhabdoviruses possess a homotrimeric membrane glycoprotein G, which is responsible for the attachment of the virus to the cellular membrane, and contain its low-pH dependent membrane fusion activity, both in mammalian (Gaudin *et al,* 1992, 1993) and fish (Lecocq-Xhonneux *et al,* 1994) rhabdoviruses.

Phospholipid-detergent micelles extracted from cellular membranes inhibited attachment to the cells and infection by rabies and vesicular stomatitis virus (VSV). Phosphatidylserine (PS) was the strongest inhibitor of VSV attachment (Schlegel *et al,* 1983). After attachment, the virus is internalized and then fusion of the viral and the cellular membranes occurs at pH ≈ 5 (low pH). The fusion peptide has not yet been identified in the G of the rhabdoviruses but the mapping of fusion defective VSV mutants has shown that different internal regions could be involved (Li *et al,* 1993; Zhang and Ghosh, 1994).

The lack of a vaccine against viral haemorrhagic septicaemia (VHS) has made this one of the most damaging diseases in the international salmoniculture industry. We are, therefore, trying to design recombinant vaccines (Leong and Fryer, 1993; Lorenzen *et al,* 1993; Estepa *et al,* 1994) and/or of viral inhibition agents by studying rhabdovirus fish cellular membrane interactions. We report here the sequence and properties of the major phospholipid-binding domain in VHSV (a fish rhabdovirus with similar general properties to other mammalian rhabdoviruses with respect to glycoprotein G mediated low pH dependent fusion) (Lecocq-Xhonneux *et al,* 1994, and results not shown).

MATERIALS AND METHODS

The VHSV 07.71 was grown and assayed for its infectious activity in epithelial papillosum cyprine (EPC) cells and radioactively labelled and purified (Basurco *et al,* 1991).

A series of 15-aa peptides (named by the 8th position) overlapping by 5-aa and spanning the cDNA derived aa sequence of G (Thiry *et al,* 1991) were synthesized (Chiron Mimotopes, Victoria, Australia). The peptides were dried at 300 pmol/well. G peptides p2 (aa 82–109), p3 (aa 110–121) and p4 (122–151) were synthesized by Clontech Lab (Palo Alto, CA, USA). Ascites obtained by injecting immunized mice with parental myelomas (Coll, 1989) were passed on a p2-activated CH-Sepharose-4B column (Sigma Chemical Co. Saint Louis) with an 8-atom spacer in order to isolate anti-p2.

The viral proteins were isolated by preparative PAGE of purified VHSV (Estepa *et al,* 1991). Soluble G (Gs) was isolated by affinity chromatography through a Sepharose–concanavalin A column from VHSV-infected VHSV-free cell culture supernatants (Ruiz-Gonzalvo and Coll, 1993). The G4 (aa 9–443) was a recombinant VHSV G fragment obtained as completely denatured inclusion bodies in yeast (Estepa *et al,* 1994).

The dried, labelled PS, L-3-phosphatidyl-[L-C3-^{14}C]serine, 1,2-dioleoyl (Amersham Buckinghamshire, UK, 53–55 mCi/mmol) was sonicated after the addition of a phosphate–citrate buffer. Viruses, viral proteins or peptides in 100 µl of distilled water were dried at 37°C in 96-well plates and washed and then PS in phosphate–citrate buffer was added (100 µl/well). After 4 h of incubation at 4°C, the plates were washed 3 times with distilled water, and incubated with 2% SDS 50 mM ethylenediamine (100 µl/well) pH 11.5 at 60°C for 30 min. The extracts were

counted on a 1450-Microbeta scintillation counter (Wallac, Oy, Turku, Finland and Pharmacia Iberica, SA). The apparent PS-binding was expressed in pmol of labelled PS bound/pmol of protein dried per well/20 pmol of initially labelled PS.

RESULTS

One peptide (p106) bound the PS about 3 times more than the other peptide background levels (fig 1, top). Maximal apparent PS-binding was again obtained with 10-fold higher amounts of peptides with p106. However, by using higher peptide concentrations, other new peptides with a PS-binding more than 3 times the background were also obtained (fig 1, bottom). Contiguous overlapping peptides p86, p96 and p106 formed a region of increasing PS-binding specific activity, which showed the highest PS-binding activity of the pepscan (table I).

The highest PS-binding specific activity was obtained with affinity-purified Gs, the only G preparation that contained about 30% of trimers and the most native monomers, suggesting that some conformation is needed for optimal binding. No significant PS-binding could be detected when virion-purified N, yeast recombinant N3 or virion-purified M1 or M2 were used to coat the plates.

The results obtained in the different experiments showed that PS-binding to solid-phase VHSV 07.71 was about 2-fold higher at pH 5.6 than at pH 7.6. Maximal apparent PS-binding to the solid-phase VHSV nucleocapsids was 2–4-fold lower than the PS-binding obtained by its corresponding purified virions. Treatment of the purified virions with either Tween or Triton to separate the G from the virions reduced the PS-binding capacity of the pelletable material to near background levels.

Table I. Position of the p2 peptide in the G sequence of VHSV.

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......FEDINKGLVSVPTRIIHLPLSVTSVSAVASGHYLHRVTYRVTCSTS.....G
                    PTRIIHLPLSVTSVS                          p86
                              VTSVSAVASGHYLHR                p96
                                        HYLHRVTYRVTCSTS      p106
                       IIHLPLSVTSVSAVASGHYLH             α-helix
                       IIHLPLSVTSVSAVASGHYLHRVTYRVT          p2
  |    |    |    |    |    |    |    |    |    |    |
 65   70   75   80   85   90   95  100  105  110  115
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G, partial sequence of the protein G (Thiry *et al*, 1991). p86, sequence of the 15-aa peptide pepscan that binds PS in this region at the high peptide concentrations (fig 1, bottom). p96, sequence of the 15-aa peptide pepscan that binds PS in this region at the high peptide concentrations (fig 1, bottom). p106, sequence of the only 15-aa peptide pepscan that binds PS at the low peptide concentrations (fig 1, top). α-Helix, predicted by the ANTIGEN program from the PCGene package. p2, synthetic peptide containing the α-helix plus the 2 Arg (R) containing sequence (RVTYRVT). Of the other 15-aa peptides of the pepscan that bound PS at the high peptide concentrations, only the p376 (^{369}YNRAQYKT**M**NN**T**WKS^{383}) was partially inside of a-d hydrophobic aa heptad-repeats from aa 377 to aa 398 (see fig 3). The hydrophobic aa of the a-d hydrophobic heptad-repeats are in bold. Numbers are the amino terminal aa positions on the G.

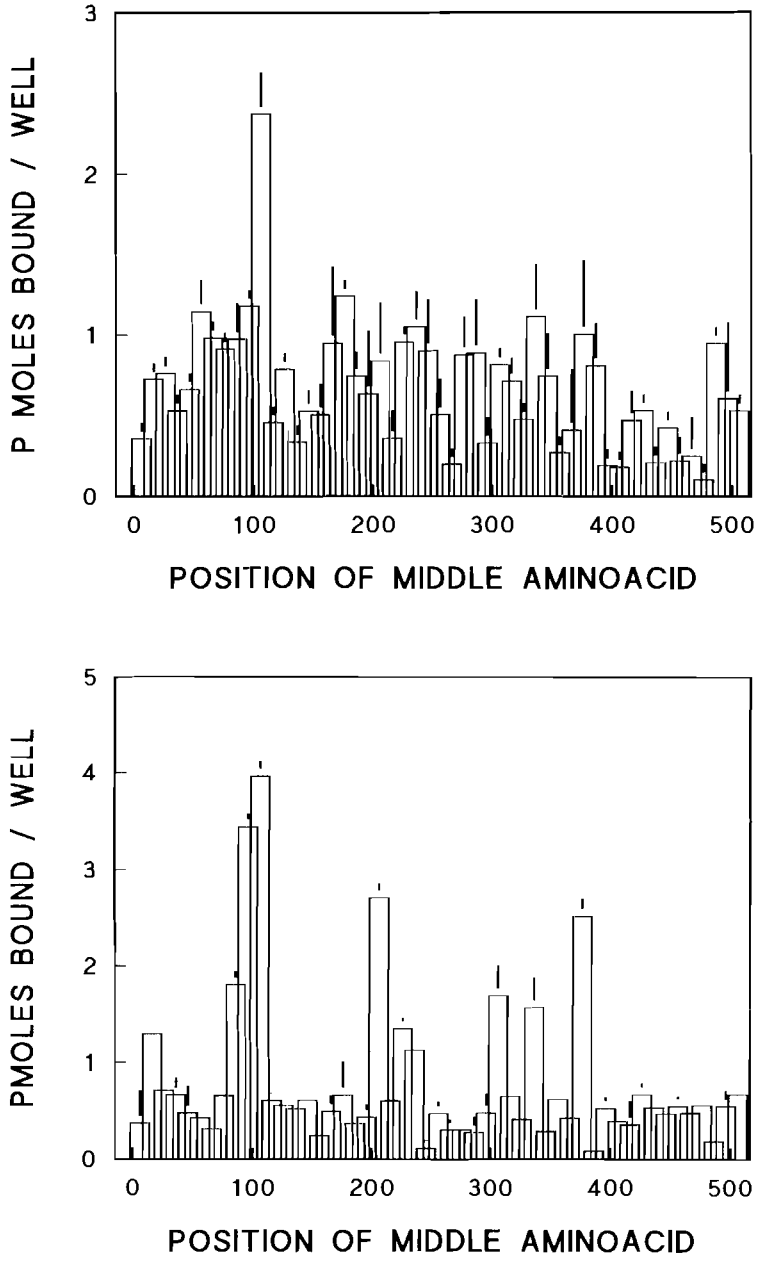

Fig 1. PS-binding to 15-aa G peptides from VHSV. PS (20 pmol/well) binding at 4°C, pH 5.6 for 4 h. About 300 pmol of peptides per well (top) or 3 000 pmol of peptides per well (bottom) coated the solid-phase depending on the peptide. Mean and standard deviations from 4 (top) or 2 (bottom) plates are given.

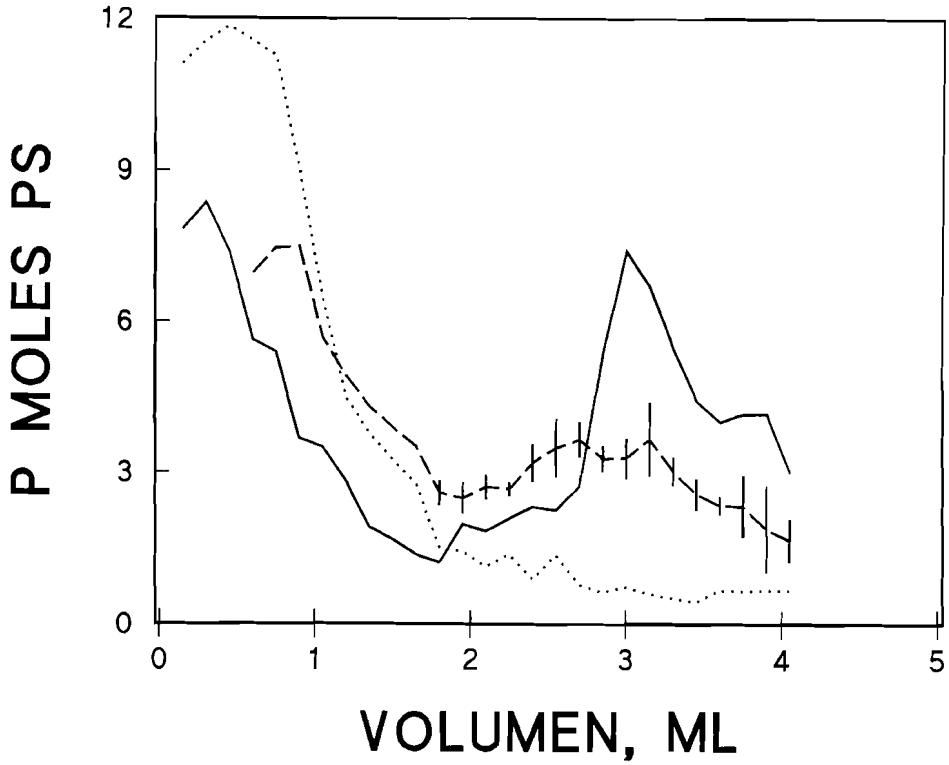

Fig 2. Inhibition of PS-binding to purified VHSV in solution by anti-p2 antibodies. Purified VHSV (40 µg containing about 60 pmol of G) and 10 µg of anti-p2 were incubated for 1 h at 20°C in 150 µl of buffer at pH 5.6, and then 500 µl of the same buffer containing 150 pmol of labelled PS was added and the mixture was incubated at 4°C for 2 h. Ultracentrifugation was as described. ••• PS alone; — — VHSV + PS; – – – VHSV + anti-p2 + PS (average and standard deviations of duplicates are represented).

The fact that the dehydrated peptides in the solid-phase would be unlikely to assume the conformation present in the native G molecule was suggested by the low molar ratio between the PS-bound and the solid-phase p2. The results obtained by ELISA at pH 5.6 with anti-p2 antibodies, showed about a 100-fold lower reaction with solid-phase p2 than with purified VHSV, when expressed in equivalent G molar bases. The inhibition of PS-binding induced by preliminary incubation of VHSV with anti-p2 could be 45.4 to 70.3% (fig 2).

In order to search for similar regions of possible phospholipid binding (p2-like regions) in other rhabdovirus glycoproteins (table II), p2-like regions were defined as belonging to an amino terminal part of the glycoprotein G with at least 3 a-d hydrophobic heptad-repeats containing any hydrophobic aa in positions a or d. Such heptad-repeats (not necessary related to coiled-coil formation) (Lupas *et al*, 1991) could be found throughout the G molecule in VHSV as well as in rabies and in vesiculoviruses (Coll, 1995a, b). The newly

Table II. Amino terminal hydrophobic heptad-repeats in the G protein sequences.

VHSGER	68	FEDINKG LVSVPTR IIHLPLS VTSVSAV ASGHYLH	RVTYR 107
VHSDK	69	FEDINKG LVSVPTK IIHLPLS VTSVSAV ASGHYLH	RVTYR 107
IHNGP	99	IHKV LYRTICS TGPFGGQ TIE	KALVEMK 126
RABMOK	140	WLRT VTTTKES LLLISPS IVEMDIY	GRTLHSP 171
RABPV	140	WLRT VKTTKES LVIISPS IADMDPY	DRSLHSR 171
RHRBGD	140	WLRT VKTTKES LVIISPS IADMDPY	DRSLHSR 171
RABSAD	140	WLRT VKTTKES LVIISPS IADMDPY	DRSLHSR 171
RABHEP	140	WLRT VKTTKES LVIISPS ITDMDPY	DRSLHSR 171
RABLEP	140	WLRT VKTTKES LVIISPS ITDMDPY	DRSLHSR 171
RABCVS	140	WLRT VRTTKES LIIISPS ITDMDCY	DRSLHSR 171
VSVGPN08	134	TVTD AEAHIIT VTPHSVK VDEYTGE WID	PHFIGGR 168
VSVGPNJA	134	TVTD AEAHIVT VTPHSVK VDEYTGE WID	PHFIGGR 168
VSVGPN29	138	AEAHIIT VTPHSVK VDEYTGE WID	PHFLGGR 168
RHGPORS	134	TVTD AEAHIVQ VTPHHVL VDEYTGE WVD	SQFINGK 167
RHGM	134	TVTD AEAHIVQ VTPHHVL VDEYTGE WVD	SQFINGK 168
RHVSVGR	134	TVTD AEAHIVQ VTPHHVL VDEYTGE WVD	SQFINGK 168

Hydrophobic residues a and d are in bold. Positively charged aa are underlined. VHSGER (Thiry *et al*, 1991), VHSDK (Lorenzen *et al*, 1993), IHNGP (Koener *et al*, 1987), rabies (Tordo *et al*, 1993), VSVGPN08, VSVGPNJA, VSVGPN29 (Nichol *et al*, 1989), RHGPORS (Gallione and Rose, 1985), RHGM (Rose and Gallione, 1981), RHVSVGR (Vandepol *et al*, 1986). IHN, infectious haematopoietic necrosis; RAB, rabies; VSVGPN, vesicular stomatitis virus, New Jersey serotype; RH, vesicular stomatitis virus, Indiana serotype. Carboxy terminal heptad-repeats were also found between aa 288–319 (VHSGER and VHSDK), aa 327–354 (IHNGP), aa 330–360 (rabies strains), aa 332–356 (VSV-NJ) and aa 328–369 (VSV-Ind). VHSGER and VHSDK contain another set of heptad-repeats between aa 377–400.

defined heptad-repeats were found between aa 68–101, 288–319 and 377–400 for VHSV, between aa 99–119 and 327–359 for IHNV, between aa 140–164 and 330–360 for all rabies strains examined, between aa 134–161 and 332–356 for VSV-NJ, and between aa 134–161 and 328–369 for VSV-Ind. Table II also shows that all the rhabdovirus G sequences examined in their amino terminal a-d hydrophobic heptad-repeats were also followed by a short 5-7 aa stretch containing 2 positively charged aa, except for IHNV and RABMOK in which only 1 positively charged aa could be found. The new heptad-repeat regions detected showed a high degree of sequence conservation among the rhabdoviruses belonging to the same families but were unrelated among members of different families (fig 3).

DISCUSSION

The main phospholipid binding domain of the G of VHSV was identified through the use of pepscan, synthetic peptides, G and purified VHSV solid-phase phospholipid binding assays. This was the first identification of a phospholipid binding domain in any rhabdovirus and it also extends the information concerning the well-known phospholipid interactions of mammalian rhabdoviruses (VSV and rabies) to fish rhabdoviruses.

The relevance of the PS-binding to solid-phase p2 to the possible PS-binding to VHSV in solution was suggested by the capacity of a p2-affinity purified anti-p2 polyclonal antibody to recognize both p2 and VHSV. Furthermore, under carefully controlled experimental conditions, anti-p2 was capable of partially inhibiting the PS-binding to VHSV in solution. However, PS-binding to solid-phase p2 was detected at the same extent at pH 5.6 and 7.6, in contrast to the binding of PS to VHSV in solution which was mostly detected at pH 5.6. This result confirms previous observations in mammalian rhabdoviruses, which showed that about 10 times more VSV or rabies bound to the cells at pH ≤ 6.5 than at higher pH (Wunner *et al*, 1984). Most probably, the pH 5.6 is needed for conformational changes in VHSV to increase its PS-binding potential.

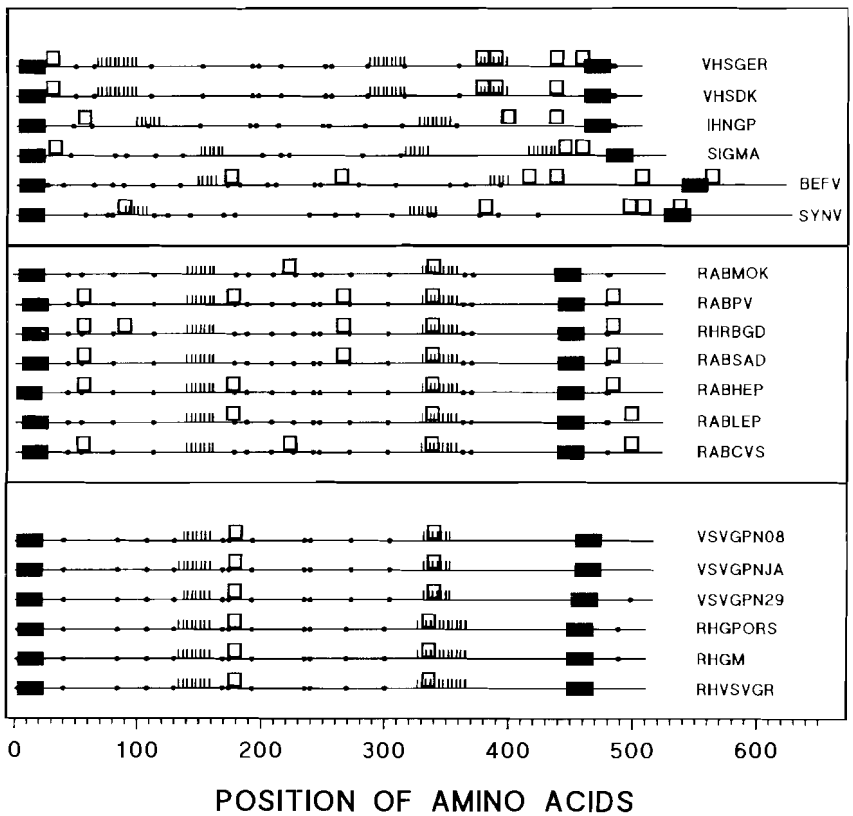

Fig 3. Sequences of amino-terminal and carboxy-terminal a-d heptad repeats in the protein G of rhabdoviruses. The heptad repeats (ıı) were located by using the program PSEARCH (PCGene package, Intelligenetics). $-\Delta G$ values ≥ 0.4 kcal/mol for transfer of the side chain from water to ethanol were used to define hydrophobic aa (single letter code aa, W, F, Y, I, L, L, C, M, A, H, T) (Schulz and Schimer, 1984). The subsequence used to search was (hydrophobic aa) XX (hydrophobic aa) XXX, (X for any aa). The highly and continuous hydrophobic aa sequences of the predicted transmembrane and signal peptide regions were not considered. Symbols: cysteine (●); putative carbohydrate ( ); and predicted transmembrane and signal peptide (▬).

It is not known at present whether the highly conserved domain of p2 and its nearby regions could be related to membrane fusion (Chambers *et al,* 1990) induced by the G of VHSV. However, similar pH dependence profiles have been shown by PS-binding to purified VHSV and by VHSV G mediated membrane fusion (Lecocq-Xhonneux *et al,* 1994; and unpublished results), suggesting that some correlation does exist between them.

The preliminary study of p2-like regions in other rhabdoviruses showed that they were present in all the studied rhabdoviruses, although there were some variations in the number of heptad-repeats, in the length of separation of the positively charged aa and in the kind of positively charged aa involved. Whether these p2-like regions also bind phospholipids or are involved in membrane fusion is unknown at present. However, some of the VSV fusion defective mutants mapped in aa 123–137 (Zhang and Ghosh, 1994), just before the VSV heptad-repeat-rich region from aa 134–161 identified in this work. Only the region 300–360 (Li *et al,* 1993) also mapped nearby another of the heptad-repeat rich regions (aa 332–356 in VSV-NJ or aa 328–369 in VSV-Ind) (Coll, 1995a,b). The fact that the putative fusion peptide of VSV, aa 123–127, is adjacent to the putative VSV PS-binding region, aa 134–161 suggested that they were functionally related.

An initial low affinity binding of VSV or VHSV to the host cells at physiological pH is most probably explained by an initial electrostatic interaction between the positively charged aa in the P-binding region of G and the negatively charged head groups of PS in the host membranes (Lenard, 1993). This initial binding was easily inhibited by PS in the mM range (Schlegel *et al,* 1983). Following internalization of the VSV or VHSV, low pH could trigger the PS-binding region to adopt a bent α-helix conformation, bent due to the presence of proline. In addition there seemed to be a low-pH-induced tendency towards spontaneous simultaneous rearrangements, which caused most of the molecules of the G of rhabdoviruses to cluster in the apical parts of the virus (Lenard, 1993). The amphipathic character (hydrophobic aa heptad-repeats) of the newly formed α-helixes caused them to interact with the fatty acid portion of the closer molecules of PS resulting in a stronger interaction. We could only speculate whether the fusion peptide was carried over by the putative low-pH triggered amphiphatic α-helix formation to interact with the membrane phospholipids or whether the PS-binding region was the one which was pulled into the membrane by the fusion peptide. It was also unclear whether the PS interaction would be essential for fusion or whether it was part of a mechanism to enhance virus binding during fusion at low pH (Konieczko *et al,* 1994).

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