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ARTICLE

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Probing the interaction between vesicular stomatitis virus and phosphatidylserine

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Abstract The entry of enveloped animal viruses into their host cells always depends on membrane fusion triggered by conformational changes in viral envelope glycoproteins. Vesicular stomatitis virus (VSV) infection is mediated by virus spike glycoprotein G, which induces membrane fusion between the viral envelope and the endosomal membrane at the acidic environment of this compartment. In this work, we evaluated VSV interactions with membranes of different phospholipid compositions, at neutral and acidic pH, using atomic force microscopy (AFM) operating in the force spectroscopy mode, isothermal calorimetry (ITC) and molecular dynamics simulation. We found that the binding forces differed dramatically depending on the membrane phospholipid composition, revealing a high specificity of G protein binding to membranes containing phosphatidylserine (PS). In a previous work, we showed that the sequence corresponding amino acid 145–164 of VSV G protein was as efficient as the virus in catalyzing membrane fusion at pH 6.0. Here, we used this sequence to explore VSV–PS interaction using ITC. We found that peptide binding to membranes was exothermic, suggesting the participation of electrostatic interactions. Peptide–membrane interaction at pH 7.5 was shown to be specific to PS and dependent on the presence of His residues in the fusion peptide. The application of the simplified continuum Gouy–Chapman theory to our system predicted a pH of 5.0 at membrane surface, suggesting that the His residues should be protonated when located close to the membrane. Molecular dynamics simulations suggested that the peptide interacts with the lipid bilayer through its N-terminal residues, especially Val^{145} and His^{148}.

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Introduction

The plasma membrane of eukaryotic cells serves as a barrier against invading parasites and viruses. To infect a cell, viruses must be capable of transporting their genome and accessory proteins into the cytosol or, in some cases, into the nucleus of the host cell, thus bypassing or modifying the barrier properties imposed by the plasma membrane. Enveloped viruses always gain entry to the cytoplasm by fusion of their lipid envelope with the plasma or endosomal membranes (Hernandez et al. 1996; Skehel and Wiley 2000; Eckert and Kim 2001), whereas nonenveloped viruses must use alternative strategies to cross the membrane. Membrane fusion induced by viruses is mediated by viral fusion glycoproteins, which have already been identified for a number of different viruses (Hernandez et al. 1996). The fusion reaction depends on conformational changes in the fusion glycoproteins that can be triggered either by

the interaction with a specific virus receptor on cell surface, or by the acidic pH of the endosomal environment.

Vesicular stomatitis virus (VSV) belongs to the *Rhabdoviridae* family, a group of enveloped negative single strand RNA viruses. The VSV envelope contains approximately 1,200 molecules of a single transmembrane glycoprotein, the G protein, that form about 400 trimeric spikes on the virus surface. VSV G protein is involved in both virus attachment to the host cell surface and in the membrane fusion mediated by the virus. VSV-induced membrane fusion occurs at the endosomal compartment where the acidic pH induces conformational changes on G protein, leading to the exposure of hydrophobic domains (Crimmins et al. 1983; Durrer et al. 1995; Pak et al. 1987), followed by dramatic structural reorganization (Carneiro et al. 2001).

The cellular receptor for VSV is still to be determined. There are several evidences in the literature suggesting an important role for phospholipids in the rhabdovirus entry into the host cell (Schlegel et al. 1982, 1983; Superti et al. 1984; Mastromarino et al. 1987). A high affinity, saturable binding site has been described for VSV on Vero cells, indicating that the binding occurs through a specific receptor (Schlegel et al. 1982). The binding could be inhibited by membrane extracts, which were resistant to protease, neuraminidase and heating, but inactivated by treatment with phospholipase C (Schlegel et al. 1983). These findings, together with the observation that only phosphatidylserine (PS) among various purified lipids was able to inhibit VSV binding to membranes, led the authors to suggest that PS could participate in the cellular binding site for VSV (Schlegel et al. 1983). On the other hand, a recent work from Coil and Miller (2004) provided consistent evidences supporting that the VSV binding to PS is not a determinant event in the context of the cellular plasma membrane. For the authors, virus recognition at cell surface must occur through an unknown cellular receptor, and the binding to PS should be important in a subsequent step of the entry process. Despite this controversy regarding the participation of PS in VSV binding site at the host cell surface, several findings indicate that PS is essential for VSV–membrane interactions. Membrane fusion mediated by VSV G protein reconstituted in lipid vesicles showed a large preference for target membranes containing phosphatidylserine or phosphatidic acid (Eidelman et al. 1984). In a previous work, we showed that the extent of pH-induced G protein conformational changes and the membrane fusion mediated by this protein could be correlated to the PS content in the vesicles (Carneiro et al. 2002). A PS-binding segment was mapped in G protein from several rhabdoviruses (Coll 1997). This sequence was first identified in viral hemorrhagic septicemia virus (VHSV), a fish rhabdovirus that infects salmonids (Estepa and Coll 1996), and then found in rabies, VSV and infectious haematopoietic necrosis virus (IHNV), another fish rhabdovirus (Coll 1997). We have demonstrated that a synthetic peptide corresponding to the VSV PS binding site (amino acids 145 to 164 of G protein) was as efficient as the whole virus to mediate fusion (Carneiro et al. 2003). This segment contains two His residues, whose substitution by Ala or modification with diethylpyrocarbonate (DEPC) inhibits the fusogenic properties of the peptide.

In the present work, we compared the interaction forces between VSV and membranes of different phospholipid composition and we found a high specificity for PS on VSV binding to vesicles. We evaluated the role of this His residues on membrane recognition at neutral bulk pH, a condition in which no fusion occurs. We show that the high specificity of VSV binding to membranes containing PS was due to His–PS interaction. In addition, a model for this interaction is proposed.

Materials and methods

Chemicals

Phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from bovine brain, phosphatidylglycerol (PG) from egg yolk lecithin, phosphatidylinositol (PI) from bovine liver, and diethylpyrocarbonate (DEPC) were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other reagents were of analytical grade.

Virus propagation and purification

VSV Indiana was propagated in monolayer cultures of BHK_{21} cells. The cells were grown at 37°C in petri dishes containing Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 0.0085% streptomycin sulfate. When the cells reached confluence, the medium was removed, and the cell monolayer was infected with VSV at a multiplicity of infection (MOI) of 0.1. The cultures were kept at 37°C for 16–20 h and the virus was harvested and purified by differential centrifugation followed by equilibrium sedimentation in a sucrose gradient as described elsewhere (Da Poian et al. 1996). For all the experiments the purified virus was dialyzed against Tris 10 mM buffer, pH 7.4 for 4 h. Purified virions were stored at −70°C.

Peptides synthesis

The VSV G protein peptide corresponding to the sequence between amino acids 145 and 164, VTPHHVLVDEYTGEWVDSQF, and the same peptide except for the substitution of then two His for Ala residues, VTPAAVLVDEYTGEWVDSQF, were synthesized by solid phase using the Fmoc methodology

and all protected amino acids were purchased from Calbiochem–Novabiochem (San Diego, USA) or from Neosystem (Strasbourg, France). The syntheses were done in an automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu). The final deprotected peptides were purified by semipreparative HPLC using an Econosil C-18 column (10 μm, 22.5 × 250 mm) and a two-solvent system: (a) trifluoroacetic acid/H_2O (1:1000, v/v) and (b) trifluoroacetic acid/acetonitrile/H_2O (1:900:100, v/v/v). The column was eluted at a flow rate of 5 ml min^{-1} with a 10 or 30 to 50 or 60% gradient of solvent B over 30 or 45 min. Analytical HPLC was performed using a binary HPLC system from Shimadzu with a SPD-10AV Shimadzu UV/vis detector, coupled to an Ultrasphere C-18 column (5 μm, 4.6 × 150 mm), which was eluted with solvent systems A1 (H_3PO_4/H_2O, 1:1000, v/v) and B1 (acetonitrile/H_2O/H_3PO_4, 900:100:1, v/v/v) at a flow rate of 1.7 ml min^{-1} and a 10–80% gradient of B1 over 15 min. The HPLC column eluted materials were monitored by their absorbance at 220 nm. The molecular mass and purity of synthesized peptides were checked by MALDI-TOF mass spectrometry (TofSpec-E, Micromass) and/or peptide sequencing using a protein sequencer PPSQ-23 (Shimadzu Tokyo, Japan).

Sample modification with DEPC

DEPC solutions were freshly prepared by dilution of the reagent in cold ethanol. The concentration of stock DEPC solution was determined by reaction with 10 mM imidazole (Miles 1977). For modification with DEPC, VSV (0.6 mg/ml) was incubated with DEPC at final concentration of 0.5 mM for 15 min at room temperature. Then VSV was diluted in 20 mM MES, 30 mM Tris buffer, pH 7.5 to a final protein concentration of 60 μg/ml. For modification of peptides, the process was carried out under the same conditions except that the initial and final protein concentrations were 10 mg/ml and 400 μg/ml, respectively.

Preparation of liposomes

Phospholipids were dissolved in chloroform and evaporated under nitrogen. The lipid film was resuspended in 20 mM MES, 30 mM Tris buffer (pH 7.5 or 6.0) in a final lipid concentration of 1 mM. The suspension was vortexed vigorously for 5 min. Small unilamellar vesicles were obtained by sonicating the turbid suspension using a Branson Sonifier (Sonic Power Company, Danbury, CT, USA) equipped with a titanium microtip probe. Sonication was performed in an ice bath, alternating cycles of 30 s at 20% full power, with 60 s resting intervals until a transparent solution was obtained (approximately ten cycles). The phospholipids used in this study were composed of PC only, PC:PE, PC:PS, PC:PI and PC:PG at the proportions indicated in the figure legends.

Atomic force microscopy

The AFM used in this work was built in collaboration with the Ludwig-Maximilians-Universität, Lehrstuhl für Angewandte Physik in München, Germany. For all the experiments, the AFM was used in force-spectroscopy mode (Florin et al. 1994; Gergely et al. 2000; Zlatanova et al. 2000). Mica coverslips were glued to magnetic stainless steel punches, and mounted in a fluid cell without using the O-ring. The mica surfaces were pre-incubated with vesicles before transferring to the fluid cell (Jass et al. 2000; Puu et al. 2000). Since the presence of calcium ions appears to facilitate as well as to increase the rate of planar membrane formation from vesicles (Puu et al. 1997; Reviakine et al. 2000), mica surfaces were incubated with 20 μl of the vesicle suspension containing 1 mM phospholipids, plus 10 μl of 20 mM MES, 30 mM Tris buffer, pH 7.4 containing 1 mM $CaCl_2$, for approximately half an hour at room temperature (25 ± 0.5°C). After incubation, the slips were washed repeatedly with the same buffer used to prepare vesicles. All experiments were performed at room temperature using standard V-shaped cantilevers, containing a silicon nitride tip with a 4 μm^2 pyramidal base (Digital Instruments Inc.). The cantilevers have a spring constant of 0.06 N/m (manufacturer's data) and were pre-incubated with VSV as follows. The cantilevers were immersed in a virus suspension (total protein concentration of 0.28 mg/ml) for 24 h at 4–6°C. The instrument allows the performance of "approach-retraction" cycles, in which the maximal contact force, interaction time and the approach-retracting rates can be controlled independently. The maximal force was limited to approximately 3 nN, the interaction time was set to zero and the approach-retracting rate was set to 7,500 nm/s. Tips can be reused but they should be cleaned soon and should not dry out before the cleaning procedure.

Calorimetric studies

Binding to lipid vesicles and membrane fusion induced by VSV or wild type and mutant peptide [145–164] were studied by isothermal titration calorimetry (ITC) using a MCS-ITC from MicroCal, Llc. (Northampton, MA, USA). Membrane fusion was studied by following the heat effect of four injections of 5 μl of a VSV suspension (60 μg/ml) or peptide solution (400 μg/ml) into the sample cell containing 1 mM PC:PS (1:3) vesicles in 20 mM MES, 30 mM Tris buffer (pH 6.0), after equilibration at 37°C. For the binding experiments, the samples were prepared at pH 7.5, and four injections (5 μl each) of the virus suspension or peptide solution were done into the sample cell (V = 1.38 ml) containing

1 mM PC:PS (1:3) or PC:PG (1:3) vesicles in 20 mM MES, 30 mM Tris buffer (pH 7.5), after equilibration at 37 ° C. The heat of dilution of the peptides was measured by injecting the same solutions into buffer only.

Molecular dynamics simulations

Initially, we decided to simulate the peptide [145–164] in solution by molecular dynamics (MD) in order to evaluate the consistency of the force field and compare it with experimental NMR data in aqueous environment (C.S. Lima et al., unpublished results). The starting atomic coordinates were parameterized with the GROMOS96 united atom force field implemented in the GROMACS MD program (Lindahl et al. 2001). The peptide [145–164] with protonated His^{148} and His^{149} was solvated in a SPC water box (Berendsen et al. 1987) with 4,737 water molecules and two sodium ions, to neutralize the -2 *e* net charge. After energy minimization with constraints of 1,000 kJ/mol applied on C, N and O atoms, we performed an unconstrained MD simulation in the NPT ensemble (fixed number of particles and constant pressure/temperature) (Berendsen et al. 1984) for 10 ns, at 298 K and 1 bar.

To gain insight into the interaction between the peptide [145–164] and charged membranes at an atomistic resolution, we chose to simulate a dimyristoylphosphosphatidylserine (DMPS) bilayer in the liquid-crystalline phase. The last configuration of the peptide [145–164] in the previous simulation was inserted in the aqueous phase of a pre-equilibrated 126 DMPS bilayer, with 7,347 SPC water molecules and 126 sodium counterions. The system was energy minimized with constraints (see above) applied on the protein. The lipids were simulated with the parameters taken from Pandit et al. (2002) and Chandrasekhar et al. (2003) in the NPT ensemble, with anisotropic pressure scaling (i.e., six box dimensions *xx, yy, zz, xy/yx, xz/zx* and *yz/zy* were independently coupled to pressure "baths") for 10 ns. In both systems, bonds were constrained with the SHAKE algorithm (Ryckaert et al. 1977) allowing a 2 fs integration step. Electrostatic forces were calculated using the particle mesh Ewald method (Darden et al. 1993) with 1.2 Å grid spacing and a fourth-order spline for interpolation. Van der Waals forces were computed with a cut-off radius of 14 Å .

Continuum electrostatics models

Assuming that the electrostatics of model lipid membranes is well described by the Gouy–Chapman approximation for interfaces of the generalized Poisson–Boltzmann equation (Cevc 1990), we can calculate the surface potential as:

$$\psi_0 = \left(\frac{2k_BT}{Ze}\right)\sinh^{-1}\left(\frac{Ze\sigma_{el}\lambda}{2\varepsilon\varepsilon_0 k_BT}\right)$$

where, Ψ_0 is the surface potential, Z is the co- and counter-ion valency, ε, ε_0 and k_B are the dielectric constant of water, the permittivity of free space and the Boltzmann constant, respectively. σ_{el} is the surface charge density and λ is the Debye screening length (or the width of the double layer), defined as:

$$\lambda = \left(\frac{\varepsilon\varepsilon_0 k_BT}{10^3 N_A e^2 \Sigma Z_i^2 c_i}\right)^{\frac{1}{2}} \quad (2)$$

According to the Boltzmann distribution, we have:

$$[\text{ion}]_0 = [\text{ion}]_{\text{bulk}} \exp\left(-\frac{Ze\Psi_0}{k_BT}\right) \quad (3)$$

where, $[\text{ion}]_0$ and $[\ ion]_{bulk}$ are the ionic species concentration near the surface and in the bulk, respectively.

Results

Probing the VSV–membrane interaction using atomic force microscopy

Force spectroscopy was used to measure the interaction forces between VSV and membranes of different phospholipid composition at pH 7.5, a condition that simulates binding to membrane but not fusion; and at pH 6.0, the optimum pH for VSV fusion. A set of five lipid film compositions was used: three negatively charged, composed of PC:PS (3:1), PC:PI (3:1), and PC:PG (3:1), and two neutral, composed of PC only and PC:PE (3:1). Sets of scans were acquired with delay times increasing from 0 to 800 ms between the approach and the retraction of the scanner (Figs. 1, 2).

At pH 7.5, the binding between the virus and lipid films containing PS was remarkably stronger when compared to that observed for other lipid compositions (Fig. 1). Moreover, as shown in Table 1, the retraction curves obtained for the interaction between VSV and PC:PS showed several rupture events that extended for hundreds of nanometers, while for other lipid films the interaction peaks appear much closer to the contact point. The interaction between the virus and films of PC:PS and PC:PG increased with contact time (Fig. 1). Although PC:PG films exhibited force curves with several rupture events after longer contact times, the force peaks were considerably smaller than that observed for PC:PS films. For PC:PI films, no significant interaction was observed even after increasing the contact time, indicating that not only the charge is important for virus–membrane interaction, but also the specific lipid head group. Films containing PC:PE showed a very small interaction peak, close to the contact point, exhibiting only a small variation with contact time. Furthermore, films containing PC only did not show any detectable interaction even after 800 ms of contact (not shown).

In order to analyze the interactions under conditions reflecting the protein–lipid interaction events that take

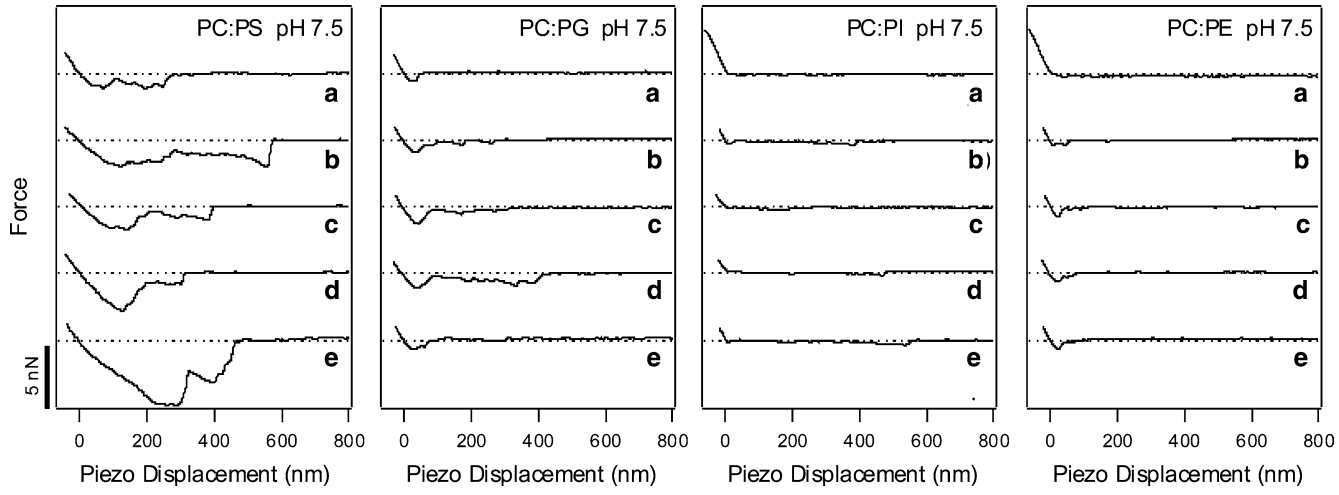

Fig. 1 Interaction forces between VSV and membranes of different phospholipid composition probed by atomic force microscopy at pH 7.5. Force–distance curves were recorded on lipid-covered mica substrates. Retracting curves were obtained with VSV adsorbed on the tip and mica substrates covered with PC:PS (3:1), PC:PG (3:1), PC:PI (3:1) or PC:PE (3:1) after a delay time of 0 (a), 200 (b), 400 (c), 600 (d) and 800 (e) ms. Data were collected in 20 mM MES, 30 mM Tris, pH 7.5, at room temperature

place during membrane fusion reaction, similar experiments were performed at pH 6.0 (Fig. 2). As observed at pH 7.5, for PS and PG containing lipid films, the interaction forces increased with the increase in the contact time and no interaction was detected between the virus and PC:PI films. A small interaction close to the contact point was observed with PC:PE films and a small increase in the force peak was observed with longer contact times. The films containing PC only did not show any response to the pH change (not shown).

All experiments were repeated with different lipid and tip preparations where a set of at least ten scans was acquired for each pH and delay time. With short contact times, we did not observe a significant variation in the adhesion peaks even after a large set of scans (20–30 scans). However, as the contact time increased, the number of reproducible scans obtained with PC:PS films was reduced when the interaction peaks become as large as 3–4 nN. One explanation for this could be a strong interaction between the virus and PS at pH 6.0 that probably involves insertion of VSV G protein into the lipid bilayer, resulting in the removal of VSV from the tip during retraction.

Calorimetric studies of VSV and peptide [145–164] interaction with vesicles

The role of PS in the interaction between membranes and VSV or G protein peptide [145–164] was also studied using microcalorimetry at the pHs of fusion and binding. Membrane fusion was studied by ITC at 37° C, by following the heat flow after injection of VSV or the peptide [145–164] into PC:PS (1:3) vesicles at pH 6.0.

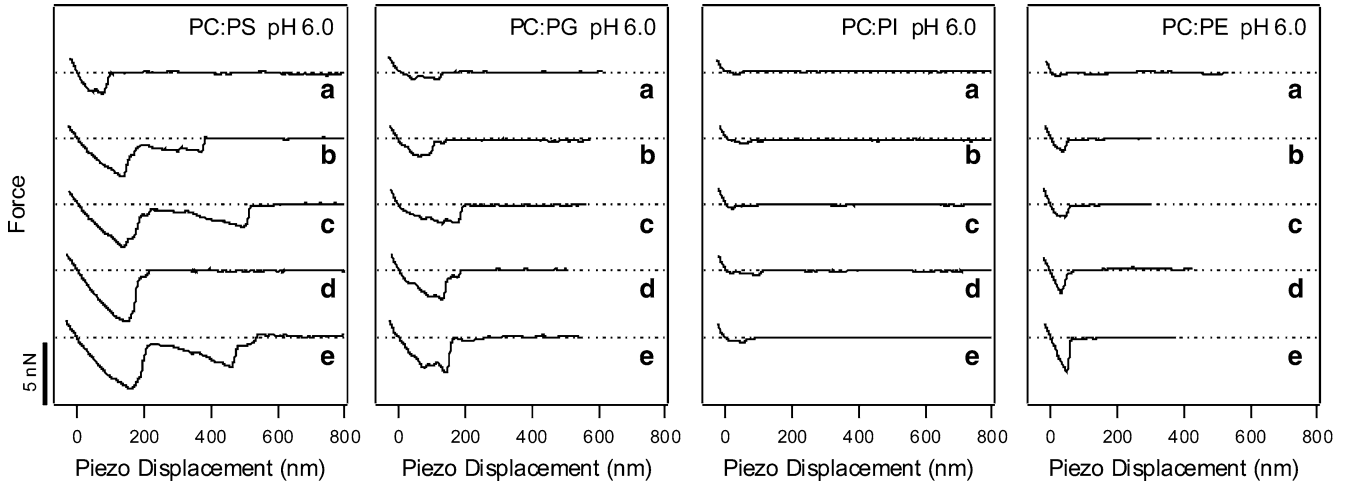

Fig. 2 Interaction forces between VSV and membranes of different phospholipid composition probed by atomic force microscopy at pH 6.0. Force–distance curves were recorded on lipid-covered mica substrates. Retracting curves were obtained with VSV adsorbed on the tip and mica substrates covered with PC:PS (3:1), PC:PG (3:1), PC:PI (3:1) or PC:PE (3:1) after a delay time of 0 (a), 200 (b), 400 (c), 600 (d) and 800 (e) ms. Data were collected in 20 mM MES, 30 mM Tris, pH 6.0, at room temperature

Table 1 Interaction between VSV and membranes of different lipid composition at pH 7.5

Film composition	Force (pN)	Maximal rupture distance (nm)	Rupture events
PC:PS	1400 ± 400	520 ± 430	Up to 8
PC:PG	660 ± 140	68 ± 22	Up to 2
PC:PI	160 ± 80	150 ± 120	2
PC:PE	450 ± 90	40 ± 15	1
PC	Not detected	–	–
Mica	250 ± 200	30 ± 25	2

Figure 3a shows that either the virus or the peptide were able to cause membrane fusion in a similar way. The kinetics of the fusion also seems to be very similar

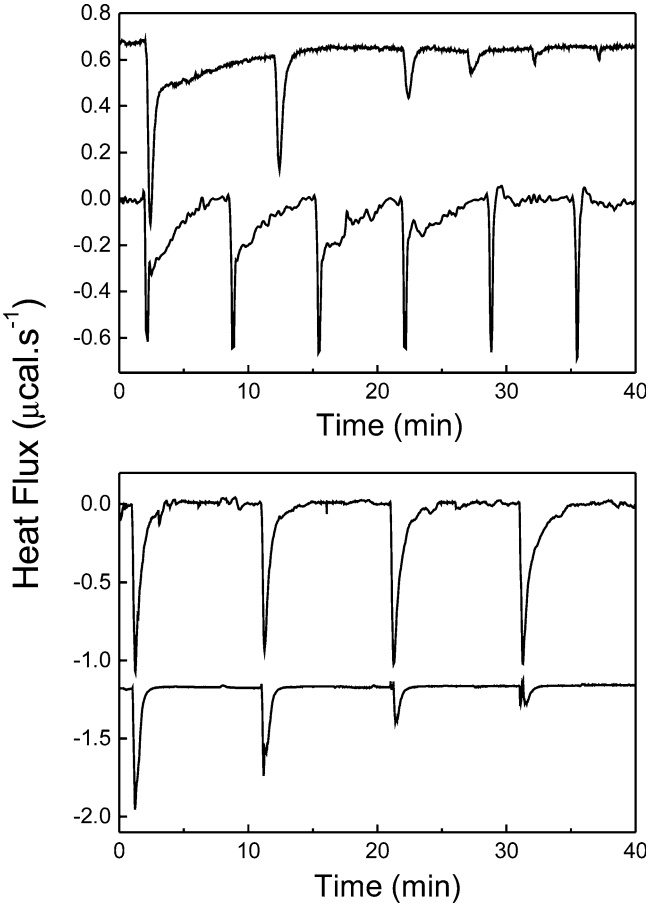

Fig. 3 Calorimetric measurements of membrane fusion (*upper panel*) and binding (*lower panel*) by VSV or peptide [145–164] at 37°C. *Upper panel*: Typical calorimetric traces (heat flow as a function of time) obtained from 6 × 5 μL injections of VSV (0.06 mg/ml) or PEP (0.4 mg/ml) into the sample cell containing 1 mM PC:PS (1:3) vesicles in 20 mM MES, 30 mM Tris, pH 6.0. The sharp peak that follows the injection is due to dilution effects, and the following slow heat effect is associated with the fusion process (broader peak). The negative heat effects indicate that the fusion is exothermic in nature. *Lower panel*: calorimetric traces for membrane binding of VSV (0.06 mg/ml) or PEP (0.4 mg/ml) into the sample cell containing 1 mM PC:PS (1:3) vesicles in vesicles in 20 mM MES, 30 mM Tris, pH 7.5. The negative peaks observed after subtraction of the heat of dilution indicate the exothermic nature of the binding

occurring in a time frame of about 10 min, in agreement with our previous observation using other techniques (Carneiro et al. 2001; 2003; Da Poian et al. 1998). The calorimetric thermograms obtained with VSV or with the peptide showed a negative displacement of the heat flow from the baseline after sample injection. Intriguingly, fusion stopped after one or two injections of viruses, suggesting the newly injected viruses were not able to fuse with the virosomes resulting from previous fusion events. This was also observed when we measured fusion by fluorescence energy transfer in labeled liposomes (not shown). However, the fusion process is probably complex. It is probably causing a change in macromolecular level to have a mixture of VSV proteins and phospholipids, resulting in a different structure than that of a lipid bilayer.

On the other hand, when the VSV or the peptide was injected into vesicles prepared at pH 7.5, no fusion was observed and the binding could be measured. In both cases we observed negative peaks indicative of an exothermic binding to the vesicles. In this case, after 2×5 μL injections of the peptide (corresponding to 4 μg of peptide in the cell), there was no significant heat effect in the thermogram indicating a binding saturation. These results suggest that besides acting in membrane fusion, the binding of peptide [145–164] to PS-containing membranes at pH 7.5 is similar to the exothermic binding between the whole VSV and membranes containing PS. This encouraged us to explore the role of His^{148} and His^{149} on membrane recognition at neutral pH. We had shown earlier that the protonation of these two His was involved in VSV fusion, since blocking these His through their reaction with DEPC or substituting these residues for Ala completely abolished peptide fusion activity (Carneiro et al. 2003). To address this point, binding experiments were done using the peptide modified with DEPC or a mutant peptide containing two Ala residues replacing His^{148} and His^{149} (Fig. 4). In both cases, as compared to the intact peptide, after the injection of either peptide to the cells containing PC:PS (1:3) vesicles at 37°C, no significant heat effect could be observed after subtraction of the heat of dilution of the peptides (Fig. 4b, c). These results are indicative that the His^{148} and His^{149} are also essential for the binding to occur. Changing PS for PG in the vesicles abolished peptide binding (Fig. 4d), indicating that Peptide–membrane interaction occurs through the binding between His and PS.

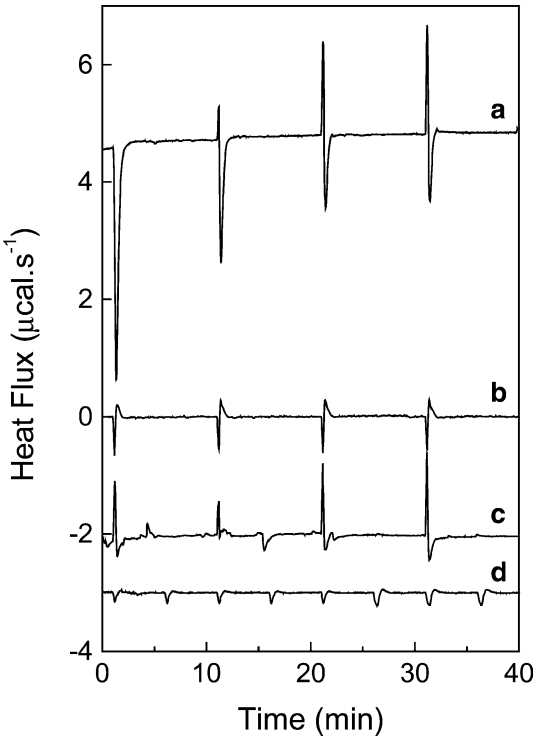

Fig. 4 Specificity of the peptide interaction with phospholipid vesicles. The heat flow as a function of temperature is shown after subtraction of the heat of dilution of the peptides. The calorimetric traces were obtained at 37°C where the peptide [145–164] was injected in the sample cell containing 1 mM PC:PS 1:3 (**a**) or PC:PG 1:3 (**d**) vesicles. Traces b and c show the importance of the His residues for the interaction as the mutant peptide (b) and the DEPC-modified peptide (**c**) since no significant heat effect is observed

Simulation of peptide [145–164] interaction with PS-containing membranes

Since classical MD techniques are carried out with fixed partial atomic charges, it was necessary to make a choice regarding the protonation state of the His^{148} and His^{149} residues. By using the simplified continuum Gouy-Chapman theory (see Material and methods) an interfacial H_3O^+ concentration was calculated and the His imidazol ring protonation state was inferred, assuming a pK_a of 6.0. Applying Eqs. 1 and 2, a Debye screening length of approximately 10 Å and a surface potential in the range of −120 mV were obtained. The parameters used in the calculations were: surface charge density, σ_{el}, of 0.2 Cm^{-2}, and ionic strength, $\Sigma\ Z_i^2\ c_i$, of 100 mM. The surface charge density was calculated assuming an ideal mixture of the PS and PC molecules and an area per lipid of 55 and 64 $Å^2$, respectively. Inserting these values into the Boltzmann distribution (Eq. 3) of H_3O^+ ion and neglecting changes in H_3O^+ ion activity coefficient, a 10^{-5} mol l^{-1} hydronium concentration was obtained, which corresponds to a local surface pH of 5.0 (Boström et al. 2004). At this pH, the His imidazol ring is mostly protonated, displaying a net charge of $+1e$.

Although the starting structure of the DMPS + peptide [145–164] system was somewhat arbitrary, a 10 ns-long MD simulation was probably sufficient to minimize any major artifacts arising from this initial choice. Moreover, using the last 2 ns of simulation, an area per lipid of 55 ± 0.7 $Å^2$ was calculated (data not shown), in accordance with other simulations (Pandit and Berkowitz 2002). This value, together with the fact that we used fully anisotropic pressure coupling (i.e., the bilayer was free to adjust its area), underlines the consistency of the lipid model.

As can be observed in Fig. 5, the peptide seems to interact with the simulated membrane patch mainly through its N-terminal residues. The rest of the peptide chain displays larger fluctuations, with the negatively charged residues (Asp and Glu) initially repelled from and afterwards approximating the water-DMPS interface. In fact, by analyzing the distance between the centers of mass of titratable aminoacids and the bilayer center (see Fig. 6), it can be seen that Val^{145} and His^{148} are stabilized in their positions. On the contrary, at least in the 10 ns time frame, the other charged residues (His^{149}, $Asp^{153,161}$ and $Glu^{154,158}$) have not reached a stable position along the normal to the membrane.

Discussion

The early events of envelope virus infection comprise at least three distinct steps: (a) the cell recognition, which occurs generally through the interaction between the virus and a specific receptor on cell surface; (b) the interaction between a viral surface protein and a cellular membrane; and (c) membrane fusion reaction induced by the viral fusion proteins. In this work, we focused on dissecting the interaction between VSV and the membrane at neutral pH, which might take place after binding to the receptor but before the events involved in the membrane fusion reaction. We have taken advantage of our previous demonstration that atomic force microscopy (AFM) operating in the force spectroscopy mode could efficiently measure the interaction forces between a virus particle and a lipid bilayer (Carneiro et al. 2002). Using this technique, we showed that VSV interacts very strongly with membranes containing PS, while no interaction was observed with membranes composed of PC only. A question not completely answered was whether VSV–membrane interaction depends only on electrostatic interaction or it was specific for PS. Although VSV fusion has already been tested varying the phospholipid composition (Eidelman et al. 1984; Hermann et al. 1990), the binding events at neutral pH were not explored so far. To address this point we measured the interaction forces between virus particles and lipid films supported on mica surfaces at pH 7.5, a condition in which binding but not fusion could occur. These experiments revealed a high specificity for membrane-containing PS, suggesting that PS is

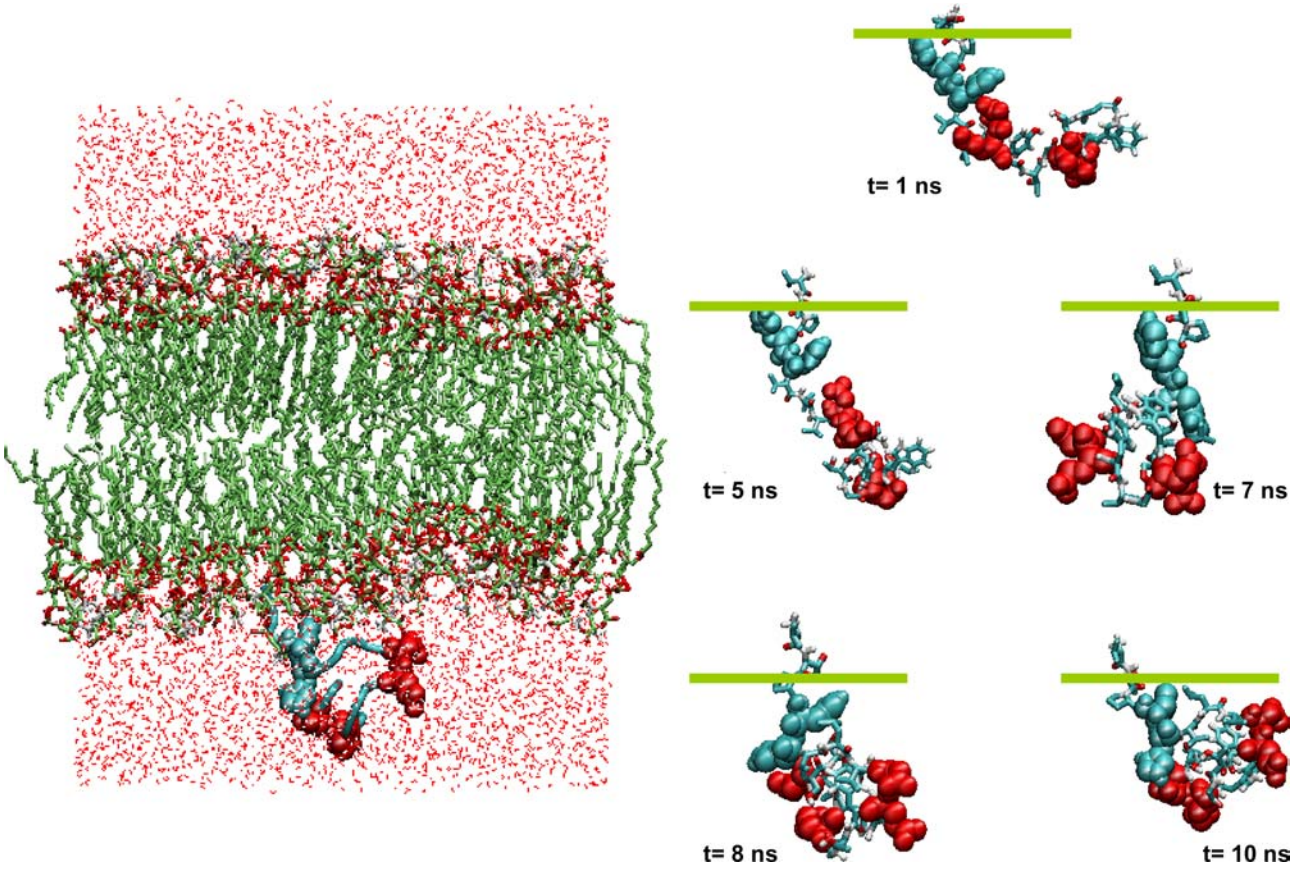

Fig. 5 Snapshots of the system configuration during 10 ns MD simulation. The protein backbone is represented as a cyan tube, except that the titratable residues are depicted as van der Waals spheres (cyan for the His and red for the Asp/Glu residues). Water is represented as *red lines*. The peptide is seen in detail along the simulation time. A representation of the water–DMPS interface is depicted as a *green line*. The DMPS bilayer is shown with the lipid tails in green, after 10 ns of simulation

also important for VSV–membrane interactions at neutral pH. This does not mean that the receptor for the virus is PS, but suggests that although other components in the cell surface might act as the VSV receptors, as indicated by the results from Coil and Miller (2004), a direct interaction between G protein and PS in the membrane could take place before the acidification inside the endosome.

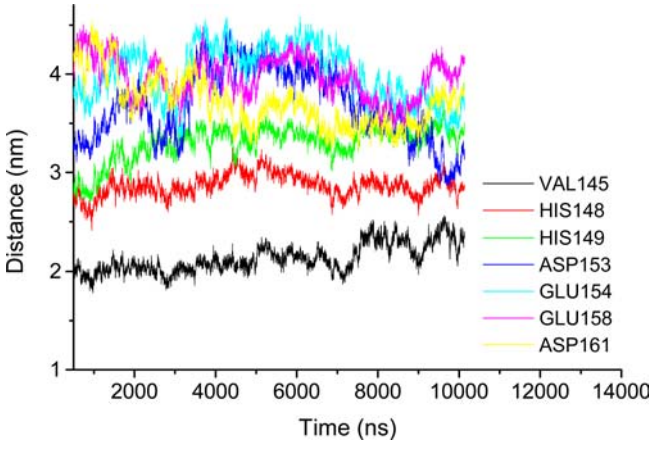

Fig. 6 Distance between titratable aminoacids and the bilayer center during the 10 ns simulation. It can be seen that Val^{145} and His^{148} display stable center of mass distances to the DMPS bilayer. The negatively charged aminoacids, on the contrary, display unstable positions in relation to the membrane as well as being more distant to the bilayer center of mass

The identification of G protein amino acid residues directly involved in VSV binding to membranes is an important point in the understanding of VSV–membrane interaction. Photolabeling studies of VSV G protein showed that its interaction with membranes strongly increases when the pH is lowered from 7.0 to 6.0 (Durrer et al. 1995). At the pH of fusion, the labeled site was located in the ectodomain comprising the amino acids 59 to 221. Based on several mutagenesis studies, the sequence between the residues 117 and 136 has been proposed as the putative fusion peptide of VSV G protein (Li et al. 1993; Zhang and Ghosh 1994; Fredericksen and Whitt 1995). However, direct evidence that this particular region interacts with the target membrane is still lacking and further investigation will be necessary to provide unambiguous evidence whether the segment between amino acids 117 and 136 of the G protein directly participates in VSV fusion or whether the substitution of its conserved amino acids affects the conformation or the exposure of other membrane-

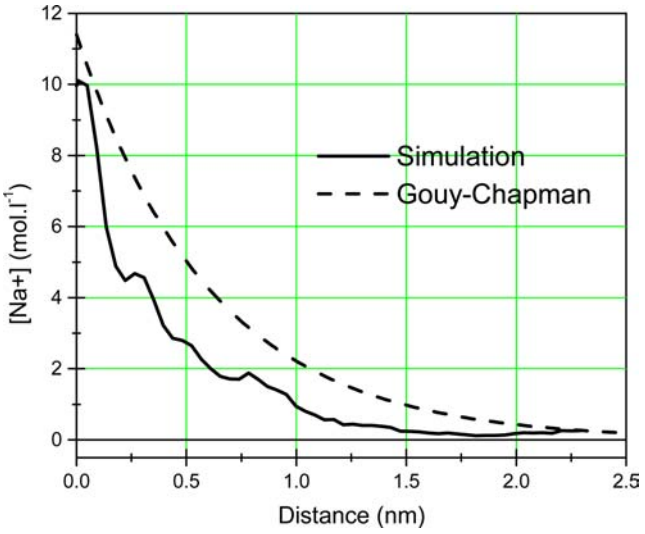

Fig. 7 Comparison of Gouy–Chapman theory and MD simulation. We divided the simulation box in slices normal to the membrane and measured the counterion Na+ concentration (*line*). Applying the Gouy–Chapman formalism we calculated the expected theoretical value (*dashed line*).

interacting sequence in the G protein. Another region of the G protein, encompassing residues 395–418 for VSV has been identified as a segment that affects the fusogenic activity of the protein by influencing the low-pH-induced conformational changes (Shokralla et al. 1998). In addition, it has also been shown that not only the ectodomain segment but also the membrane anchoring domain is required for VSV fusion activity (Odell et al. 1997; Cleverly and Lenard 1998).

Recently, we identified a specific sequence in the VSV G protein directly involved in membrane interaction and fusion (Carneiro et al. 2003). This segment has been previously characterized as the PS-binding site in the VSV G protein together with similar regions of G proteins from other rhabdoviruses (Coll et al. 1997). We showed that this segment, which corresponds to the sequence between amino acids 145 to 164 of the VSV G protein, is very efficient in catalyzing membrane fusion (Carneiro et al. 2003). Here we used the peptide 145–164 to explore G protein–membrane interaction. The study of the interaction between membrane and synthetic peptides corresponding to the fusogenic domain of the fusion protein is sometimes an important strategy to adopt. Although the results obtained with the peptides have to be considered cautiously since many features of the complex viral full length protein are not present in the isolated peptide–vesicle model system, analysis of the molecular mechanisms underlying fusion peptide activity in the whole protein would be not viable in most cases. As revised by Nieva and Agirre (2003), several findings support the view that synthetic peptides are useful models to study viral cell fusion. In our case, we were encouraged to use this peptide by the similarity between peptide- and whole virus-induced fusion. Besides showing the same kinetics of whole virus fusion, the peptide-induced fusion was dependent on pH and on the presence of PS in the target membrane.

Here we have shown that the peptide [145–164] binding to PS-containing vesicles is exothermic, suggesting the electrostatic nature of the binding. Therefore, taking into account the requirement of the negatively charged PS in the vesicles for VSV binding, our results suggest that the binding process may be mediated by a direct interaction between the positively charged His and the negative charges of PS in the membranes. Indeed, calorimetric experiments using the mutant peptide or the wild-type peptide modified with DEPC revealed that PS–peptide interaction occurs through the His residues. This proposition is further substantiated by the results of MD simulations, which show that His^{148} interacts strongly with the DMPS bilayer. The positive charge of His residues at pH 7.5 could be explained by the proximity of the peptide to the negativelly charged headgroups of the lipids, which can lower the pH at membrane surface, as suggested by the Gouy–Chapman theory. Despite the simplicity of the Gouy–Chapman theory and its mean-field nature, its use seems justifiable because the charge density σ_{el} of 0.2 C m^{-2} is in accordance with reported values by Cevc (1990) and with our own MD simulations. In fact, integrating the average charge density per 1 Å -long slices of the system along the bilayer normal in the last 2 ns of simulation, we obtained a value of 0.3 C m^{-2} concentrated in the phosphate region of the DMPS lipid (data not shown), in accordance with a mean charge of 1 *e* per 55 $Å^2$ for a pure PS bilayer. In addition, to further check the validity of the Gouy-Chapman model, we applied it to the calculation of Na^+ counter ion concentration near a surface of 0.3 C m^{-2} charge density and compared it to the MD simulation. As can be seen in Fig. 7, the agreement is reasonable.

Taking together, our results suggest that during VSV entry into the host cell binding to the receptor at cell surface could be essential for bringing the active His of G protein fusion peptide to the proximity of membrane surface, allowing its protonation and the interaction of the fusion protein with the target membrane.

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