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Phosphatidylserine binding to solid-phase rhabdoviral peptides: a new method to study phospholipid/viral protein interactions

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Abstract

A new method is described for the study of phosphatidylserine binding to rhabdoviral peptides by using solid-phase assays. This new assay could probably be extended to study the interactions between host membrane phospholipid and viral proteins in other viruses. By using labeled and hydrated phosphatidylserine (PS), PS-binding to solid-phase 15-mer peptides (pepscan) could map putative phospholipid-binding regions of the glycoprotein G of viral haemorrhagic septicaemia virus (VHSV), a salmonid rhabdovirus. The major PS-binding region of 27 aa (aa82–109, p2) did not only bind PS, but also phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Extraction of the PS bound to solid-phase p2 by a variety of chemical compounds and competition experiments with several phospholipid-related compounds showed that PS-binding to p2 was dependent on not only hydrophobic, but also ionic interactions, as suggested by prior work on phospholipid interactions in other rhabdoviruses. Saturation/competition experiments with labeled and cold PS, PE and PC also showed that the reaction probably takes place between high molecular weight aggregates of hydrated phospholipids and several molecules of solid-phase p2. This assay has been used previously to detect hydrophobic amino acid heptad-repeats in rhabdoviruses and when anti-p2 antibodies to VHSV were obtained they were capable of inhibiting VHSV-induced cell to cell fusion.

Keywords: Phosphatidylserine; Rhabdoviral peptides; PS-binding; Hydrophobic

1. Introduction

The entry of some enveloped viruses to host cells is related to viral/host membrane-membrane interactions (Bentz, 1993; White, 1992). Among other possibilities, these interactions could involve

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viral proteins and host phospholipids (Carr and Kim, 1993; Kemble et al., 1994; Lear and Rafalski, 1993). To investigate the possible existence of these types of interactions, we made use of the properties and advantages of solid-phase binding assays. Either phospholipids or viral peptides could be used to coat the solid-phases, however, we only obtained results using viral immobilized peptides (Coll, 1995a,b,c; Estepa and Coll, 1996).

A rhabdovirus was chosen as a model since there is evidence that rhabdoviruses interact with the phospholipids of the host membranes (Lenard, 1993), through its homotrimeric protein G (Gaudin et al., 1992). The rhabdovirus causing viral haemorrhagic septicaemia (VHSV) was chosen because it is a significant pathogen in European trout farms. The protein G is responsible not only for attachment but also for the low-pH dependent membrane fusion activity (Rigaut et al., 1991; Schlegel et al., 1982; Superti et al., 1984) both in mammalian (Gaudin et al., 1992, 1993) and in fish (Lecocq-Xhonneux et al., 1994; Estepa and Coll, 1996) rhabdoviruses. Furthermore, phospholipids inhibited attachment and infection of rabies (Superti et al., 1984) and of vesicular stomatitis virus (VSV) (Bailey et al., 1984; Conti et al., 1988; Mastromarino et al., 1987). Inhibition of VSV attachment and infection by phosphatidylserine (PS) (Schlegel et al., 1982) and binding of PS by VHSV (Coll, 1995d) have been also reported. Because of the detection of PS-binding-like sequences in all rhabdoviruses (Coll, 1995a) and of the relation between VHSV PS-binding sequences and fusion (Estepa and Coll, 1996), we studied some of the variables of phospholipid binding to solid-phase VHSV peptides so that this technique could be applied to other viral/host systems.

2. Materials and methods

2.1. Synthetic peptides from the G sequence of VHSV

A series of 15-mer peptides overlapping 5 aa and spanning the cDNA derived aa sequence of G (Thiry et al., 1991) of VHSV 07.71 were synthesized chemically (Chiron Mimotopes, Victoria, Australia). Amino acid numbering used throughout the paper, correspond to the protein before signal sequence cleavage as deduced from the cDNA sequences. The peptides were named by the protein sequence amino terminal position of their middle aa (8th position), the first peptide being number 6, due to needs of synthesis (it contained 2 additional aa before the initial methionine according to the cDNA sequence). The peptides diluted in 5 mM Hepes, pH 7, were dried in 100 μl/well. G peptides p2 (aa82–109), p3 (aa110–121) and p4 (aa122–151) were obtained from Clontech (Palo Alto, CA, USA). Irrelevant peptides were pA (H_2N-TWKEYNHNLQLDDGTC and pB (H_2N-PYRRDCVTTVENED).

2.2. Solid-phase phospholipid binding assays

Prior to its use, the synthetic labeled phospholipids were chromatographed by thin layer chromatography to demonstrate a higher than 95% purity. The labeled phospholipids, 53–55 mCi/mmol, L-3-phosphatidyl-L-[C3-^{14}C]Serine, 1,2-dioleoyl (PS); L-3-phosphatidylcholine, 1-palmitoyl-2-[1-^{14}C]linoleoyl (PC) or L-3-phosphatidylethanolamine, 1-palmitoyl-2-[1-^{14}C]linoleoyl (PE) (Amershan, Buckinghamshire, England), were dissolved in organic solvents, dried in glass tubes, phosphate-citrate buffer (Gaudin et al., 1993) added and the mixture sonicated three times at 4°C for periods of 1 min, immediately before use.

For the competition experiments, tissue-extracted, purified phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA.) and from Sigma Chemical Co. (St. Louis, USA.) with ≥ 90% purity as estimated by thin layer chromatography. Different amounts of phospholipids dissolved in organic solvents were dried in separate tubes and sonicated in phosphate-citrate buffer immediately before use.

One hundred μl/well of viral peptides were dried at 37°C in 96-well plates (polystyrene from Costar/Nunc). Just prior to use, the coated plates were washed with the phosphate-citrate buffer. The amount of peptides that remain bound to the polystyrene solid-phases was about 50% of the

initial amount pipetted and dried (input) as estimated by using the Bradford reagent (Bio Rad, Richmond, VI, USA) before and after the coating. Then, phospholipids in phosphate-citrate buffer were pipetted in 100 μl/well. After 4 h of incubation at 4°C, the plates were washed three times with distilled water. To extract the bound phospholipids, 100 μl/well of 2% SDS 50 mM ethylenediamine, pH 12.5, were added and incubated at 60°C for 30 min. The extracts were then transferred to 96-well polyethylene terephthalate plates (Wallac-Pharmacia), 100 μl of Hiload-scintillation liquid (LKB, Loughtrough, England) added per well, mixed and counted on a 1450-Microbeta scintillation counter (Wallac, Oy, Turku, Finland and Pharmacia Ibérica, SA). Backgrounds (maximum of 0.5 pmol of PS/well) were estimated by PS-binding to non-coated wells. The apparent PS-binding was expressed as specific activity calculated in pmol of labeled PS bound/pmol of input peptide per well/20 pmol of PS.

For the blotting experiments, 10 μl containing 1000 pmol of peptide were spotted and dried onto an IPVH-20200 Immobilon-P transfer membrane (Millipore, Bedford, MA). The membrane was blocked with 1% defatted milk in phosphate/citrate buffer at pH 5.6, washed and incubated at 20°C with 10 ml of 1500 pmol of labeled PS for 4 h. After washing and drying, the membrane was exposed to X-Ray film and developed according to the manufacturer's instructions.

3. Results

3.1. Hydratation of the phospholipids

In order to reduce the hydrated PS to aggregates of the smallest possible size, sonication in the citrate/phosphate buffers was carried out with the aid of a sonication probe (New, 1992). The estimated size of the PS aggregates after sonication ranged from 8 to 80 $\times$ 10^3 KDa as estimated by chromatography over Sepharose 4B, most being of 20 $\times$ 10^3 KDa or about 25 000 PS molecules (M_r of PS = 788) per aggregate (Fig. 1 shows the elution profile obtained with PS).

3.2. PS-binding to 15-mer peptides

Maximal apparent PS-binding to 15-mer peptides was obtained with p106 (Table 1). However, peptides with significant PS-binding $\geq$ 3 times the background, p96, p206 and p376 and peptides with significant PS-binding $\leq$ 3-fold the background, p16, p86, p226, p306 and p336 were also obtained. The extent of PS-binding of all the above mentioned peptides were confirmed when individually tested by solid-phase assays in the range of 100–10 000 pmol/well (not shown). PS binding by peptides p86, p96, p106 (most intense spot), p206, p226, p236, p336 and p376 was also confirmed by autoradiography of blotted peptides after incubation with labeled PS (not shown). The contiguous overlapping peptides p86, p96 and p106 form a region of increasing PS-binding specific activity which together shows the highest PS binding activity.

No obvious similarities appear among all the detectable PS-binding peptide sequences nor could any correlation of the PS binding with its hydrophobicity index be shown. Peptides p466, p476 and p486, that define the transmembrane segment, did not show any PS-binding above the

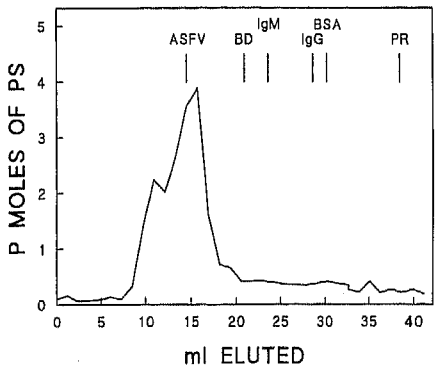

Fig. 1. Chromatography over Sepharose 4B of hydrated and sonicated PS. PS aggregates were prepared by sonication of dried PS in aqueous buffers as described in Materials and methods. PS sonicated aggregates (1 ml) were chromatographed in a 100 $\times$ 1 cm Sepharose 4B column in 1 M ammonium bicarbonate. Markers were as follows: ASFV, purified African swine fever virus; BD, blue dextran of 2 $\times$ 10^3 KDa; IgM, trout immunoglobulins of 750 KDa; IgG, mice immunoglobulin G of 150 KDa; BSA, bovine serum albumin of 65 KDa; PR, phenol red.

Table 1
15-mer peptides from the G of VHSV showing detectable PS-binding

p[a](aa[b] first-last)	Sequence	Hydrophobicity	pmol (PS/well)
p16(9–23)	VILIIIIKSTTPGIT	0.93	1.29 ± 0.04
p86(79–93)[d]	PTRIIHLPLLSVTSVS	0.69	1.80 ± 0.14
p96(89–103)[d]	VTSVSAVASGHYLHR	0.42	3.43 ± 0.13
p106(99–113)[d]	HYLHRVTYRVTCSTS	0.44	23.95 ± 1.50[c]
p206(199–213)[d]	THWQGVYWVGATPKA	0.58	2.70 ± 0.15
p226(219–233)[d]	ETLEGHLFIRTHDHR	0.26	1.35 ± 0.11
p236(229–243)[d]	THDHRVVKAIVAGHH	0.27	1.13 ± 0.18
p306(299–313)[d]	FSYLNHLITNMAQRT	0.51	1.69 ± 0.32
p336(329–343)[d]	SSFLLSKFRPSHPGP	0.47	1.57 ± 0.31
p376(368–383)[d]	YNRAQYKTMNNTWKS	0.08	2.50 ± 0.18
p466(456–463)	NWSLWPSLSGMGVVG	0.77	0.24 ± 0.24
p476(466–473)	MGVVGGAFLLLVLCC	1.12	0.10 ± 0.10
p486(476–493)	LVLCCCCKASPPIPN	0.89	0.94 ± 0.10

Positively charged aa in bold. Predicted α-helix (aa82–102) underlined. The putative transmembrane domain (aa462–482) includes p466, p467 and p486. Hydrophobicity index as described by Kyte and Doolittle, 1982. p2 = predicted α-helix + the sequence RVTYRVT.
[a]Peptide.
[b]Amino acid. PS-binding (20 pmol/well) was at 4°C, pH 5.6 during 4 h to 3000 pmol of peptides/well.
[c]Calculated from Fig 2. The other 38 G peptides bound an average of 0.46 ± 0.15 pmol of PS/well. Mean and standard deviations from two plates are represented.
[d]Indicates positive autoradiography of blotted peptides after PS-binding.

background levels (Table 1), most probably due to the absence of charged aa in the sequences of the putative transmembrane domain (aa474–494). On the other hand, the transmembrane domain must interact mostly with other phospholipids such as PC and PE and/or cholesterol, which are usually the principal components of animal membranes and not only with PS.

3.3. Binding of PS to p2

The highest PS-binding sequence, the p106 (aa99–113) contained two Arginines (aa103 and aa107) in the carboxy-terminal part (Table 1). In addition, the PS-binding G region (p86 + p96 + p106) showed a putative α-helix (aa82–102) contained in a longer region made of hydrophobic (a–d hydrophobic) heptad-repeats (aa68–102) (Coll, 1995a,b). The presence of hydrophobic aa in the a–d positions of aa heptads would make an amphipathic α-helix because of the clustering of hydrophobic aa on one side only. The presence of proline at the aa86 would probably make it a bent α-helix. The so called p2 peptide (aa82–109) containing both the predicted putative amphipathic α-helix and the aa stretch with the two Arginines was synthesized.

As foreseen from the properties of the 15-mer peptides, p2 bound PS with about a 10-fold higher apparent specific activity than the highest PS-binding 15-mer p106 (Fig. 2). PS-binding to solid-phase p2 in the presence of ≥ 1000-fold molar excess of p2 showed background levels of binding but it was unaffected by the presence of a similar molar excess of p4. The apparent PS-binding increased with the input concentration of p2 bound to the solid-phase to about 100 pmol of p2 per well and the plateaus (> 200 pmol/well). Only background levels of PS-binding were obtained with p3, p4 or with the irrelevant peptides pA or pB (Fig. 2). Similar profiles of the peptide concentration dependence on PS-binding were obtained at pH 5.6 or 7.6 (not shown). The PS-binding to solid-phase p2 but not to p4 (aa122–151) nor to p3 (aa110–121), increased with time, still slowly increasing after 4 h of incubation (Fig. 3). The kinetics of PS-binding to p2 were identical whether they were performed at pH 7.6 or pH 5.6 and at 4°C or 20°C.

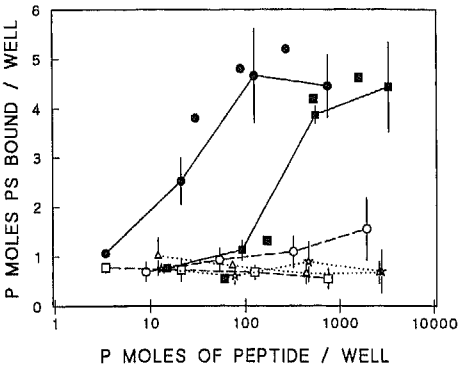

Fig. 2. Dependence of PS-binding on VHSV peptide concentrations. PS-binding (20 pmol/well) to different amounts of solid-phase peptides was performed at 4°C, pH 5.6: ●——●, p2 (aa82–109); ●, p2 from a different experiment; ■——■, p106 (aa 99–113); ■, p106 from a different experiment; ○—-○, p3 (aa110–121); □—-□, p4 (aa122–151). The irrelevant peptides were, △····△, pA and ☆····☆, pB. Averages and standard deviations of duplicates are represented.

To saturate the solid-phase containing 150 pmol of input p2 (equivalent to 75 pmol of p2 bound to the solid-phase), more than 10^5 pmol of PS (equivalent to ≥ 4 'pmol' of hydrated PS aggregates) have to be added (Fig. 4).

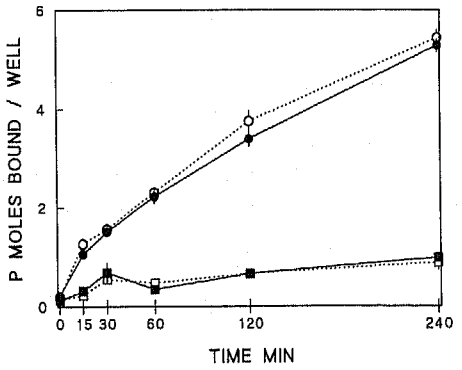

Fig. 3. Time course of PS-binding to solid-phase p2 or p4. PS-binding (20 pmol/well) was performed at 4°C. Plates were coated with 150 pmol of either p2 (aa82–109) or p4 (aa122–151): ●——●, PS-binding to p2 at pH 5.6; ○—-○, PS-binding to p2 at pH 7.6; ■——■, PS-binding to p4 at pH 5.6; □—-□, PS-binding to p4 at pH 7.6. Results of PS-binding to p3 were similar to those obtained with p4 (not shown). Averages and standard deviations of duplicates are represented.

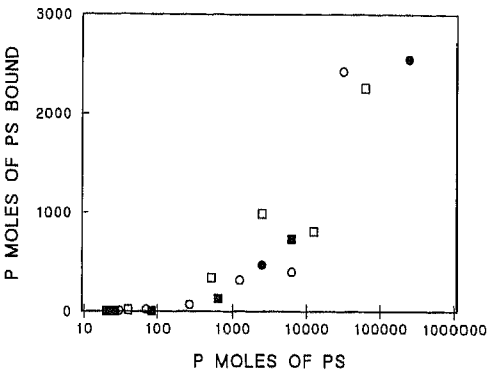

Fig. 4. Dependence of PS-binding to solid-phase p2 on the initial PS concentration. PS-binding at high concentrations was estimated by mixing labeled PS with different amounts of cold PS (Avanti Polar Lipids). PS-binding was performed at 4°C, pH 5.6 using plates coated with 150 pmol of p2/well. ●, ○, ■, □, results obtained in different experiments.

3.4. Preliminary characterization of PS-binding to solid-phase p2

Inhibition of PS-binding (78.6 ± 3.4%) was obtained when solid-phase p2 was treated with 1 mg/ml of pepsin at pH 5.7 during 2 h of incubation at 20°C prior to the addition of labeled PS (pepsin cleaves after aromatic and other hydrophobic aa) (Beynon and Bond, 1993), suggesting that the PS-binding to solid-phase p2 is, at least, partially dependent on the integrity of the p2 structure. Pepsin digestion had no effect if it were undertaken after the PS was bound to p2. Protease V8 digestion at pH 8.4 (cleaves after aa E or D, neither of which is present in p2) had no effect on posterior PS-binding (not shown).

A series of experiments focused on the extraction of the PS bound to solid-phase p2 were conducted. The results showed that once bound to p2, PS was resistant to agents known to denature proteins such as urea, guanidine isothiocyanate, NaCl, EDTA, pH 3, and pH 11.5 but it was extracted by any of the detergents used (2% of SDS, 10% of CHAPS, 1% of TX-100, 1% of TX-114, 1% of Tween 20, 1% of Tween 80, 10% of Sarcosil or 1% of octylglucoside) or by lipid solvents such as DMSO (Table 2). Furthermore, buffers with very high pH (pH $\geq$ 12.5, the highest PK of arginine) were also capable of extracting

Table 2
PS remaining bound to solid-phase p2 after extraction with several compounds

Compound	Concentration	Bound PS (%)	Compound	Concentration	Bound PS (%)
Urea	10 M	97.0 ± 4.5	EDTA[c]	0.5 M	82.1 ± 5.4
GI[a]	10%	87.3 ± 11.4	PEG6000[d]	35%	85.3 ± 5.6
NaCl	5 M	95.7 ± 2.6	CHAPS[e]	10%	5.3 ± 0.8
Tris	0.2M (pH 3)	94.3 ± 6.8	SDS[f]	2%	6.9 ± 1.7
H2O	-pH5	95.6 ± 0.5	+SDS	0.1%	90.9+2.6
ETDM[b]	50mM (pH 11)	80.6 ± 14.7	+SDS	0.1%	45.1 ± 25.1
NaOH	50mM (pH 12)	75.8 ± 4.7	+SDS	0.1%	12.4 ± 4.2
NaOH	0.5N (pH 14)	7.5 ± 1.9	+SDS	0.1%	5.5 ± 4.2

PS (20 pmol/well) were incubated with solid-phase p2 (150 pmoles/well) at 4°C, pH 5.6 during 4 h. After washing, about 5 pmoles of PS were bound per well, then different compounds in 100 μl volume per well were pipetted and incubated during 1 h at 20°C (treated wells). Plates were again washed and the bound radioactivity extracted with 2% SDS, pH 12.5, 60°C during 30 min and counted. Bound PS as calculated by the formula, cpm extracted in treated wells/cpm extracted in control non-treated wells × 100. Averages and standard deviations from triplicates are represented.
[a]Guanidine isothiocyanate.
[b]Ethylenediamine.
[c]Ethylendiaminotriacetate.
[d]Polyethylenglycol 6000.
[e]3-cholamidopropyl-dimethylammonio-1 propane sulfonate.
[f]Sodium dodecyl sulfate.

the bound PS. It was also suggested that the PS-binding to p2 depends both on hydrophobic and electrostatic interactions by the synergic results obtained between SDS at subextracting concentration (0.1%) and different pH's. Thus, at 0.1% SDS and pH 5, most of the bound PS remained associated to the solid-phase p2, however by increasing the pH, less and less PS remained bound to the solid-phase p2, a result that could not be obtained in the absence of SDS (Table 2).

A molar excess of ≥ 10 000–100 000 fold over the 5 pmol of PS bound to p2, of free fatty acid (linoleic acid) or glycero-phosphorylserine reduced the PS-binding to about 65 or 40% of its initial value, respectively. In contrast, neither serine nor phosphorylserine were able to reduce the PS-binding to p2 when used at similar concentrations (Fig. 5).

PS-binding to solid-phase p2 was 2–3 fold higher than that of PC or PE-binding (not shown). A series of experiments using labeled PS, PC or PE and competition with tissue extracted phospholipids, confirmed that solid-phase p2 was also capable of binding labeled PE and PC and that all phospholipids PS, PC or PE could compete with each other (Fig. 6). About ≥ 10 000 pmol of cold PS (a 2000 molar excess over the 5 pmol of PS bound to p2) abruptly reduced to 20% the bound PS. Maximal phospholipid competition as a function of the concentration of cold phospholipid competitors was also abrupt with ≥ 10 000 pmol of PC but it was more continuous when PE was used. Binding of labeled PE or PC were similarly reduced to 20% of the initial counts by the addition of ≥ 10 000 pmol of cold PS. Binding of labeled PE was also abruptly reduced to 20% by the addition of ≥ 10 000 pmol of cold PC but the reduction was more continuous when cold PE was added. Binding of labeled PC was reduced continuously as the concentrations of either cold PC or cold PE were increased (Fig. 6).

4. Discussion

The binding of radioactively labeled phospholipids, especially of PS to solid-phase viral peptides has been characterized preliminarily by using peptides from the protein G of VHSV, a salmonid rhabdovirus, as a model.

Since hydrophobic molecules, including phospholipids, stick to some solid-phases made of different plastics and sometimes to those made of glass, specificity of the labeled phospholipid binding to solid-phase peptides was a key point for the interpretation of the results. Evidence that the PS-binding reaction to the solid-phase peptides was specific includes: absence of significant binding when no peptides or irrelevant peptides were used to coat the wells (Fig. 2), competition of PS-binding to solid-phase p2 with soluble p2 but not with p3 or p4, solid-phase p2 concentration dependence of PS-binding (Fig. 2), inhibition of PS-binding by pepsin digestion of solid-phase p2, 10-fold increase of PS-binding using p2 compared to smaller 15-mer peptides from the p2 region (p86, p96 and p106) (Fig. 2), competition to PS-binding with molecules chemically related to PS (Fig. 6) and properties of the compounds capable of the extraction of p2 bound PS (Table 2).

The highest PS-binding peptide p106 (aa99–113) was at the carboxy-terminal part of a pre-

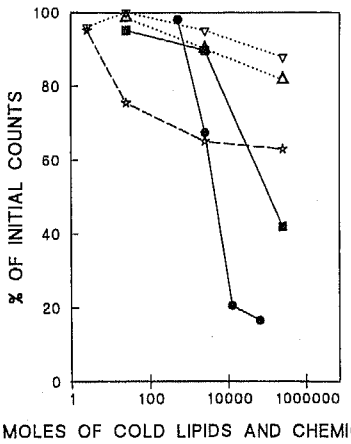

Fig. 5. Competition of PS-related chemicals to PS-binding to solid-phase p2. Different concentrations of sonicated cold tissue-extracted PS or other chemicals (competitors) were pipetted (50 μl/well) into p2 coated plates (150 pmol/well). Then, 50 μl of sonicated labeled PS (20 pmol/well) was added and plates were incubated for 4 h at 4°C, pH 5.6. Plates were washed and the bound radioactivity counted. Control, no competitor added wells, showed 5 pmol of PS bound/well. Results were calculated by the formula, cpm in the presence of cold competitors/cpm in control × 100. ●——●, cold bovine brain PS; ■——■, L-α-glycerolphosphorylserine; ☆—-☆, linoleic acid; △····△, serine; ▽····▽, phosphorylserine.

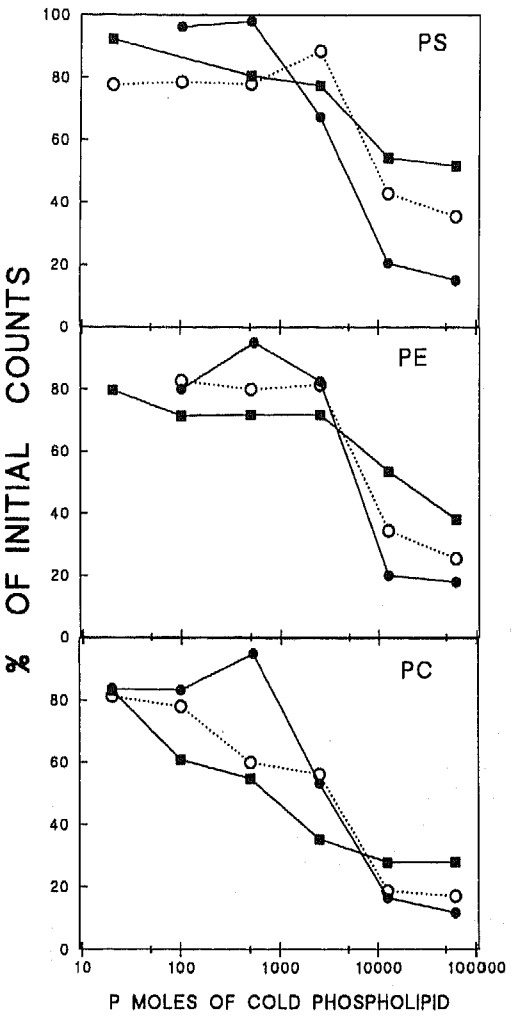

Fig. 6. Labeled PS, PE and PC-binding to solid-phase p2 in the presence of cold PS, PE and PC. Different concentrations of sonicated cold tissue-extracted phospholipids were pipetted (50 μl/well) into p2 coated plates (150 pmol/well). Then 50 μl of sonicated labeled PS, PE or PC (20, 60 and 60 pmol/well, respectively) were added and plates were incubated during 4 h at 4°C. Plates were washed and the bound radioactivity counted. Control, no phospholipid added wells, showed ~5 pmol of labeled phospholipid bound/well. Results were calculated by the formula, cpm in the presence of cold phospholipids/cpm in control × 100. A representative experiment of 3 is shown in the figure: ●——●, cold bovine brain PS; ○—-○, cold egg-yolk PC; □—-□, cold sheep brain PE. In the upper right corner are shown the labeled phospholipids used (PS, PE and PC).

dicted α-helix within a domain with a–d hydrophobic aa heptad-repeats (Coll, 1995d). That this was the major PS-binding region was shown by the two other highest PS- binding peptides overlapping with p106 (p86, aa79–93 and p96, aa89–103) (Table 1). Its PS-binding was further confirmed by blotting/autoradiography and by ELISA of the individual peptides. All these results, lead to the design of p2 (Table 1) containing both the part of the heptad-repeat with a putative amphipathic α-helix and the part with two Arginines (Arg 103 and 107). Confirming that the above defined region was the main PS-binding region, p2 increased 10-fold the specific activity of PS-binding to p106, the highest PS-binding peptide of the pepscan (Fig. 2).

Experimental evidence showed that the physicochemical nature of the PS-p2 bond seems to be both hydrophobic (destroyed by detergents and competed by fatty acid) and ionic (destroyed at pH > 12.5 and competed by glycerolphosphorylserine) (Table 2 and Fig. 5). Because from aa82–102 there is a putative α-helix with hydrophobic aa heptad- repeats, it would be an amphipathic α-helix (Coll, 1995b). An amphipathic α-helix followed by two positively charged aa (R) could interact with both the long hydrophobic fatty acids and the two negative charges of the PS molecules. This could confirm previous work using spin-labeled phospholipids which showed a distinct requirement of fatty acids for VSV fusion and of the head group for VSV binding (Yamada and Ohnishi, 1986). Hypothetically, the PS-binding to solid-phase p2, could be explained by groups of p2 molecules with its hydrophobic sides facing either the hydrophobic solid-phase or the PS fatty acid chains and its charged residues facing either the aqueous solvent or the PS negative charges.

What is the meaning of the binding of PS hydrated aggregates containing ~25 000 molecules per aggregate to solid-phase peptides in terms of molarity? If ~2500 pmol of PS is the maximal PS-binding capacity of the 75 pmol of solid-phase p2 (Fig. 4), to bind one PS aggregate several p2 molecules are needed. Alternatively, the aggregated state of PS could be destroyed after it is bound to solid-phase p2, as suggested by preliminary evidence that indicates leaking of PS liposomes induced by p2 in solution (not shown). On the other hand, 10^4–10^5 pmol of cold PS have to be added to compete with 5 pmol of PS bound to 75 pmol of solid-phase p2 (Fig. 6), suggesting that the need of such a large excess of cold PS could be explained by its aggregated nature, at least at high PS concentrations. Thus because one average PS aggregate of 20×10^3 KDa contains about 25 000 molecules of PS, 10^4–10^5 pmol of cold PS would only be 0.4–4 'pmol' of aggregated PS, the same order of magnitude that the 5 pmol of PS bound to p2, with which the cold PS aggregates have to compete. In this case each of the aggregates of cold-PS would compete with one molecule of bound PS suggesting also that the PS once bound to solid-phase p2 has been disaggregated. However, because very high amounts of the cold phospholipids were necessary to inhibit each of the phospholipid bindings, it cannot be excluded that the results obtained could be due to the presence of trace amounts of impurities. Therefore, more experimental evidence is needed to interpret the p2-PS reaction in terms of molarity.

Whether or not the labeled PS-binding to solid-phase peptide assays could be used to study other viral domains with some biological significance remains to be demonstrated. However, these assays have already allowed the detection of a–d hydrophobic aa heptad repeats in all rhabdoviruses (Coll, 1995a). Moreover, the mapping of fusion defective VSV mutants or of hydrophobic photolabeled regions have shown that the regions aa123–137 (Li et al., 1993; Whitt et al., 1990; Zhang and Ghosh, 1994) or aa 74–237 for VSV and aa 122–198 for rabies (Gaudin et al., 1995; Durrer et al., 1995), respectively, are located nearby or inside similar PS-binding regions (p2-like) from aa134–168 in VSV and from aa 140–171 in rabies (Coll, 1995d). Furthermore, anti-p2 antibodies inhibit VHSV fusion and bind to VHSV only at the fusion pH (pH 5.5) (Estepa and Coll, 1996).

It is expected that phospholipid binding to solid-phase peptide assays could be used to study other viral-phospholipid interactions to help clarify some of the mechanisms of viral entry into the cells. On the other hand, theoretical studies can be carried out between phospholipid liposomes and p2 from VHSV in solid as well as in liquid phases as a new model to study phospholipid/peptide physicochemical interactions.

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