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Phospholipid Interactions of a Peptide from the Fusion-Related Domain of the Glycoprotein of VHSV, a Fish Rhabdovirus

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Previous studies mapped a p2 domain (aa 82–109) which binds phosphatidylserine (PS) (Estepa and Coll, 1996a) and contains three contiguous hydrophobic amino acid heptad repeats followed by a positively charged stretch (Coll, 1995b) in the glycoprotein G of the viral hemorrhagic septicemia virus (VHSV), a fish rhabdovirus. Anti-p2 antibodies inhibited low-pH VHSV-induced fusion (Estepa and Coll, 1997) and low-pH PS binding to VHSV (Estepa and Coll, 1996a). We report here further studies on the interaction of the synthetic peptide p2 with phospholipid vesicles. The synthetic p2 peptide was able to mediate aggregation, lipid mixing, and leakage of contents only with negatively charged phospholipid vesicles and in a concentration-dependent manner. As shown by its effect on lipid phase transitions deduced from data with fluorescence polarization and differential scanning calorimetry, the p2 peptide becomes inserted into the hydrophobic negatively charged phospholipid vesicle bilayers. In addition, data based on circular dichroism showed that the p2 peptide folds as a structure with a high content of β-sheets stabilized by interaction with anionic phospholipids. These studies are potentially relevant to viral fusion in VHSV.

INTRODUCTION

The fusion of the membrane of enveloped viruses with the membrane of their target cells is one of the first steps of the viral infection cycle (Hernandez *et al.,* 1996). Only after the fusion of the viral and cellular membranes can the viral nucleocapsid enter the cytoplasm to continue its infective events (White, 1990, 1992). The unique glycoprotein G is responsible for the binding to cellular membranes and for the subsequent low-pH (pH 5–6) dependent fusion with them (Rigaut *et al.,* 1991; Schlegel *et al.,* 1982; Superti *et al.,* 1984), both in mammalian (Gaudin *et al.,* 1993, 1992) and in fish (Estepa and Coll, 1996a; Lecocq-Xhonneux *et al.,* 1994) rhabdoviruses. Glycoprotein G of rhabdoviruses interacts with the phospholipid components of the cellular membranes (Gaudin *et al.,* 1992; Lenard, 1993). Furthermore, phospholipids inhibited binding 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). Moreover, inhibition of VSV attachment and infection by phosphatidylserine (PS) (Schlegel *et al.,* 1982) and binding of PS by viral hemorrhagic septicemia (VHS) rhabdovirus (Estepa and Coll, 1996b) have also been reported.

PS binding to solid-phase 15-mer peptides (pepscan) mapped the major PS-binding domain of glycoprotein G of the viral hemorrhagic septicemia virus (VHSV) to the region of aa 82–109 (p2) (Coll, 1997; Estepa and Coll, 1996a,b). That the PS binding to p2 in solid phase could be relevant to the infective cycle of VHSV (Coll, 1996) was supported by the capacity of anti-p2 polyclonal antibodies to recognize both p2 and VHSV only at the low pH required for viral fusion (Estepa and Coll, 1996b), to inhibit PS binding to VHSV in solution only at low pH (Estepa and Coll, 1996a), and to inhibit low-pH VHSV-induced cell to cell fusion (Estepa and Coll, 1997). Furthermore, similar pH-dependence profiles were shown by PS binding to purified VHSV (Estepa and Coll, 1996a) and by glycoprotein G-mediated cell to cell membrane fusion (Lecocq-Xhonneux *et al.,* 1994). The PS-binding peptide sequence (p2) of glycoprotein G of VHSV contained three contiguous a–d hydrophobic heptad repeats followed by a charged head with two arginines (Coll, 1995a,b). The p2 sequence was totally conserved in two published (Lorenzen *et al.,* 1993; Thiry *et al.,* 1991a) and in seven other unpublished (A. Benmansour, personal communication) sequences of glycoprotein G from VHSV (Coll, 1997). On the other hand, p2-like regions were present in all rhabdoviruses (Coll, 1995b), synthetic peptides derived from them also bound PS (Coll, 1997), and the mapping of VSV fusion-defective mutants and of VSV and rabies virus hydrophobic photolabeled regions (Gaudin *et al.,* 1995a; Li *et al.,* 1993; Powers *et al.,* 1991; Whitt *et al.,* 1990) were located near to the p2-like regions (Coll,

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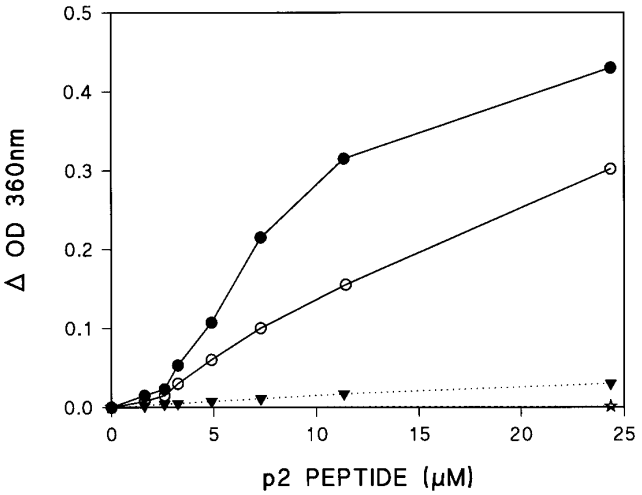

FIG. 1. Changes in the absorbance at 360 nm induced by the interaction of the p2 peptide with phospholipid vesicles. To 1 ml of vesicles (0.14 mM) of PS or PC in medium buffer at the appropriate pH, aliquots of peptide p2 from a 5 mg/ml stock solution in DMSO were added. (○) PS at pH 7.0; (●) PS at pH 5.0; (▲) PC at pH 5.0; (*), DMSO alone at the concentration corresponding to the highest p2 concentration used. The absorbance was measured after incubating the sample for 1 h at 37°C. The results shown are representative of those obtained for three different experiments.

1995b). All the above-mentioned results suggest that both p2-like regions and fusion might be functionally related in all rhabdoviruses.

The ability of synthetic peptides corresponding to sequences potentially involved in fusion to penetrate into membranes and to destabilize them, as well as the requirements of peptide structure for this function, might be adequate models that can reveal some of the molecular mechanisms of the fusion mediated by viral fusion proteins. Similar approaches in other viral glycoproteins have already been reported (Duzgunes and Shavnin, 1992). Thus, a synthetic peptide from glycoprotein G of the rhabdovirus causing VHS, a significant disease in European salmonid farms, comprising the above-mentioned p2 sequence (Estepa and Coll, 1996b) has been used in this work to study its interaction with phospholipids by employing a variety of biophysical assays. The results are discussed in terms of the possible interaction of this sequence with the target cellular membrane during the initial rhabdoviral infective steps.

RESULTS

Membrane destabilization induced by the p2 peptide

Figure 1 shows the changes in the absorbance at 360 nm after mixing PS vesicles with the p2 peptide at various phospholipid/peptide ratios. Since isolated PS or p2 had no adsorbing species at 360 nm, the apparent absorbance values that appear when p2 is added to the PS vesicles should be due to the scattered light produced by the p2-induced aggregation or increase in size of PS vesicles. At neutral pH the peptide interacts with PS, although to a lower extent than at acidic pH. In the presence of PC the increase in absorbance was almost negligible even at acidic pH, indicating the specificity of the observed peptide–phospholipid interaction in solution.

The p2 peptide was also able to induce membrane phospholipid mixing between the NBD-PE/RH-PE fluorescent probes containing PS vesicles (labeled) and the fluorescent probe-free PS vesicles (unlabeled). Figure 2 shows that the final extent of phospholipid mixing was dependent on the peptide concentration and was slightly higher at acidic pH. The fact that the observed change in energy transfer represents a decrease in the acceptor surface density of approximately 7- to 10-fold (from 74 to 16% at pH 7.0 and from 74 to 10% at pH 5.0) would indicate that the p2 peptide has induced a complete fusion between the labeled and unlabeled vesicles. The mere aggregation of vesicles would not result in such a change in energy transfer (Blumenthal *et al.,* 1983). Moreover, this assay of phospholipid mixing has been taken as a reliable indicator of membrane fusion (Duzgunes *et al.,* 1987).

The ability of the peptide to destabilize lipid bilayers was further assessed by measuring the p2-induced leakage of the intravesicular aqueous content from phospholipid vesicles. Vesicle aqueous content leakage was monitored by the dilution of coencapsulated ANTS and DPX that leads to a rapid increase in the ANTS fluorescence at 520 nm (Ellens *et al.,* 1985). Figure 3 shows the aqueous content leakage induced by the p2 peptide

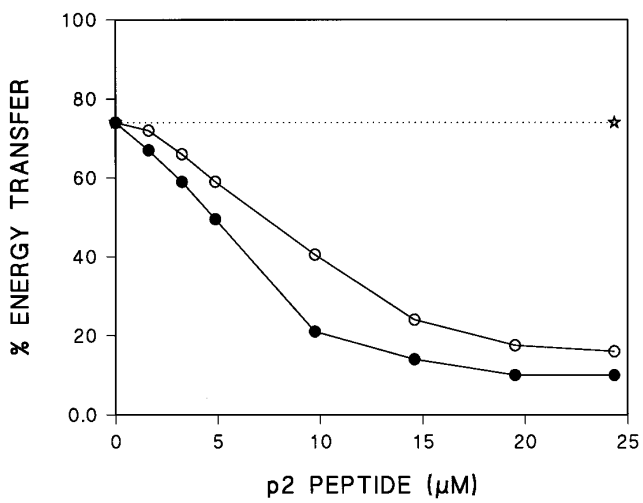

FIG. 2. Lipid mixing induced by the p2 peptide. The p2 peptide dissolved in DMSO (5 mg/ml) was added to 0.14 mM of PS vesicles in medium buffer at the appropriate pH and the mixture incubated for 1 h at 37°C. (○) pH 7.0; (●) pH 5.0; (*), DMSO alone. The energy transfer between NBD-PE and RH-PE was calculated as indicated under Methods. The results shown are representative of those obtained for three different experiments.

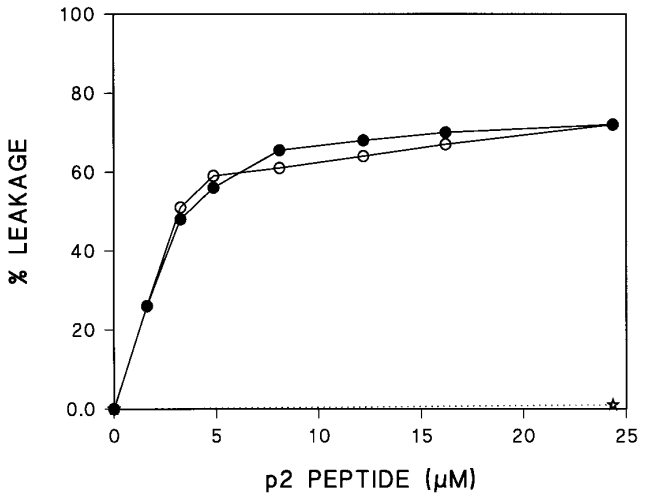

FIG. 3. Leakage of ANTS/DPX from phospholipid vesicles induced by the p2 peptide. The p2 peptide dissolved in DMSO (5 mg/ml) was added to 1 ml of PS vesicles (0.14 mM) loaded with ANTS and DPX in medium buffer adjusted at the appropriate pH. (○) pH 7; (●) pH 5; (*), DMSO alone. The mixtures were incubated at 37°C for 1 h and the fluorescence intensity was measured at 520 nm. Maximal fluorescence was obtained upon addition of 0.5% Triton X-100. The results shown are representative of those obtained for three different experiments.

added to a PS vesicle suspension. At the two pH values tested, the peptide was able to induce a rapid release of the internal content of the vesicles in a concentration-dependent manner. Under the conditions employed in this assay no differences were found at the two pH values tested. Also, the maximum effect on vesicle aqueous content leakage was reached at a slightly lower peptide concentration than that needed to induce maximum aggregation or lipid mixing.

The results shown in Figs. 1–3 were obtained upon interaction at 37°C. Nevertheless, similar results were obtained after incubation at 20°C in the three assays.

Studies of peptide insertion into phospholipid bilayers

The fact that the p2 peptide does not induce aggregation of neutral phospholipids (Fig. 1) indicates the importance of electrostatic interactions in eliciting the observed vesicle destabilization effects but gives no information on the possible relevance of hydrophobic interactions in the association of the peptide with the vesicles. To explore such possibility we choose to use DMPG vesicles because this synthetic phospholipid has a convenient transition temperature of 23.3°C. Thus, the possible effect of increasing temperatures on the peptide conformation is minimized compared to what it would have been in the case of DMPS, which has a transition temperature of 37°C.

The interaction of the p2 peptide with phospholipid vesicles has a great effect on the thermotropic behavior of DMPG vesicles. Figure 4 shows the fluorescence polarization of DPH-labeled DMPG vesicles in the presence of p2 peptide at a phospholipid to peptide molar ratio of 7.5 to 1. The main transition temperature for the pure phospholipid is observed at 23–24°C, while the addition of the p2 peptide completely abolished the transition at both pH 7.0 and 5.0. The behavior observed is that of an integral molecule which strongly restricts the mobility of the phospholipid acyl chains, suggesting the insertion of the peptide into the membrane.

The possible insertion of the p2 peptide in the membrane was also assessed by differential scanning calorimetry (DSC). DSC was only performed at pH 5.0 due to the high amounts of peptide that this technique demands and the availability of the peptide. Figure 5 shows that the p2 peptide causes a concentration-dependent decrease in the transition enthalpy without modifying the phase transition temperature. At a peptide/DMPG molar ratio of 0.1 the transition is completely inhibited. This inhibition indicates that the p2 peptide becomes inserted into the lipid bilayer, interacting hydrophobically with the phospholipid acyl chains, preventing part of the phospholipid molecules from undergoing the phase transition characteristics of the "pure" phospholipid species (Papahadjopoulos *et al.,* 1975).

A plot of the observed changes in the transition enthalpies versus the peptide/phospholipid molar ratios used in these studies (insert of Fig. 5) predicts that each

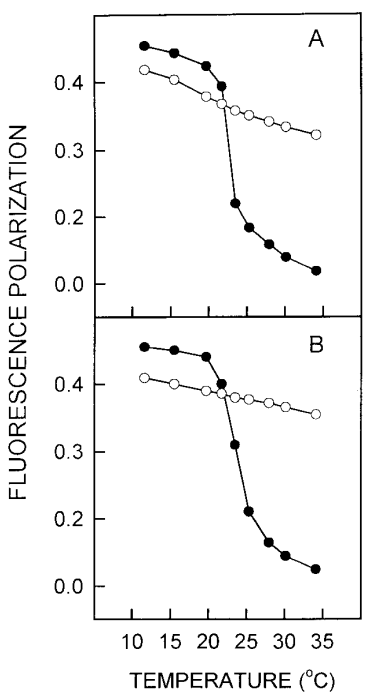

FIG. 4. Temperature dependence of fluorescence polarization of DPH-labeled DMPG vesicles. Phospholipid vesicles were labeled with DPH and fluorescence polarization was measured at the indicated temperature. The phospholipid concentration was 0.14 mM and the DPH probe to lipid molar ratio was 1 to 500. (●) DMPG vesicles alone. (○) DMPG in the presence of peptide p2 at a peptide molar ratio of 0.13. (A) pH 7.0 and (B) pH 5.0.

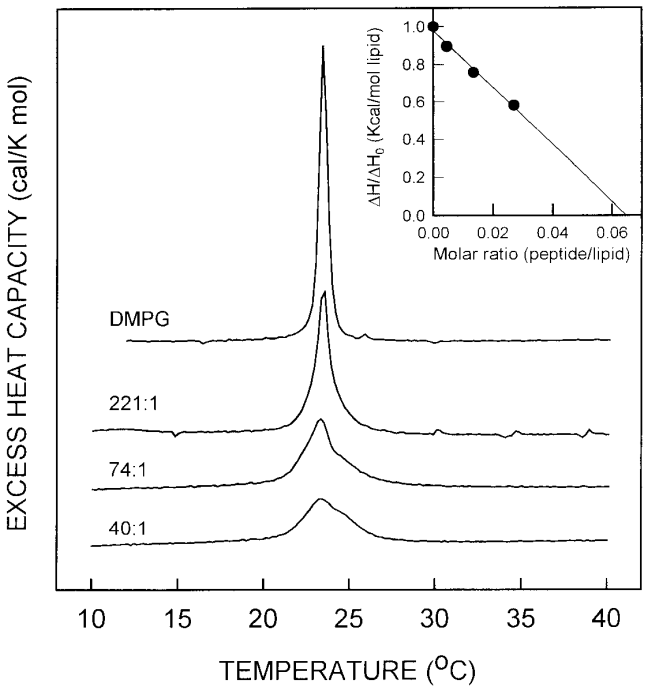

FIG. 5. Differential scanning calorimetry (DSC) of p2 peptide mixed with DMPG. Hydrated vesicles of DMPG containing different molar ratios DMPG:p2 peptide were heated from 10° to 40°C and thermograms recorded. The insert shows the changes in the transition enthalpies versus the peptide/lipid molar ratios.

p2 peptide molecule prevents an average of 15–17 phospholipid molecules from undergoing the phase transition.

CD studies of peptide conformation

Figure 6 shows the CD spectra of the p2 peptide both at pH 7.0 (A) and 5.0 (B) in the absence and in the presence of PS vesicles. At pH 7.0 the spectrum shows a minimum at 220 nm, which is characteristic of β-sheet extended conformation (Perczel *et al.,* 1992). Lowering the pH value to 5.0 modifies the spectrum in such a way that a minimum at 200 nm and a shoulder at 220 nm are observed. These features are indicative of the presence of both nonordered conformations and β-sheet. Deconvolution of these spectra by the CCA method (Perctzel *et al.,* 199l) renders the data shown in Table 2. At pH 7.0, β-sheet is the major component (66%) and at pH 5.0 the percentage of this conformation is lower (28%). The conformational features observed for the p2 peptide alone should be primarily those of the p2 peptide in monomeric form because of the low peptide concentration used in these studies and the fact that the solution are not turbid cloudy but translucent.

The presence of PS vesicles induces a p2 peptide spectrum with a minimum at around 220 nm and a maximum at 200 nm, very similar both at pH 7.0 and 5.0. Hence, the presence of the PS vesicles increases the proportion of β-sheet conformation on the p2 peptide. At pH 7.0 the increase in β-sheet is lower (from 66 to 71%) than at pH 5.0, where the latter conformation is practically the only one observed (91%) (Table 2). Decreasing the peptide/lipid molar ratio from 0.05 to 0.025 does not modify the spectrum any further (not shown). Also, a displacement of the position of the minimum, from 221 in the absence of PS to 224–225 in the presence of PS, was observed. This displacement could be due to a change in the polarity of the local environment of the β-sheet adopted.

The existence of extended conformations was further confirmed by Fourier transform infrared spectroscopy. However, due to the high peptide concentration used in this technique (16–3.2 mM) large aggregates were formed and the high intensity of the band at 1617 cm^{-1} characteristic of such aggregates masked the rest of the minor conformations, thus preventing the carrying out of a detailed study by this means (not shown).

DISCUSSION

Because we have previously shown that a 27-aa peptide (p2) from the glycoprotein G of VHS rhabdovirus binds anionic phospholipids in solid (Estepa and Coll, 1996a,b) as well as in liquid phases (Coll, 1997) and

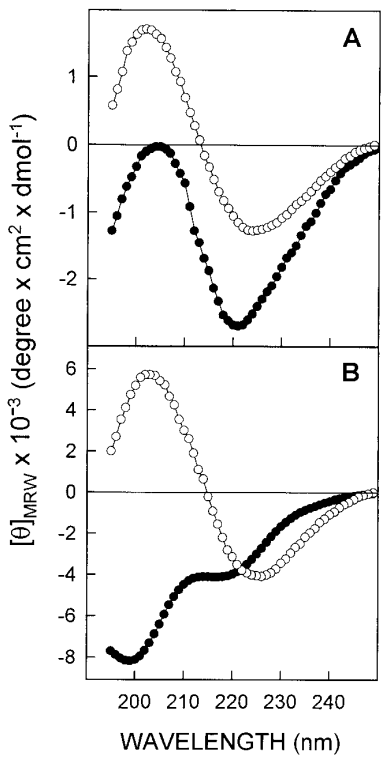

FIG. 6. Circular dichroism (CD) spectra of the p2 peptide incubated with PS vesicles. The p2 peptide in medium buffer at pH 7.0 (A) and 5.0 (B) was incubated with PS vesicles for 1 h at 37°C. ●, p2 peptide in the absence of lipid. ○, p2 peptide in the presence of PS at a peptide/lipid molar ratio of 0.05.

anti-p2 antibodies inhibited VHSV-induced fusion (Estepa and Coll, 1997) and PS binding to VHSV at low pH (Estepa and Coll, 1996a), we have further studied the interaction between the p2 peptide and anionic phospholipid vesicles, a model target that contains a negatively charged surface and a hydrophobic bilayer. Our expectations are that by exploring in detail the interaction of the viral synthetic peptides with such a model target, we might be able to infer the occurrence of similar molecular events involved in the rhabdoviral–cellular membrane first steps that lead to rhabdoviral–cellular fusion. The p2 peptide turns out to do far more than simply bind, it promotes vesicle aggregation, leakage, and lipid exchange. It penetrates the lipid bilayer, as shown by its effect on lipid phase transitions, and CD measurements report a β-sheet conformation, stabilized by interaction with anionic lipids.

The p2 peptide was able to induce lipid vesicle aggregation, lipid mixing, and rapid leakage of lipid vesicle-trapped fluorescent probes in a peptide concentration-dependent manner. The vesicle–vesicle interactions that lead to the appearance of vesicle aggregates did not take place with neutral phospholipids, indicating the specificity of the peptide–phospholipid interaction and therefore the importance of its electrostatic components. The above-mentioned results confirm previous evidence obtained from solid-phase assays (Estepa and Coll, 1996b) that showed that PS–p2 binding occurs because of both hydrophobic and ionic interactions, a result also demonstrated for phospholipid–glycoprotein G interactions in other rhabdoviruses (Yamada and Ohnishi, 1986). Lipid vesicle aggregation, lipid mixing to a lesser extent, but not lipid vesicle leakage increased at low pH, suggesting that the lipid vesicle aggregation, which has to occur first to allow lipid mixing, could be the major step that is favored by the low-pH requirement for fusion.

Both fluorescence depolarization and DSC studies demonstrate that the p2 peptide inserts into the hydrophobic core of the anionic phospholipid bilayer. Both at pH 7.0 and at pH 5.0, the CD studies indicate that the β-sheet conformation is practically the only one observed upon interaction with PS. In this regard there is increasing evidence that a β-sheet conformation of fusion-related peptides could also be involved in the fusion events mediated by other viruses. Thus, a human immunodeficiency virus (HIV) peptide (Nieva *et al.,* 1994) or the fusion peptide of measles virus (Epand *et al.,* 1992) adopt a β-sheet structure under conditions supporting fusion or when interacting with liposomes. On the contrary, synthetic peptides containing heptad repeats contiguous to the fusion peptide of the HIV-gp41 have been shown to form an α-helix characteristic of a coiled coil under physiological conditions (Wild *et al.,* 1992), while in the influenza virus hemagglutinin (HA) the α-helix conformation of a similar stretch of protein was pH dependent (Bullough *et al.,* 1994; Carr and Kim, 1993). Since computer programs predict a putative α-helix inside the p2 peptide, it is possible that the p2 peptide would be in an α-helix conformation in the native glycoprotein G. According to the data obtained with the synthetic p2 peptide, the p2 region in the glycoprotein G could acquire a β-sheet structure when interacting with the anionic phospholipids. A conformational change in the glycoprotein G of VHSV when exposed to low-pH seems to be occurring because the binding of anti-p2 antibodies with purified VHSV occurs only at low pH (Estepa and Coll, 1996a). Moreover, similar low-pH-induced conformational changes in the glycoprotein G of rabies and VSV have also been reported (Gaudin *et al.,* 1993). Independently of a possible previous viral–cellular receptor-mediated specific binding, the PS–p2 interaction studied here could, most probably, start only after internalization of the virus. Thus, only after internalization could the lowering of the pH trigger the conformational changes in glycoprotein G (Gaudin *et al.,* 1993), which could expose the p2 peptide to increase the interaction of viral and cellular membranes to favor subsequent steps that will provoke membrane destabilization. Whether the so-called fusion peptide is carried over by the putative low-pH-triggered p2 peptide to interact with other membrane phospholipids or whether the p2 region is pulled into the membrane by the fusion peptide is not known. On the other hand and since the p2 peptide is behaving exactly like the fusion peptides of influenza HA and gp41, it could, therefore, likely play a similar role in VHSV fusion. More experimental evidence is needed to decide among these possibilities.

Although a peptide corresponding to part of the well-characterized coiled-coil from the proteolytically activated influenza HA subunit binds to lipid vesicles (Yu *et al.,* 1994), this is not accepted as being biologically relevant to fusion or infection. This is because the low-pH form of HA lacking the fusion peptide does not bind to lipid vesicles (Chen *et al.,* 1995) and whereas the fusion peptide of HA becomes photolabeled at low pH, the coiled-coil region was not (Durrer *et al.,* 1996). However, this might be different for the glycoprotein G from rhabdoviruses, which is not known to be proteolytically activated before fusion, and both the fusion putative peptide and the heptad-repeat regions become photolabeled at low pH when virus is incubated with liposomes (Durrer *et al.,* 1995; Gaudin *et al.,* 1995a). Interactions of the regions containing heptad repeats with phospholipids have also been demonstrated in HIV-gp41 by membrane-binding studies (Rabenstein and Shin, 1995) and in the rhabdoviruses VHSV (Estepa and Coll, 1996a,b), VSV, and rabies (Coll, 1997) by solid-phase and liquid-phase binding assays. Mutations in the hydrophobic aa of the heptad repeats of the glycoproteins of several paramyxoviruses (Rapaport *et al.,* 1995; Reitter *et al.,* 1995; Sergel-Germano *et al.,* 1994), baculovirus (Monsma and Blissard, 1995), HIV (Wild *et al.,* 1994) and murine leukemia virus

TABLE 1

Position of the p2 Peptide in the G Sequence of VHSV

```
..RPAQLRCPHEFEDINKGLVSVPTRIIHLPLSVTSVSAVASGHYLHRVTYRVTC...  G
                            IIHLPLSVTSVSAVASGHYLH             α-helix
                            IIHLPLSVTSVSAVASGHYLHRVTYRVT      P2
|   |    |    |    |    |    |    |    |    |    |    |
55  60   65   70   75   80   85   90   95  100  105  110
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Note. G, partial sequence of the G gene from VHSV 07.71 (Thiry *et al.,* 1991b). α-Helix, putative α-helix predicted by the HELIXMEM program from the PCGene package. p2, designed synthetic peptide containing the putative α-helix plus the 2 Arg (R) containing carboxy-terminal sequence (RVTYRVT) called "head." This sequence (aa 82–109) was shown to contain the major binding domain of the glycoprotein G of the VHSV (Coll, 1997; Estepa and Coll, 1996a; Estepa and Coll, 1996b). The region containing only heptad repeats (aa 68–99) is not predicted to form coiled-coils by the algorithm of Lupas (Coll, 1995b; Lupas *et al.,* 1991). The hydrophobic aa of the a–d hydrophobic heptad repeats are in bold. Numbers are the amino-terminal aa positions on glycoprotein G by including the signal peptide.

(Ramsdale *et al.,* 1996) abolished viral fusion. Thus, all the above-mentioned results suggest that not only the fusion peptide but also adjacent downstream heptad repeats might be important for fusion (Reitter *et al.,* 1995), at least in some viruses.

In conclusion, the p2 peptide corresponding to the amino-terminal heptad repeat and positively charged head of glycoprotein G of VHSV has been shown to induce aggregation of lipid vesicles, close apposition of membranes, and destabilization of anionic phospholipid bilayers, most of the essential steps required for fusion (Bentz *et al.,* 1983). Although there is not yet evidence that the interaction being studied here is really relevant to VHSV entry, these findings and the fact that anti-p2 antibodies inhibited fusion (Estepa and Coll, 1997) and PS binding to VHSV (Estepa and Coll, 1996a) support the idea that this region of the G protein might play an active role in the viral fusogenic processes and that it might be considered an important target for the design of strategies for the prevention of VHSV infection *in vivo.*

METHODS

Reagents

Bovine brain phosphatidylserine (PS), egg phosphatidylcholine (PC), 1,2-dimyristoilphosphatidylglycerol (DMPG), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dimyristoylphosphatidylethanolamine (NBD-PE), and *N*-(lissamine rhodamine B sulfonyl)-diacylphosphatidylethanolamine (RH-PE) were provided by Avanti Polar Lipids (Alabaster, AL). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Aldrich. 8-Aminonaphtalene-1,3,6-trisulfonic acid (ANTS) and *p*-xylenebis (pyridium) bromide (DPX) were obtained from Molecular Probes. Triton X-100 was purchased from Boehringer Mannheim (Penzberg, Germany).

Peptide synthesis

The 27-amino-acid (aa) peptide covering the major amino-terminal PS-binding region of the glycoprotein G of VHSV with the sequence: ^{82}IIPHLPLSVTSVSAVAS-GHYLHRVTYRVT^{109} (Table 1) was synthesized (Clontech, Palo Alto, CA). Due to the low solubility of the peptide in aqueous buffers, a 5 mg/ml stock solution of the peptide in dimethyl sulfoxide (DMSO) or trifluoroethanol (TFE) was kept at −20°C and employed in the various assays. The final organic solvent concentration was kept under 1.5% (v/v) in all the assays since it had no measurable effect on lipid vesicle stability.

Lipid vesicle preparation

A phospholipid film was obtained upon overnight drying of a chloroform solution under vacuum. The phospholipids were resuspended at a concentration of 1 mg/ml in medium buffer (100 mM NaCl, 5 mM MES, 5 mM sodium citrate, 5 mM Tris, 1 mM EDTA) at the appropriate pH value for 1 h at 37°C and vortexed vigorously. This suspension was subjected to 19 cycles of extrusion in a LoposoFast Basic extrusion apparatus with 100-nm polycarbonate filters (Avestin Inc.). When encapsulation was necessary, an additional step of four freeze–thawing cycles was included after the hydration process (see below). A 0.14 mM phospholipid final concentration was used in all the experiments.

Vesicle aggregation assay

The optical density (OD) variation at 360 nm produced by the addition of the p2 peptide from the DMSO stock solution to a phospholipid vesicle suspension in medium buffer at the appropriate pH was measured on a Beck-

TABLE 2

Secondary Structure of the p2 Peptide from CD Data

	pH 7.0		pH 5.0	
	p2	p2 + PS	p2	p2 + PS
β-Sheet	66	71	28	91
β-Turn	16	17	43	1
Nonordered	18	12	31	8

Note. Data are expressed as percentages.

man DU-7 spectrophotometer after 1 h incubation at 37°C. To account for the absorbance of phospholipid vesicles and peptide alone, control samples containing equal amounts of DMSO, but in the absence of peptide or phospholipid, were included.

Lipid vesicles mixing assay

The p2-induced decrease in the efficiency of the fluorescence energy transfer due to dilution between NBD-PE (energy donor) and RH-PE (energy acceptor) incorporated into lipid vesicles as a consequence of lipid mixing was measured (Struck *et al.,* 1981). Lipid vesicles, in medium buffer at the appropriate pH, labeled with 1 mol% NBD-PE and 0.6 mol% RH-PE were mixed with unlabeled lipid vesicles in a 1:9 molar ratio. The assay was initiated by addition of the p2 peptide from the DMSO stock solution. The samples were incubated for 1 h at 37°C and the emission spectra were recorded in a SLM Amino 8000 spectrofluorimeter at an excitation wavelength of 450 nm. Both the excitation and emission slits were set at 4 nm. The excitation and emission polarizers were kept constant at 90° and 0°, respectively, to minimize dispersive interference. The efficiency of the energy transfer was calculated from the ratio of the emission intensities at 530 and 585 nm and the appropriate calibration curve.

Leakage assay

Leakage of vesicles was determined by the ANTS/DPX assay (Ellens *et al.,* 1985), based on the dequenching of ANTS fluorescence caused by its dilution upon release of the aqueous contents of lipid vesicle containing both ANTS and DPX. It was performed coencapsulating 12.5 mM ANTS and 45 mM DPX in 20 mM NaCl, 10 mM Tris, pH 7.2, into PS vesicles. PS was first hydrated for 1 h at 37°C, and then the PS vesicles were subjected to four cycles of freeze–thawing in liquid nitrogen and passed through a LiposoFast Basic extrusion apparatus at least 19 times with 100-nm polycarbonate filters (Avestin, Inc.). The sample was passed through a Sephadex G-75 column (Pharmacia) in the above-mentioned buffer to separate the PS vesicles from the nonencapsulated material (Duzgunes and Shavnin, 1992). Leakage was initiated by addition of the p2 peptide from the DMSO stock solution. The samples were incubated for 1 h at 37°C. The suspensions were excited at 385 nm and ANTS emission was monitored at 520 nm. Both the excitation and emission slits were set at 4 nm. The excitation polarizer was kept constant at 90° and the emission polarizer was kept constant at 0° to minimize dispersive interference. The fluorescence scale was set to 100% by addition of 0.5% Triton X-100 and the 0% leakage was obtained by measuring the fluorescence of the control vesicles after the addition of DMSO.

Fluorescence polarization

Fluorescence polarization measurements of DPH were made on a SLM Aminco 8000C spectrofluorimeter equipped with 10-mm Glan-Thompson polarizers. DMPG (0.14 mM) containing DPH at a ratio of 1 molecule of DPH for every 500 DMPG molecules was hydrated at the appropriate pH and extruded as indicated above. The peptide was added from the DMSO stock solution and the peptide–vesicle mixtures were incubated for 1 h at 37°C and then cooled down. The excitation wavelength was set at 365 nm and the emission was measured at 425 nm after equilibration of the samples at the required temperature. The temperature in the cuvette was maintained with a Polystat Huber circulating water bath.

Differential scanning calorimetry (DSC)

Large multilamellar vesicles made from synthetic DMPG were used for the calorimetric measurements. Dried lipid films were suspended in medium buffer at pH 5.0 to give a final concentration of 15 mM in terms of lipid phosphorus. The suspended lipids were kept for 90 min above their phase transition temperature and vortexed. The resulting liposomes were stored overnight at 4°C, to assure a complete hydration of the sample prior to the DSC measurements. Thermograms were recorded on a high-resolution Microcal MC-2 differential scanning microcalorimeter, equipped with the DA-2 digital interface and a data acquisition utility for automatic data collection as described earlier (Villar *et al.,* 1988). The lipid dispersions containing added p2 peptide at different molar ratios and the corresponding buffer in the reference cell were thermally equilibrated in the microcalorimeter at $\sim$ 10°C for 45 min before heat was applied. Differences in the heat capacity between the sample and the reference cell were obtained by raising the temperature at a constant rate of 45°C/h. Transition temperatures and enthalpies were calculated by fitting the observed transitions to a single van't Hoff component. Reported transition temperatures correspond to those at which there is a maximum differential heat capacity as observed in the thermograms.

Circular dichroism (CD)

Aliquots of the p2 peptide from a stock solution at 5 mg/ml in trifluoroethanol (TFE) were added to medium buffer (100 mM NaCl, 5 mM Tris, 5 mM citrate, 5 mM MES, 1 mM EDTA) at the appropriate pH to give a final concentration of the peptide of 32.5 μM. The peptide was dissolved in TFE because the high absorbance of DMSO precludes its use in CD. The effect of the interaction with PS vesicles on the p2 conformation was determined by adding increasing amounts of PS to peptide aliquots. The mixtures were allowed to react for 1 h at 37°C and then were subjected to bath sonication. The clear sonicated

solutions were then analyzed by CD. The CD spectra were recorded on a JASCO 715 dichrograph at 25°C and with a 1-mm pathlength cell at a scanning speed of 0.5 nm/s. The temperature in the cuvette was maintained with a Neslab RTE-111 circulating water bath. A minimum of four spectra were accumulated for each sample. The peptide concentration was determined by amino acid analysis on a Beckman 6300 automatic amino acid analyzer. Deconvolution of the CD data was performed according to the convex constraint analysis method (Perczel *et al.,* 1991). This method relies on an algorithm that calculates the contribution of the secondary structure elements that give rise to the original spectral curve without referring to spectra from model systems. This analysis provides evidence that the CD spectrum of a protein contains independent information of at least four different additive secondary structures.

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REFERENCES

Bailey, C. A., Miller, D. K., and Lenard, J. (1984). Effects of DEAE-dextran on infection and hemolysis by VSV. Evidence that nonspecific electrostatic interactions mediate effective binding of VSV to cells. *Virology* 133, 111–117.

Bentz, J., Duzgunes, N., and Nir, S. (1983). Kinetics of divalent cation induced fusion of phosphatidylserine vesicles: Correlation between fusogenic properties and binding affinities. *Biochemistry* 22, 3320–3330.

Blumenthal, R., Henkart, M., and Steer, C. J. (1983). Clathrin-induced pH-dependent fusion of phosphatidylcholine vesicles. *J. Biol. Chem.* 258, 3409–3415.

Bullough, P. A., Hughson, F. M., Skehel, J. J., and Wiley, D. C. (1994). Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* 371, 37–43.

Carr, C. M., and Kim, P. S. (1993). A spring-loaded mechanism for the conformational change of influenza hemagglutinin. *Cell* 73, 823–832.

Chen, J., Wharton, S. A., Weissenhorn, W., Calder, L. J., Hughson, F. M., Skehel, J. J., and Wiley, D. C. (1995). A soluble domain of the membrane-anchoring chain of the influenza virus hemagglutinin (HA2) folds in *Escherichia coli* into the low-pH-induced conformation. *Proc. Natl. Acad. Sci. USA* 92, 12205–12209.

Coll, J. M. (1995a). The glycoprotein G of rhabdoviruses. *Arch. Virol.* 140, 827–851.

Coll, J. M. (1995b). Heptad-repeat sequences in the glycoprotein of rhabdoviruses. *Virus Genes* 10, 107–114.

Coll, J. M. (1996). Low-pH increases the binding of haemorrhagic septicemia rhabdovirus to membrane phospholipids. *J. Fish Dis.* 18, 519–527.

Coll, J. M. (1997). Synthetic peptides from the heptad repeats of the glycoproteins of rabies, vesicular stomatitis and fish rhabdoviruses bind phosphatidylserine. *Arch. Virol.* 142, 2089–2097.

Conti, C., Hauttecoeur, B., Morelec, M. J., Bizzini, B., Orsi, N., and Tsiang, H. (1988). Inhibition of rabies virus infection by a soluble membrane fraction from the rat central nervous system. *Arch. Virol.* 98, 73–86.

Durrer, P., Galli, C., Hoenke, S., Corti, C., Gluck, R., Vorherr, T., and Brunner, J. (1996). H+-induced membrane insertion of influenza virus hemagglutinin involves the HA2 amino-terminal fusion peptide but not the coiled coil region. *J. Biol. Chem.* 271, 13417–13421.

Durrer, P., Gaudin, Y., Ruigrok, R. W. H., Graf, R., and Brunner, J. (1995). Photolabeling identifies a putative fusion domain in the envelope glycoprotein of rabies and vesicular stomatitis viruses. *J. Biol. Chem.* 270, 17575–17581.

Duzgunes, N., Allen, T. M., Fedor, J., and Papahadjopoulos, D. (1987). Lipid mixing during membrane aggregation and fusion: Why fusion assays disagree. *Biochemistry* 26, 8435–8442.

Duzgunes, N., and Shavnin, S. A. (1992). Membrane destabilization by N-terminal peptides of viral envelope proteins. *J. Membr. Biol.* 128, 71–80.

Ellens, H., Bentz, J., and Szoka, F. C. (1985). H+ and Ca++ -induced fusion and destabilization of liposomes. *Biochemistry* 24, 3099–3106.

Epand, R. M., Cheetam, J. J., Epand, R. F., Yeagle, P. L., Richardson, C. D., Rockwell, A., and DeGrado, W. F. (1992). Peptide models for the membrane destabilizing actions of viral fusion proteins. *Biopolymers* 32, 309–314.

Estepa, A., and Coll, J. M. (1996a). Pepscan mapping and fusion related properties of the major phosphatidylserine-binding domain of the glycoprotein of viral hemorrhagic septicemia virus, a salmonid rhabdovirus. *Virology* 216, 60–70.

Estepa, A., and Coll, J. M. (1996b). Phosphatidylserine binding to solid-phase peptides: A new method to study phospholipid/viral protein interactions. *J. Virol. Methods* 61, 37–45.

Estepa, A., and Coll, J. M. (1997). Temperature and pH requirements for viral haemorrhagic septicemia virus induced cell fusion. *Dis. Aquatic Organisms* 28, 185–189.

Gaudin, Y., Ruigrok, R. W. H., and Brunner, J. (1995a). Low-pH induced conformational changes in viral fusion proteins: Implications for the fusion mechanism. *J. Gen. Virol.* 76, 1541–1556.

Gaudin, Y., Ruigrok, R. W. H., Knossow, M., and Flamand, A. (1993). Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. *J. Virol.* 67, 1365–1372.

Gaudin, Y., Ruigrok, R. W. H., Tuffereau, C., Knossow, M., and Flamand, A. (1992). Rabies virus glycoprotein is a trimer. *Virology* 187, 627–632.

Gaudin, Y., Tuffereau, C., Durrer, P., Flamand, A., and Ruigrok, R. W. H. (1995b). Biological function of the low-pH, fusion-inactive conformation of rabies virus glycoprotein (G): G is transported in a fusion-inactive state-like conformation. *J. Virol.* 69, 5528–5534.

Hernandez, L. D., Hoffman, L. R., Wolfsberg, T. G., and White, J. M. (1996). Virus-cell and cell-cell fusion. *Annu. Rev. Cell Dev. Biol.* 12, 627–661.

Lecocq-Xhonneux, F., Thiry, M., Dheur, I., Rossius, M., Vanderheijden, N., Martial, J., and DeKinkelin, P. (1994). A recombinant viral haemorrhagic septicaemia virus glycoprotein expressed in insect cells induces protective immunity in rainbow trout. *J. Gen. Virol.* 75, 1579–1587.

Lenard, J. (1993). Vesicular stomatitis virus fusion. *In* "Viral Fusion Mechanisms" (J. Bentz, Ed.), pp. 425–435. CRC Press, Boca Raton.

Li, Y., Drone, C., Sat, E., and Ghosh, H. P. (1993). Mutational analysis of the vesicular stomatitis virus glycoprotein G for membrane fusion domains. *J. Virol.* 67, 4070–4077.

Lorenzen, N., Olesen, N. J., Vestergaard-Jorgensen, P. E., Etzerodt, M., Holtet, T. L., and Thorgersen, M. C. (1993). Molecular cloning and expression in *Escherichia coli* of the glycoprotein gene of VHS virus and immunization of rainbow trout with the recombinant protein. *J. Gen. Virol.* 74, 623–630.

Lupas, A., Van Dyke, M., and Stock, J. (1991). Predicting coiled coils from protein sequences. *Science* 252, 1162–1169.

Mastromarino, P., Conti, C., Goldoni, P., Hauttecouer, B., and Orsi, N. (1987). Characterization of membrane components of the erythrocyte involved in vesicular stomatitis virus attachment and fusion at acidic pH. *J. Gen. Virol.* 68, 2359–2369.

Monsma, S. A., and Blissard, G. W. (1995). Identification of a membrane

fusion domain and a oligomerization domain in the baculovirus GP64 envelope fusion protein. *J. Virol.* 69, 2583–2595.

Nieva, J. L., Nir, S., Muga, A., Goñi, F. M., and Wilschut, J. (1994). Interaction of the HIV-1 fusion peptide with phospholipid vesicles: Different structural requirements for fusion and leakage. *Biochemistry* 33, 3201–3209.

Papahadjopoulos, D., Moscarello, M., Eylar, E. H., and Isac, T. (1975). Effects of proteins on thermotropic phase transitions of phospholipid membranes. *Biochim. Biophys. Acta* 401, 317–335.

Perczel, A., Hollosi, M., Tusnady, G., and Fasman, G. (1991). Convex constrain analysis: A natural deconvolution of circular dichroism curves of proteins. *Protein Eng.* 4, 669–679.

Perczel, A., Park, K., and Fasman, G. D. (1992). Deconvolution of the circular dichroism spectra of proteins: the circular dichroism spectra of the antiparallel b-sheet in proteins. *Protein Struct. Funct. Genet.* 13, 57–69.

Powers, D. A., Gonzalez-Villaseñor, L. I., Zhang, P. J., Chen, T. T., and Dunham, R. A. (1991). Studies on transgenic fish: Gene transfer, expression and inheritance. *Biotechnology* 16, 307–324.

Rabenstein, M., and Shin, Y. K. (1995). A peptide from the heptad repeat of human immunodeficiency virus gp41 shows both membrane binding and coiled-coil formation. *Biochemistry* 34, 13390–13397.

Ramsdale, E. E., Kingsman, S. M., and Kingsman, A. J. (1996). The "putative" leucine zipper region of murine leukemia virus transmembrane protein (p15e) is essential for viral infectivity. *Virology* 220, 100–108.

Rapaport, D., Ovadia, M., and Shai, Y. (1995). A synthetic peptide corresponding to a conserved heptad repeat domain is a potent inhibitor of Sendai virus-cell fusion: An emerging similarity with functional domains of other viruses. *EMBO J.* 14, 5524–5531.

Reitter, J. N., Sergel, T., and Morrison, T. G. (1995). Mutational analysis of the leucine zipper motif in the Newcastle disease virus fusion protein. *J. Virol.* 69, 5995–6004.

Rigaut, K. D., Birk, D. E., and Lenard, J. (1991). Intracellular distribution of input vesicular stomatitis virus proteins after uncoating. *J. Virol.* 65, 2622–2628.

Schlegel, R., Willigan, M. C., and Pastan, I. H. (1982). Saturable binding sites for vesicular stomatitis virus on the surface of Vero cells. *J. Virol.* 43, 871–875.

Sergel-Germano, T., McQuain, C., and Morrison, T. (1994). Mutations in the fusion peptide and heptad repeat regions of the Newcastle disease virus fusion protein block fusion. *J. Virol.* 68, 7654–7658.

Struck, D. K., Hoekstra, D., and Pagano, R. E. (1981). Use of resonance energy transfer to monitor fusion. *Biochemistry* 20, 4093–4099.

Superti, J., Seganti, L., Tsiang, H., and Orsi, N. (1984). Role of phospholipids in rhabdovirus attachment to Cer cells:Brief report. *Arch. Virol.* 81, 321–328.

Thiry, M., Lecoq-Xhonneux, F., Dheur, I., Renard, A., and Kinkelin, D. (1991a). Molecular cloning of the m-RNA coding for the G protein of the viral haemorrhagic septicaemia (VHS) of salmonids. *J. Vet. Microbiol.* 23, 221–226.

Thiry, M., Lecoq-Xhonneux, F., Dheur, I., Renard, A., and Kinkelin, D. (1991b). Sequence of a cDNA carrying the glycoprotein gene and part of the matrix protein M2 gene of viral haemorrhagic septicaemia virus, a fish rhabdovirus. *Biochim. Biophys. Acta* 1090, 345–347.

Villar, M. T., Artigues, J. A., Ferragut, J. A., and Gonzalez-Ros, J. M. (1988). Phospholipase A2 hydrolysis of membrane phospholipids causes structural alteration of the nicotinic acetylcholine receptor. *Biochim. Biophys. Acta* 938, 35–43.

White, J. M. (1990). Viral and cellular membrane fusion proteins. *Annu. Rev. Physiol.* 52, 675–697.

White, J. M. (1992). Membrane fusion. *Science* 258, 917–924.

Whitt, M. A., Zagouras, P., Crise, B., and Rose, J. K. (1990). A fusion-defective mutant of the vesicular stomatitis virus glycoprotein. *J. Virol.* 64, 4907–4913.

Wild, C., Dubay, J. W., Greenwell, T., Baird, T., Oas, T. G., McDanal, C., Hunter, E., and Matthews, T. (1994). Propensity for a leucine zipper-like domain of human immunodeficiency virus type 1 gp41 to form oligomers correlates with a role in virus-induced fusion rather than assembly of the glycoprotein complex. *Proc. Natl. Acad. Sci. USA* 91, 12676–12680.

Wild, C., McDanal, C., Bolognesi, D., and Mathews, T. (1992). A synthetic peptide inhibitor of human immunodeficiency virus replication: Correlation between solution structure and viral inhibition. *Proc. Natl. Acad. Sci. USA* 77, 3273–3277.

Yamada, S., and Ohnishi, S. (1986). Vesicular stomatitis virus binds and fuses with phospholipid domain in target cell membranes. *Biochemistry* 25, 3703–3708.

Yu, Y. G., King, D. S., and Shin, Y. K. (1994). Insertion of a coiled-coil peptide from influenza virus hemagglutinin into membranes. *Science* 266, 274–276.