Structure of a Membrane-binding Domain from a Non-enveloped Animal Virus: Insights into the Mechanism of Membrane Permeability and Cellular Entry

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Running Title: Kinked structure of the membrane-permeabilizing γ-peptide of Flock House virus
γ1-Peptide is a 21-residue lipid-binding domain from the non-enveloped Flock House virus (FHV). Unlike enveloped viruses, the entry of non-enveloped viruses into cells is believed to occur without membrane fusion. In the present study, we performed NMR experiments to establish the solution structure of a membrane-binding peptide from a small non-enveloped, icosahedral virus. The three-dimensional structure of FHV γ1-domain was determined at pH 6.5 and 4.0 in a hydrophobic environment. The secondary and tertiary structures were evaluated in the context of the peptides’ capacity for permeabilizing membrane vesicles of different lipid composition, as measured by fluorescence assays. At both pHs, the peptide has a kinked structure, similar to the fusion domain from enveloped viruses. The secondary structure was similar in three different hydrophobic environments: water/TFE; SDS and membrane vesicles of different compositions. The ability of the peptide to induce vesicle leakage was highly dependent on the membrane composition. Although the gamma peptide shares some structural properties to fusion domains of enveloped viruses, it did not induce membrane fusion. Our results suggest that small protein components such as the gamma peptide in nodaviruses (such as FHV) and VP4 in picornaviruses have a crucial role in conducting nucleic acids through cellular membranes and that their structures resemble the fusion domains of membrane proteins from enveloped viruses.

Animal viruses require a built-in membrane penetration mechanism. The strategies of infection adopted by enveloped and non-enveloped viruses are different, depending on the way the virus enters and leaves a cell (1). The cellular entry by enveloped viruses, such as influenza virus and retroviruses, is the most thoroughly studied (2, 3). In general, infection by an enveloped virus starts with the fusion of viral and cellular membranes, mediated by viral envelope glycoproteins that contain well-defined fusion domains. Fusion appears to be triggered by an optimum pH (such as the low pH at lysosomes) or by binding to a specific receptor (2, 3). Recent studies have suggested that fusion peptides tend to present a kinked region, regardless of the overall secondary structure proposed (2, 4, 5). The kink confers to the domain a boomerang-like structure, which can act as anchor of the virus particle to the membrane.

Non-enveloped viruses do not require membrane fusion for entry into cells, but a membrane-binding motif is present in some of them (6-9). In Flock House virus (FHV), a non-enveloped RNA insect nodavirus, γ1-peptide has a membrane-binding activity. FHV has been used as a model for the investigation of animal virus assembly, maturation, structure and evolution (7, 8, 10-14). The γ1-peptide contains the 21 N-terminal residues of the 44-residue γ-peptide, a cleavage product of the coat precursor protein α. The cleavage of α occurs after assembly of FHV particles and is required for acquisition of virion infectivity, which also results in a significant increase in particle stability (15). It has been proposed that this lipophilic domain could be a membrane-permeabilizing agent in the viral RNA translocation process (8). The γ1-domain has an amphipathic character and an amino-acid sequence containing several residues with short side chains, which are a common feature of fusion peptides (2, 16, 17). Although there are few structures of fusion peptides from enveloped viruses, no solution structure is available for membrane-binding domains from small icosahedral viruses. Previous studies by nuclear magnetic resonance (NMR) and biophysical methods with Flock House virus-like particles (VLPs) indicated that the cleaved wt VLP has a greater mobility than the uncleaved mutant because of the sharp lines of the cleaved γ-peptide (12).

NMR studies of fusion-peptide sequences from enveloped viruses have provided valuable information about the relationship between structure and membrane-binding and
fusion activity, such as herpesvirus (18), Influenza hemagglutinin (4, 19), and sea urchin fertilization protein (20). However, no data are available for isosahedral viruses. In the present study, we perform CD and NMR experiments to establish, for the first time, the solution structure of a lipid-binding domain from a small non-enveloped virus. The three-dimensional structures of \( \gamma_1 \) peptide at pH 6.5 and pH 4.0 in a hydrophobic environment are reported. We evaluate conformational variability and membrane-permeabilizing activity by fluorescence spectroscopy at different pHs and different membrane compositions. Our results shed light into the mechanisms of how small non-enveloped particles bind to membranes and insert their genomes into the host cells.

Materials and Methods

Peptides - The synthetic \( \gamma_1 \) peptide composed of 21 residues (ASMWERV-KSIKSS-LAAAASNI) was purchased from Genemed Synthesis, Inc (San Francisco, CA, USA).

CD spectroscopy - CD data were performed using a Jasco J-715 spectrophotometer with a 2-mm path length cuvette. The spectra were recorded from 190 to 250 nm at a scanning rate of 50 nm/min with a wavelength step of 1.0 nm. The samples of \( \gamma_1 \) peptide (200 \( \mu \)M) were prepared in 10 mM of Na phosphate buffer, pH 7.0 in different concentrations of trifluoroethanol, in 60 mM of SDS micelles or containing lipid vesicles. Samples were prepared by dissolving 1 mM of \( \gamma_1 \) peptide in buffered water (pH 7.0 and 4.0) containing 160 and 300 mM SDS micelles, and 2.5 mM of \( \gamma_1 \) peptide in buffered water (pH 7.0 and 5.5) containing 120, 200 and 300 mM DPC micelles, to each sample was added 10 % D$_2$O. SDS-TOCSY (spin lock time of 70 ms) using the MLEV-17 pulse sequence (21) and NOESY (mixing time of 120 ms) spectra (22, 23). The NOESY spectra were collected with 512 data points in F1 and 4096 data points in F2. Water suppression for the samples was achieved by a pre-saturation pulse at the water frequency (24). All spectra were recorded in time-proportioned phase increment (TTP1) mode (25). The NMR data were processed by NMRPIPE (26). All NMR spectra were analyzed using NMRVIEW software package version 5.0.3 (27).

NMR spectroscopy - NMR measurements were carried out at 20 °C on a Bruker Avance DRX 600 or DRX 400 spectrometer operating at 600.04 MHz. Lyophilized synthetic \( \gamma_1 \) peptide was prepared in 5 mM Na phosphate buffer at pH 7.0 and pH 4.0 containing D$_3$-trifluoroethanol/water (1:1, v/v) and 10 % D$_2$O. Final samples contained 4.2 mM peptide at pH 6.5 and 3.6 mM peptide at pH 4.0. Resonances of the sample at pH 6.5 were assigned from TOCSY (spin lock time of 70 ms) using the MLEV-17 pulse sequence (21), COSY-GP and NOESY (mixing time of 150 ms) spectra (Supplementary Figs. 1-3) (22, 23). The NOESY spectra were collected with 200 data points in F1 and 4096 data points in F2. The TOCSY spectra were collected with 400 data points in F1 and 4096 data points in F2. Resonances of the sample at pH 4.0 were assigned from TOCSY (MLEV-17 pulse sequence, spin lock time of 70 ms) and NOESY (mixing time 160 ms). The NOESY spectra were collected with 512 data points in F1 and 2048 data points in F2. The TOCSY spectra were collected with 300 data points in F1 and 2048 data points in F2. NMR data sets were also collected for \( \gamma_1 \)-peptide incorporated into SDS and DPC micelles. Samples were prepared by dissolving 1 mM of \( \gamma_1 \)-peptide in buffered water (pH 7.0 and 4.0) containing 160 and 300 mM SDS micelles, and 2.5 mM of \( \gamma_1 \)-peptide in buffered water (pH 7.0 and 5.5) containing 120, 200 and 300 mM DPC micelles, to each sample was added 10 % D$_2$O. SDS-TOCSY (spin lock time of 70 ms) using the MLEV-17 pulse sequence (21) and NOESY (mixing time of 120 ms) spectra (22, 23). The NOESY spectra were collected with 512 data points in F1 and 4096 data points in F2. Water suppression for the samples was achieved by a pre-saturation pulse at the water frequency (24). All spectra were recorded in time-proportioned phase increment (TTP1) mode (25). The NMR data were processed by NMRPIPE (26). All NMR spectra were analyzed using NMRVIEW software package version 5.0.3 (27).

Structure calculation - Distance restraints were derived from the 150 ms NOESY spectrum of the peptide at pH 6.5 and the 160 ms NOESY spectrum at pH 4.0. NOE cross-peak intensities were measured and converted into distances. Structures were calculated with the program CNS-solve version 1.1. One hundred structures were calculated for each sample, using simulated annealing protocol, applying cartesian-cartesian angle molecular dynamics. The stereochemical quality of the lowest energy structures was analysed by PROCHECK-NMR (28). The display, analysis, and manipulation of the three-dimensional structures were performed with the program MOLMOL (29).

ANTS/DPX release assay - Peptide-induced release of aqueous vesicle content was measured
by using the ANTS (8-aminonaphthalene-1,3,6-trisulphonic acid)/DPX (N,N'p-xylene-bis-pyrimidinium bromide) assay (30). Large unilamellar vesicles (LUV) were prepared according to the extrusion method of Hope et al. (31) in 10 mM Na phosphate, 100 mM NaCl (pH 7.0). LUVs containing 12.5 mM ANTS, 45 mM DPX, 100 mM NaCl, and 10 mM Na phosphate were obtained by separating the unencapsulated material by gel filtration in a GPC300 column (SynChropak, Micra Scientific, Inc) eluted with 10 mM Na phosphate, in 100 mM NaCl (pH 7.0). Fluorescence measurements were performed by setting the ANTS emission at 523 nm and the excitation at 353 nm with of a 500 nm cutoff filter in the emission beam. The absence of leakage (0%) corresponded to fluorescence of the vesicles at time zero; 100% leakage was taken as the fluorescence value obtained after addition of 1% (v/v) Triton X-100. The degree of permeabilization was then inferred from Equation 1 where \( F \) is the fluorescence intensity after the addition of protein, \( F_0 \) is the initial fluorescence of the intact LUV suspension, and \( F_T \) is the fluorescence after the addition of Triton X-100.

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\text{% Leakage} = \left( \frac{(F-F_0)}{(F_T-F_0)} \right) \times 100
\]

Equation 1

Carboxyfluorescein release assay - For fluorescent probe release experiments, a self-quenching solution (100 mM) of carboxyfluorescein (2',7'-bis-(2-carboxyethyl)-5- (and-6)-carboxyfluorescein (BCECF acid) (Molecular Probes), 10 mM Na phosphate buffer at pH 7, was entrapped in L-\( \alpha \)-phosphatidylcholine large unilamelar vesicles (LUVs). The vesicles obtained (0.2 \( \mu \)M diameter) were separated from the non-incorporated probe by gel filtration using a G-75 Sephadex column (Pharmacia). Measurements were performed in Na phosphate buffer at pH 6.5 and 7.0. The fluorescence measurements were performed in 1 mL of buffer composed of 10 mM Na phosphate at pH 7.0, in a quartz cuvette with stirring. Fluorescence was recorded as a function of time using an excitation wavelength of 490 nm and an emission wavelength of 518 nm with 2.5 nm bandwidth slits. Release was initiated by the addition of peptide and monitored by the fluorescence intensity increase after the addition of the peptide as described for the ANTS/DPX assay.

RESULTS

\( \gamma_1 \)-peptide binding domain structure

In water (10 mM Na phosphate buffer at pH 7.0), the CD spectrum of \( \gamma_1 \)-peptide was that of a typical “random coil” with only one band at ~200 nm (Fig. 1). A change from random coil to an \( \alpha \)-helical conformation was observed when increasing amounts of trifluoroethanol (TFE, up to 50 %) were added to the peptide in phosphate buffer pH 7.0 (Fig. 1). Double minimum bands at about 222 nm and 208-210 nm and a maximum band at 191-193 nm, which are characteristic of an \( \alpha \)-helix, were observed in concentrations of 20 % TFE and above. \( \alpha \)-helix pattern was also observed with the sample in 60 mM of SDS (Fig. 2A) or when inserted in membrane vesicles of different compositions (Fig. 2B). The high similarity of the spectra indicates that the peptide is assuming a very similar conformation in the three different media.

NMR studies were conducted in three different media: TFE/water; SDS and dodecylphosphocoline (DPC). For the TFE/water condition, The NMR experiments were performed with \( \gamma_1 \)-peptide 50 % TFE/buffered water solution at pH 6.5 and at pH 4.0. pH 4.0 is close to the values of the acidic lysosomal vesicular compartments. The sequence-specific assignments of \( \gamma_1 \)-peptide at pH 6.5 and pH 4.0 were carried out using NOESY, TOCSY and COSY spectra (Fig. 3; Supplementary Figs. 1S, 2S and 3S), as proposed by Wuthrich (32). Despite the complexity of the spectra obtained, all residues of each peptide could be sequentially assigned via \( \delta \alpha N(i+1), \delta \alpha N(i+1) \) and \( \alpha NN \) NOEs. The \( \alpha \)-proton (\( \alpha \)H) resonances were unambiguously identified on the basis of COSY spectra at pH 6.5 (Fig. 4A). To locate the elements of secondary structure, chemical shifts of the \( \alpha \)H were compared with statistical chemical-shift values typical of a random-coil conformation to calculate chemical-shift index deviation. The differences between the measured chemical shifts of \( \alpha \)H and the standard values
for a random-coil polypeptide reveal an upfield shift of the residues Met 3 to Ile 10 and Ile 11 to Leu 15 (Fig. 5), suggesting two helical regions connected by a flexible hinge. Secondary structure content was also evaluated by medium- and long-range backbone H'H distances (Table 1).

The NMR data set (TOCSY and NOESY) obtained from γ1-peptide incorporated into DPC (Supplementary Figs. 4S-7S) and SDS (Figs. 8S, 9S) micelles, displayed peaks with sharp lines but a weak dispersion of signals, not allowing complete sequence-specific assignment. In Fig. 6, the NOESY spectra of γ1-peptide in the presence of DPC micelles at two different pHs are superimposed. Although, we were able to identify some of the peaks, there was less dispersion than in TFE/water. Particularly, at pH 7.0, the lines were less sharp, which could be explained by protein association (Fig. 6).

To try to elucidate whether the increased peptide linewidths in micelles are due to oligomerization of the peptide rather than the result of intermediate exchange between different conformations, we used different temperatures and two detergent concentrations (Supplementary Figs. 6S, 7S). The data show no significant changes in the linewidths at different temperatures, which would be expected in case the increased linewidths were due to intermediate exchange between different conformations. The increase in detergent concentration did not result in any considerable change in linewidth as well.

**Structure description**

In general, the observation of dαN(ii+3) together with dαβ(ii+3) and dαN(ii+4) connectivities suggests a stabilization of α-helical structure (Fig. 4). The NOE connectivities at pH 6.5 showed a continuous pattern of αN(ii+3) from Trp 4 to Leu 15 and αβ(ii+3) from Trp 4 to Val 7 and Lys 8 to Ile 11 (Fig. 4A). At pH 4.0 a continuous stretch of αN (ii+3) was observed from Trp 4 to Ile 11 (Fig. 4B). The αβ(ii+3) connectivities occurred from Trp 4 to Val 7 and Ile 11 to Ser 14. In addition αN (ii+2) connectivities from Met 3 to Ser 9 also indicated a folded conformation for the N-terminal portion. αN(ii+4) interaction was observed from Ile 11 to Leu 15 only at pH 6.5. Side-chain H'H distances of (ii + 3), (ii + 4) and long-range connectivities at both pHs were also very useful for the description of the structure (Table 1).

One hundred structures were calculated for each pH utilizing 179 (pH 6.5) and 208 (pH 4.0) interproton distance restraints (Table 1). The chemical-shift index data and NOE connectivities showed a tendency toward helix formation in the N-terminal segment followed by a break and a second helix in the C-terminal half, at both pHs. The backbone structures of the 20 lowest-energy conformers in 50% TFE at pH 6.5 and 4.0 are shown (Fig. 7). At both pHs, the peptides present a kinked structure, similar to the fusion domain from Influenza hemagglutinin (4). A helical content in the N-terminal portion and a sharp bend in the middle of the domain occur at both pHs. However, the C-terminal half has fewer observable d(ii+3) NOE interactions, suggesting a less regular structure than the N-terminal half. There were significant differences between the structure observed at pH 6.5 and that seen at pH 4.0 with respect to the extent and position of the elements of secondary structure. At pH 6.5 the NOE connectivities indicate two distinct helical regions, from Trp 4 to Arg 6 and from Lys 12 to Leu 15. The first region shows a tendency to form a short 310-helix, which is stabilized by hydrogen bonds from the amides of Val 7 and Glu 5 to the carbonyls of Trp 4 and Met 3, respectively. In the second region, the presence of NOE connectivities between αN(ii+3) of Ile 10 and Ser 13, and of Lys 12 and Leu 15; and between αN(ii+4) of Ile 11 and Leu 15, strongly supports the observation of an α-helix in the 20 structures of lowest energy. However, we did not find hydrogen bonds that could stabilize a regular structure in the C-terminal portion. The residues in the center of the peptide have a slight tendency toward a helical or turn conformation based on the NOEs αN(ii+3) between Val 7 and Ile 10, Ile 10 and Ser 13 and αβ,αγ,αδ(ii+3) between Lys 8 and Ile 11. Indeed, this region exhibits a bend, or kink, previously found in the
fusogenic fertilization peptide from sea urchin fertilization protein bindin (20) and in the fusion domain of the influenza hemagglutinin (4, 19). At pH 4.0, the N-terminal domain displays a 3_10-helix that extends from Glu 5 to Ser 9. This motif is stabilized by hydrogen bonds from the NHs of Ser 9, Val 7 and Met 3 to the carboxyls of Arg 6, Trp 4 and the hydroxyl of Ser 2, respectively. The NOEs \(\alpha\beta_{ii+3}\) between Ile 11 and Ser 14, \(\alpha\beta_{ii+4}\) between Lys 8 and Lys 12, and a long-range connectivity between Ile 10 and Ala 16, and between Ile 11 and Leu 15 were observed exclusively at pH 4.0.

The structure of the C-terminal portion of the peptide at pH 4.0 differs substantially from that proposed for pH 6.5, since there was no helical content in any of the 20 lowest-energy structures. The structural difference between pH 6.5 and pH 4.0 can be attributed to neutralization of Glu 5 (protonation at low pH). The number of observed NOEs involving Glu 5 in \(\gamma_1\)-peptide at pH 4.0 is greater than at pH 6.5 (Fig. 3; Supplementary Fig. 2S). The presence of extra NOEs for Glu 5 and for other residues at pH 4.0 provides strong evidence for the conformational differences between the two pHs. It is quite likely that negatively charged Glu 5 limits the conformational flexibility of the C-terminal portion that is more positively charged. Lipophilicity surface-potential maps of the peptides at pH 6.5 and 4.0 showed the amphipathic nature of the two structures (Fig. 7). Figure 7 also shows the arrangement of the hydrophobic residues based on the lowest energy structures at each pH (Fig. 7c, d). At pH 6.5, the more hydrophobic residues were observed in two major regions on each face of the molecule. Residues Trp 4, Ile 11 and Leu 15 appear on one face and residues Met 3, Trp 4, Val 7 and Ile 10 on the other (Fig. 7). At low pH, the polar and apolar regions are segregated, so that the more hydrophobic residues mentioned above all lie on one side of the molecule (Fig. 7 right) with the more hydrophilic residues on the opposite side.

Interestingly, the conformational change between pH 6.5 and pH 4.0 did not seem to affect the interaction between side-chains from Lys 8 and Lys 12; they face each other at both pHs despite the differences of chemical environment (Fig. 6). Lysine 8 and Lysine 12 are of particular interest, since X-ray crystallography data from Flock House virus (33) shows that both side chains from these lysines contact the phosphodiester backbone of the packaged viral RNA, and this interaction may be required to position the RNA correctly between the capsid subunits. It seems that this contact confers a certain organization on the RNA within the virion, and may also be critical for formation of the protein subunit interactions that are established during assembly of the virion (15).

**Effect of pH on \(\gamma_1\)-peptide-mediated leakage of dyes from liposomes**

In order to obtain more information about the interaction of \(\gamma\)-peptide with biological membranes, the leakage of encapsulated DPX/ANTS from liposomes was monitored. The fluorescent dye leakage assay is a well-established method for the study of pore formation in membranes (34, 35) Figure 8 shows the release of DPX/ANTS from liposomes of different composition, induced by addition of 50 μM of \(\gamma_1\)-peptide. The effects of \(\gamma_1\)-peptide were highly dependent on lipid composition. A much greater release was observed with vesicles composed of PC:PE:SPM:Cho (1:1:1:0.2) when compared with vesicles enriched with cholesterol (PC:PE:SPM:Cho (1:1:1:1.5)) (Fig. 8). For the two conditions (low and high cholesterol), there was no significant difference between pHs 4.0 and 6.5. Therefore, cholesterol shows a great importance for the interaction of FHV \(\gamma_1\)-peptide with the target membrane and the induction of permeabilization. However, in both conditions, \(\gamma_1\)-peptide binds to the membrane (Fig. 2B). This is relevant since the plasma membrane of insect cells contains around 10 times less cholesterol than those isolated from mammalian cells (36). Membrane with less cholesterol are more fluid, which may favor lateral association between the peptides. The critical dependence on peptide concentration for the leakage (Table 2) corroborates the hypothesis of oligomerization in the liposomes with low cholesterol. On the other hand, the membrane destabilization cannot be associated with membrane fusion, owing to lack of mixing of internal aqueous content of
vesicles-entrapped DPX and ANTS (data not shown). The same results of leakage were obtained when another assay (carboxyfluorescein) was used (data not shown).

DISCUSSION

Here we show the solution NMR structure of a membrane-binding peptide contained in the protein shell of a small, non-enveloped icosahedral virus. The three-dimensional structure of FHV $\gamma_1$-domain was determined at pH 6.5 and 4.0 in a hydrophobic environment. The three-dimensional structure determined by X-ray crystallography for Flock House virus (11, 33) showed that the first 15 residues of the 21 residues of $\gamma_1$-peptide (residues 364-385 of precursor protein $\gamma$) are in an amphipathic $\alpha$-helical conformation while the remaining 28 carboxy-terminal residues (385-407) of $\gamma$-peptide were not visible in the X-ray electron density map. Although the atomic structure of the C-terminal portion of the $\gamma$-peptide is still unknown, it plays a significant role in recognition of FHV RNA during assembly (37). The NMR solution structures of $\gamma_1$-peptide also show an amphipathic helix, but it is less extensive and structurally different than the one determined by crystallography. The structural difference observed in the X-ray and NMR analyses are not surprising given that $\gamma$-peptides associated with virus particles interact with the packaged viral genome. Specifically, the side chains of lysines 8 and 12 contact the negatively charged phosphodiester backbone of the RNA. During cell entry, the $\gamma$-peptides are likely to be released from the virion and their structure could change to that observed in our NMR analysis before insertion into a cellular membrane.

The observation of kinked NMR structures found in $\gamma_1$-peptide in solution are remarkably similar to the structures determined for class I fusion peptides such as Influenza hemagglutinin (4, 19), HIV-1 gp41 (38) and the sea urchin fertilization protein bindin “B18” (20). On the other hand, they differ from the NMR structure reported for the reptilian reovirus p14 fusion peptide, which has a loop structure found in class II fusion peptides (39). However, no fusion activity was detected for the $\gamma_1$-peptide whereas a high leakage activity was observed. The structure similarity indicates a common mechanism for the interaction between leakage-inducing domains of non-enveloped viruses and fusion domains of enveloped viruses. Indeed, Lai et al. (40) have recently demonstrated that a specific angle in the boomerang structure is required for membrane fusion. Fusion peptides have only to insert into the outer leaflet of the lipid bilayer whereas the leakage activity requires formation of a channel across it.

The circular dichroism data demonstrated that the peptide adopts similar secondary structures in TFE/H$_2$O, SDS and in lipid vesicles. The NMR experiments in SDS and DPC micelles also point in the same direction, even though we were not able to solve the atomic structure in the micelles. Altogether the NMR and CD data do show that the TFE/water structure can mimic what is seen with lipids.

As the pH is decreased from 6.5 to 4.0, $\gamma_1$-peptide undergoes a conformational change, which leads to a less ordered structure at the C-terminal. The NOE patterns observed for the N-terminal of both peptides evidence the presence of a 3$_{10}$-helix, which has been proposed to be an intermediate structure in the folding/unfolding of $\alpha$-helices (41, 42). Based on helix-coil theory, 3$_{10}$-helix should be populated in the helix-coil transition (43). The pH-dependent structural change of $\gamma_1$-peptide may reflect a conformational equilibrium between 3$_{10}$-helix and random coil structures. The increase of pH favors the hydrogen bonding interaction between residues that stabilize 3$_{10}$- and $\alpha$-helical structures.

The combination of NMR and fluorescence results at both pHs shows that a change in pH promotes alteration in protein conformation, but do not affect the ability of $\gamma_1$-peptide to perturb mimetic membranes. In both pHs, Lys 8 and Lys 12 are spatially related, which may be a pre-requisite for the interaction with the sugar-phosphate backbone during transfer of the encapsidated RNA. Based on these data we hypothesize that the interaction of $\gamma_1$-peptide with mimetic membranes may not
require a specific pH. On the other hand, we did find a high dependence on the lipid composition of the membrane (Fig. 8). Thus, the hydrophobic environment and pH were necessary conditions for the membrane binding but lipid composition was the determining factor for leakage. An enticing hypothesis is that lipid constitution of the vesicles would affect oligomerization of the peptide, which would open the channel. The oligomerization may explain why we had much more difficulty in assigning the spectra in DPC micelles.

Altogether, the structural and fluorescence data indicate that the insertion of a protein subunit from an icosahedral virus into a target membrane is carried out by a structural motif similar to that found in enveloped viruses. However, it induces vesicle leakage and no fusion. Picornaviruses are closely related P= 3 icosahedral viruses that cause diseases in humans and other mammals (44-47). They contain a small protein (VP4) derived from maturation-proteolytic cleavage of VP0 that seems to assume a conformation similar to that of γ-peptide in nodaviruses (44, 46). Our results strongly suggest that small protein components such as α protein in nodaviruses and VP4 in picornaviruses have crucial role in disrupting membranes during viral entry.

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**FOOTNOTES**

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**FIGURE LEGENDS**

**Fig. 1.** Secondary structure of γ₁-peptide. CD spectra of γ₁-peptide in 10 mM Na phosphate buffer at pH 7.0, 25º C in the absence of TFE (*purple line*); 10% TFE (*black line*); 20% TFE (*green line*); 30% TFE (*blue line*) and 50% TFE (*red line*).

**Fig 2.** Secondary structure of γ₁-peptide. (A) CD spectra of γ₁-peptide in 10 mM Na phosphate buffer at pH 7.0, 25º C (*dashed line*) and 60 mM of SDS (*solid line*). (B) CD spectra of γ₁-peptide in the absence of vesicles at pH 6.5 (*blue solid line*) and pH 4.0 (*red solid line*) or in the presence of vesicles composed of PC:PE:SPM:Cho (1:1:1:1.5) at pH 6.5 (*red dashed line*) and pH 4.0 (*blue dashed line*) or in the presence of vesicles composed of PC:PE:SPM:Cho (1:1:1:0.2) at pH 6.5 (*blue dotted line*) and pH 4.0 (*red dotted line*). All spectra were acquired at room temperature.

**Fig. 3.** Amide region of ¹H-¹H NOESY spectra of gamma peptide in TFE:H₂O 1:1 at pH 6.5, 20ºC. Spectrum A is an expansion of the H^N^ region of the NOESY spectrum, B is an expansion of the HN region with some assigned H^N^-H^N^ connections.

**Fig 4.** Summary of NOESY connectivities of γ₁-peptide in TFE:H₂O 1:1 at pH 6.5 (A) and pH 4.0 (B), 20ºC. The position and thickness of the bar represent the J coupling value and its estimated accuracy, respectively.

**Fig. 5.** Chemical-shift differences between α-protons of γ₁-peptide and the standard values of a random coil peptide. **Black bars** are values obtained at pH 6.5 and **grey bars** at pH 4.0.

**Fig. 6.** Superposition of amide region of ¹H-¹H NOESY spectra of gamma peptide in 120 mM DPC at pH 5.5 (black) and 7.0 (red). Spectrum A is an expansion of the H^N^ region of the NOESY spectrum.

**Fig. 7.** Structures of the γ₁-peptide in 50% TFE at 6.5 and pH 4.0. a, View of the backbone superimposition of the 20 lowest-energy structures between residues Met₃-Ala₁₈ at 6.5 and pH 4.0. b, Ribbon diagrams of the representative conformer at pH 6.5 and pH 4.0. Sidechains for Lys 8 and Lys 12 are shown in blue. c, d, show the surface plot in two views, following the axis. Surface potential maps are colored according to a lipophilicity scale, where brown is more lipophilic and blue is more hydrophilic.

**Fig. 8.** Liposome leakage mediated by γ₁-peptide. Kinetics of γ₁-peptide-induced ANTS/DPX leakage from vesicles composed of PC:PE:SPM:Cho (1:1:1:1.5) at pH 6.5 (green line) and pH 4.0 (purple line) or in the presence of vesicles composed of PC:PE:SPM:Cho (1:1:1:0.2) at pH 6.5 (red line) and pH 4.0 (blue line). The experimental control is indicated by black line, in which were added 8.8 μL of TFE 50%. Fluorescence dequenching was monitored at an emission wavelength of 523 nm (excitation wavelength at 353 nm). 100% is the fluorescence intensity in the presence of 1% of Triton-X 100. The peptide concentration was 50 μM.
Table 1. Summary of structural statistics of $\gamma_1$-peptide at pH 6.5 and 4.0 in 50% TFE.

|                        | pH 6.5 | pH 4.0 |
|------------------------|--------|--------|
| Total number of distance constraints | 179    | 208    |
| Number of intrareside constraint | 128    | 129    |
| Number of sequential constraints | 30     | 41     |
| Number of medium to long range constraints | 21     | 38     |
| Energies (kcal/mol)    |        |        |
| Overall                | 79.78 ± 18.57 | 220.71 ± 47.42 |
| Bond                   | 4.18 ± 1.12  | 12.79 ± 2.44  |
| Angle                  | 29.69 ± 5.97 | 66.51 ± 13.17 |
| Improper               | 3.29 ± 1.28  | 10.65 ± 3.66  |
| VDW                    | 26.70 ± 6.63 | 52.48 ± 22.80 |
| NOE                    | 14.25 ± 4.29 | 66.56 ± 14.37 |
| Backbone rmse (%)      |        |        |
| residues 1-21          | 2.925  | 2.455  |
| residues 3-18          | 1.778  | 1.347  |
| residues 3-9           | 0.639  | 0.567  |
| residues 10-15         | 0.585  | 0.456  |
| Heavy rmse (%)         |        |        |
| residues 1-21          | 3.571  | 2.971  |
| residues 3-18          | 2.345  | 1.888  |
| residues 3-9           | 1.300  | 1.372  |
| residues 10-15         | 1.044  | 0.911  |
| % of residues in allowed region of Ramachandran plot | 97.4 | 99.2 |

*a RMSD values from molmol 41
*b Data from PROCHECK-NMR 40

Errors shown are standard deviations.
Table 2. Dependence on peptide concentration of the fraction of leakage.

| Peptide Concentration (μM) | Plateau Fraction of Leakage (%)* |
|----------------------------|----------------------------------|
| 4.8                        | 10                               |
| 9.6                        | 36                               |
| 19.1                       | 54                               |
| 50                         | 68                               |

*γ1-peptide-induced leakage of liposome vesicles composed of PC:PE:SPM:Cho (1:1:1:0.2) at pH 6.5 was measured as described in Fig. 8.
Figure 1
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Figure 2
Maia et al., 2006
Figure 3
Maia et al., 2006
Figure 4
Maia et al., 2006
Figure 5
Maia et al., 2006
Figure 7
Maia et al., 2006
Figure 8
Maia et al., 2006
Structure of a membrane-binding domain from a non-enveloped animal virus: Insights into the mechanism of membrane permeability and cellular entry

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