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Synthetic peptides from the heptad repeats of the glycoproteins of rabies, vesicular stomatitis and fish rhabdoviruses bind phosphatidylserine

Brief Report

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Summary. This work follows up on observations previously published concerning phosphatidylserine (PS) binding properties of synthetic peptides (p2) from the hydrophobic heptad repeats of the glycoprotein of viral haemorrhagic septicemia (VHS) rhabdovirus and the presence of similar repeats in the sequences of the glycoproteins of four separate rhabdoviruses. Similar p2-like peptides are now synthesized according to the corresponding cDNA sequences of infectious haematopoietic necrosis (IHN), rabies and vesicular stomatitis (VSV) viruses and shown to bind phosphatidylserine (PS) by solid-phase as well as from liquid-phase assays. The PS-binding peptides located in the amino-terminal part of the glycoproteins contained 3–5 contiguous heptad repeats (abcdefg) of hydrophobic amino acids (aa) in positions a and d followed by a short aa stretch containing positively charged aa and not belonging to the heptad repeats. The rhabdoviral PS-binding regions had low sequence variability among the members of each of the rhabdoviral genus but show no sequence similarity among the different genera.

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Rhabdoviruses possess an homotrimeric membrane glycoprotein G that forms protruding spikes [14, 15], initiates virus attachment to host cells [28], reacts with neutralizing antibodies [2] and induces low-pH dependent fusion [10, 14, 17, 26, 27].

Previous studies showed that phospholipids from cellular membranes inhibited attachment and infection of rabies virus [24] and of vesicular stomatitis virus (VSV) [1, 7, 19, 22]. On the other hand, phosphatidylserine (PS) was the strongest inhibitor of VSV attachment and infection [21]. Only in the viral haemorrhagic septicemia virus (VHSV), a fish rhabdovirus, has the major

PS-binding domain of its glycoprotein G been mapped by pepsan solid-phase binding assays to its amino terminal region [10]. The PS-binding domain was shown to contain amino acid (aa) heptad (abcdefg) repeats containing hydrophobic aa in positions a and d followed by a short aa stretch with two positively charged aa (p2 peptide) [4]. The hydrophobic heptad repeats do not contain the positively charged sites, but rather the charged aa occur after the completion of the heptad repeats. Heptad repeats followed by two charged aa (p2-like peptides) were also found in all rhabdoviruses with variations in the number of heptad repeats, in the length of the charged aa stretch, and in the kind of charged aa involved [5, 6, 10]. Whether or not all the p2-like peptides also bind PS is not known. In this work, we show that the synthetic p2-like peptides obtained from the sequences of rabies, VSV and IHNV glycoproteins also bind PS despite of the fact that no similarity of aa sequences exist among them.

The glycoprotein G sequences (accession numbers from the GenBank data libraries) used to define the p2-like sequences were obtained for VHSV (8 sequences, accession numbers X59148 and X66134 and unpublished sequences kindly provided by Dr. A. Benmansour), infectious haematopoietic necrosis viruses (IHNV) (12 sequences, accession numbers L40871-L40882, kindly provided by Dr. J. Winton), rabies viruses (7 sequences, accession numbers S59447, M32751, J02293, M31046, M32751 and X69122) and VSV (60 sequences, accession numbers M35207-M35232, J04326, M21424, M21416, M21565, M11048, J02430 and X03633). The aa numbering used, corresponds to the protein before signal sequence cleavage as deduced from its cDNA sequence. Synthetic peptides p2 (aa 82–109) and p4 (aa 122–151), from VHSV were obtained from Clontech (Palo Alto, CA, USA). The p2-like peptides from the other rhabdoviral glycoproteins were defined as sequences containing ≥ 3 contiguous heptad repeats of aa with any hydrophobic aa (aa with $-\Delta$ G, Gibbs free energy values $\geq 0.4\,\mathrm{kcal/mol}$ for transfer of the aa side chain from water to ethanol) [23] in positions a and d, followed by a stretch of 5–7 aa containing one or two positively charged aa. P2-like regions could be found between aa 99–119 for IHNV, between aa 140–164 for rabies, and between aa 134–161 for VSV [5, 10]. Most of the charged aa were R as in VHSV, but K and H were also found in the other rhabdoviruses studied (Table 1). To study PS-binding, p2-like peptides were designed to contain as many heptad repeats as feasible for peptide synthesis including the charged aa stretch or charged head, since shorter 15-mer p2 peptides from VHSV were also capable of PS-binding [10, 11]. Taking into account the above mentioned considerations, the p2-like peptides that were synthesized were from aa 103 to 126 for IHNV, from aa 147 to 171 for rabies and from aa 145 to 168 for VSV (Table 1). They were obtained from Chiron Mimotopes (Victoria, Australia). Purity ranged from 35–60% and identity was confirmed by mass spectroscopy. Peptide concentrations showed in the figures were corrected according to the purity values of each peptide.

To perform the solid-phase PS-binding assays, samples of peptides in $100\,\mu\mathrm{l/well}$ were dried at $37\,^{\circ}\mathrm{C}$ in 96-well plates (polystyrene from Costar/Nunc) and just prior to use, the coated plates were washed with phosphate-

Table 1. Upstream regions, a–d hydrophobic aa heptad repeats and positively charged heads of rhabdoviruses

Virus (#seq)		Upstream regions	a–d hydrophobic aa heptad repeats + heads	
VHSV (8)	58	*R*PAQL*R*CPHE**F**FED**I**NKG **T**	**L**VS**V**PTR AQ K	**I**IH**L**PLS **V**TS**V**SAV **A**SG**H**YLH *R*VTY*R* 107 N **V**N
IHNGP (12)	75	AYPTSI*R*SLS**V**GND**L**GD**I**HTQ A E K	GNH**I**HKV	**L**YR**T**ICS **T**GF**F**GGQ **T**IE *K*ALVEM*K* 126 T V
RABSAD (7)	120	*K*VAGDP*R*YEESLHNPYP MS Q**T**	DYR**W**LRT SS H	**V**KT**T**KES **L**VI**I**SPS **V**AD**L**DPY D*R*SLHS<u>R</u> 171 R L IVEM I G*K*T P T I T
VSVGPN08 (60)	113	*K*SY*K*DGVSFNPGFPPQSCGYG**T**VTD fdm EAT Q AWLI S Q I T	**A**EA**H**IVT **A** IQ V Q	VTP**H**SVK **V**DE**Y**TGE **W**ID PHFIGG*R* 168 H L V SQ LN *K*

The positions of the aa counted from the start of the peptide signal sequence are shown by numbers. Number of sequences screened in parenthesis (#seq). Each heptad is separated by one space. a–d hydrophobic aa are indicated via bold type. The positively charged aa in the extreme positions of the shown sequences are in italics. – p2 and p2-like peptides, VHSV, aa 82–107, IHNV aa 103–126; rabies, aa 147–171 and VSV aa 145–168. – *fdm* Fusion defective mutants of VSV aa 123–137 [29]. Under each of the partial glycoprotein G sequences, the aa variations found in the rest of the sequences of the same genus have been represented. *VHSV* Viral haemorrhagic septicemia 07.71 [25]; *IHNGP* infectious haematopoietic necrosis RB-1 strain [16]; *RABSAD* rabies rabased B19 strain [8]; *VSVGPN08* vesicular stomatitis virus New Jersey strain [20]

citrate buffer [11]. After washing, the amount of the peptides remaining bound to the solid-phase was about 50% of the input peptide concentration (initial solution for coating) as measured with the Bradford reagent on the coated surface [11]. Peptide concentrations shown in Fig. 1A were corrected according to the final bound peptide concentration. Labelled PS (53–55 mCi/mmol, L-3-phosphatidyl-[L-C3-^{14}C]Serine,1,2-dioleoyl, Amersham, Buckinghamshire, England) was dissolved in organic solvents, dried in glass tubes and hydrated with phosphate-citrate buffer. The mixture was then sonicated at 4 °C for 3, 1 min periods and 100 µl containing 20 pmoles of labelled PS were added to the peptide containing wells. After 4 h of incubation at 4 °C, the plates were washed 3 times with distilled water, and extracted with 100 µl/well of 2% SDS 50 mM ethylenediamine pH 12.5 at 60 °C during 30 min. The extracts were transferred to 96-well polyethylene terephthalate plates (Wallac-Pharmacia), 100 µl per well of Hiload-scintillation liquid (LKB, Loughtrough, England) added, the contents mixed by shaking and the plates counted on a 1450-Microbeta counter (Wallac, Turku, Finland & Pharmacia). Backgrounds estimated by performing the PS-binding assay in empty polystyrene wells were $\sim$ 0.5 pmoles of PS/well.

The PS-binding to p2 from VHSV increased with the concentration of the peptide bound to the solid-phase up to $\geq$ 100 pmoles of p2 per well (Fig. 1A), confirming previous observations [10]. As a specificity control, the PS-binding to solid-phase p2 performed in the presence of $\geq$ 1000-fold molar excess of p2 showed only background levels of PS-binding but it was unaffected by the presence of a similar excess of p4 (not shown). An identical profile (not shown) was obtained by using p2 from VHSV Makah, a non virulent isolate [3] which has only one ^{97}S $\rightarrow$ N mutation in the p2 region (A. Benmansour, pers. comm.). Similar profiles of PS-binding dependence on p2 peptide concentration were obtained at pH 5.6 or 7.6 (not shown). The PS-binding also increased with the concentration of p2-like peptides from IHNV but at $\sim$ 10-fold higher peptide concentrations. P2-like peptides from rabies and VSV also showed an increase of PS-binding with increasing peptide concentrations, with a different slope and with a lower maximal PS-binding capacity than the other rhabdoviruses. All, p2 and p2-like rhabdoviral peptides showed an increase of PS-binding to about 100 pmoles of peptides per well (Fig. 1A) and then a plateau and/or a fall in their PS-binding extent with higher peptide concentrations bound to the solid phase (not shown). Though small, the PS-binding data to solid-phase p2 and p2-like peptides was in contrast with the PS-binding shown to control p4 peptide from VHSV (^{122}KTILEAKLSRQEATDEASKDHEYPFFPEPS) and to the irrelevant peptides (pA, TWKEYNHNLQLDDGTC and pB, PYRRDCVTTTVENED) (Fig. 1A), since none of those peptides showed by PS-binding values above background levels when used in the same range of concentrations.

To investigate the possible contribution of the positively charged aa residues ^{103}R and ^{107}R of the p2 peptide to the PS-binding, 15-mer peptides were synthesized and tested after replacing them with other positively (K), slightly positive (H) or negatively (E) charged aa residues. Table 1 shows that PS-binding could be slightly increased or not changed by most of the combinations

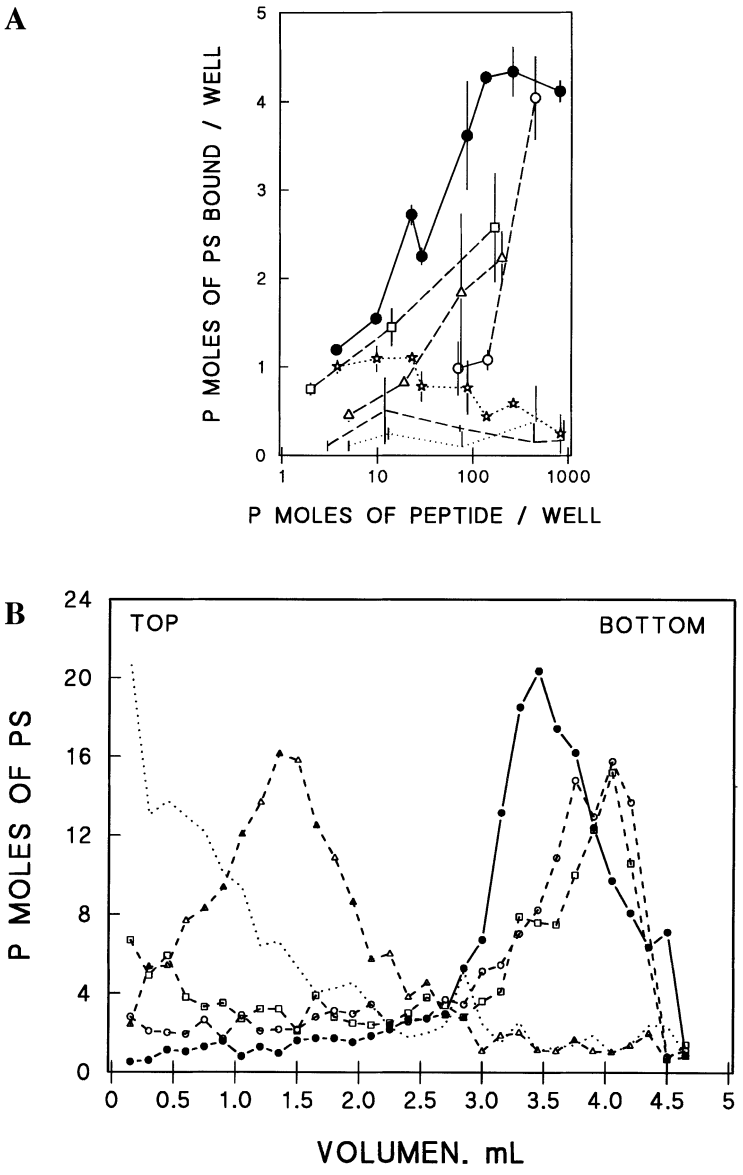

Fig. 1. A PS-binding to rhabdoviral solid-phase peptides. PS-binding (20 pmol/well) to different amounts of solid-phase peptides was performed at 4 °C, pH 5.6. Higher peptide concentrations than those represented showed lower PS binding (they have been omitted here for clarity). ●–● p2 from VHSV; *·····* p4 from VHSV; ◯---◯ p2-like from IHNV; □---□ p2-like from rabies; △---△ p2-like from VSV; - - -,· · · irrelevant peptides pA and pB respectively. **B** PS-binding to rhabdoviral peptides in solution. After several preliminary experiments, different optimal amounts of rhabdoviral p2 and p2-like peptides were incubated overnight in 1 ml of buffer citrate/phosphate pH 5.6 at 4 °C with 0.2 nmoles of labelled PS. Then they were ultracentrifuged in siliconized tubes containing a sucrose gradient at 90 000 **g** during 20 h. ●–● 7 nmoles of p2 from VHSV + 0.2 nmoles of PS; · · · 70 nmoles of p4 VHSV + 0.2 nmoles of PS; ◯---◯ 20 nmoles of p2-like from IHNV +0.2 nmoles of ps; □---□ 60 nmoles of p2-like from rabies +0.2 nmoles of PS; △---△ 100 nmoles of p2-like from VSV +0.2 nmoles of PS. A representative result from several experiments is represented

Table 2. PS-binding by 15-mer peptides derived from p2 after replacing the charged aa residues at positions 103 and/or 107

Position 103–107	PS-binding, pmoles
R–R	1.51±0.16
R–K	3.22±0.03
K–R	2.37±0.12
K–K	0.86±0.03
R–H	2.62±0.16
H–R	2.10±0.08
H–H	2.32±0.02
R–E	2.74±0.12
E–R	1.98±0.19
E–E	0.40±0.05

The 15-mer p2 derived peptide ^{93}SAVASGHYLHR^{103}VTYR^{107} containing some of the heptad repeat and the positive-head region of p2 was synthesized with the replacements in positions 103 and 107 indicated in the table. PS-binding was estimated as indicated. Background of the PS-binding was substracted from all the results obtained

tested but it was inhibited by either the K–K or E–E (aa in the single letter coding) combinations, suggesting that charge is indeed important in this interaction confirming previous results obtained in competition, pH and detergent experiments [10, 11].

Nevertheless and because PS-binding to solid-phase p2-like peptides was weaker than the PS-binding to p2, PS-binding assays in liquid-phase were also tried. To perform the liquid-phase PS-binding assays, a separation of the p2 and the p2-like peptide-PS complexes was attempted by ultracentrifugation over sucrose gradients. About 0.2 nmoles of PS were incubated in the presence or absence of different amounts of p2 or p2-like peptides at 4 °C overnight in 1 ml of citrate/phosphate buffer at pH 5.6. The mixtures were then applied to the top of a 3 ml discontinuous sucrose gradient in citrate/phosphate buffer at pH 5.6 (1 ml of 40% sucrose, plus 1 ml of 16% sucrose, plus 1 ml of 8% sucrose) made in siliconized tubes. Mixtures were then ultracentrifuged at 90 000 **g** during 20 h at 4 °C in a Sorvall ultracentrifuge. After ultracentrifugation, the gradients were collected in 150 µl fractions from the top of the tube and assayed for the presence of radioactivity and for the presence of protein with the Bradford reagent (BioRad, Richmond, VI, USA). Isolated PS (as measured by radioactivity), p4, p2 and p2-like peptides (as measured by the Bradford reagent) remained in the top of the tube (first 1–1.5 ml) of the sucrose gradient after ultracentrifugation under the conditions employed (not shown). Mixtures

of peptides p2 and p2-like from IHNV, rabies or VSV incubated with labelled PS when ultracentrifuged through the sucrose gradients migrated into the gradient in contrast to control peptide p4 incubated with labelled PS. The extent of migration into the sucrose gradient was dependent on the peptide/phospholipid concentration ratios for every p2-like peptide tested. Different peptide concentrations ratios had to be tested until they showed about the same extent of migration of labelled PS into the sucrose gradient except for VSV which was more retarded than all the others at all concentrations tested. Figure 1B shows the results of a representative experiment. The different concentrations of p2-like peptides required for migration of the peptide/phospholipid complexes into the sucrose gradients might be a reflection of intrinsic peptide properties and/or different sizes or packaging densities of the complexes. Alternatively this could also be due to the interactions with the plastic tube, since the peptide/phospholipid complexes bound to non siliconized tubes (not shown).

Basically what has been shown is that peptides with one hydrophobic end and a more positively charged opposite end (p2 and p2-like peptides), can bind a negatively charged phospholipid, which is not surprising. The sequences of the control peptides (p4, pA and pB) are much less hydrophobic and/or rather more negatively charged and thus bind no PS, which is also not surprising. Therefore, it could be suspected that it is the amino acid composition rather than the specific sequence that might account for this degree of PS-binding and that the particular sequences in each rhabdovirus might only be required for structural purposes and/or needed for conformational changes. On the other hand, although indicative, our results do not demonstrate that the rhabdoviral peptides will bind to PS when in the context of the whole glycoprotein. These possibilities remain to be further investigated.

No attempts have been made in the present work to examine the possible biological role of the binding of p2-like rhabdoviral peptides to PS; however, several lines of published evidence suggest that all the rhabdoviral p2-like domains could be somehow related to membrane fusion with host membranes. For instance, viral regions interacting with the host membrane were photolabeled from aa 74–237 in VSV and from aa 122–198 in rabies [9, 13], both these regions containing the p2-like peptides studied here. Moreover mapping of fusion defective VSV mutants identified the aa 123–137 stretch (situated immediately before the p2-like peptide of VSV) as putative fusion peptide [12, 18, 27, 29]. Finally, anti-p2 antibodies were capable of inhibiting VHSV induced fusion [10]. The PS-binding data reported here are therefore likely to be relevant to the early steps of rhabdoviral infections.

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