Triatoma virus recombinant VP4 protein induces membrane permeability through dynamic pores

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ABSTRACT

In naked viruses, membrane breaching is a key step that must be performed for genome transfer into the target cells. Despite its importance, the mechanisms behind this process remain poorly understood. The small protein VP4, codified in the genome of most Picornavirales order viruses, has been shown to be involved in membrane alterations. Here we have analyzed the permeabilization activity of the natively non-myristoylated VP4 protein from Triatoma virus (TrV), a virus belonging to the Dicistroviridae family within the Picornavirales order. The VP4 protein was produced as a C-terminal Maltose Binding Protein (MBP) fusion to achieve its successful expression. This recombinant VP4 protein is able to produce membrane permeabilization in model membranes in a membrane composition-dependent manner. The induced permeability was also influenced by the pH, being greater at higher pH values. We demonstrate that the permeabilization activity elicited by the protein occurs through discrete pores that are inserted on the membrane. Sizing experiments using fluorescent dextrans, cryo-electron microscopy imaging and other additional techniques showed that recombinant VP4 forms heterogeneous proteo-lipidic pores rather than common proteinaceous channels. These results suggest that VP4 protein may be involved in the membrane alterations required for genome transfer or cell entry steps during dicistrovirus infection.

IMPORTANCE

During viral infection, viruses need to overcome the membrane barrier in order to enter the cell and replicate their genome. In non-enveloped viruses membrane fusion is not possible and hence, other mechanisms are
implemented. Among other proteins, like the capsid forming proteins and the proteins required for viral replication, several Picornaviral order viruses contain a small protein called VP4 that has been shown to be involved in membrane alterations. Here we show that Triatoma virus VP4 protein is able to produce membrane permeabilization in model membranes by the formation of heterogeneous dynamic pores. These pores formed by VP4 may be involved in the genome transfer or cell entry steps during viral infection.
INTRODUCTION

The positive single-stranded RNA (+ssRNA) virus *Triatoma virus* (TrV) belongs to the *Dicistroviridae* family in the *Picornavirales* order. It is a lethal pathogen of the bloodsucking insect *Triatoma infestans* and of other insect species belonging to the *Triatominae* subfamily (1, 2). These insects are the main vectors for the transmission of the protozoon *Trypanosoma cruzi*, the causative agent of the Chagas disease (American Trypanosomiasis), a neglected tropical endemic disease affecting Latin America (3). Thus TrV has been proposed as a biological control agent against the spread of this pathology (4–6).

Dicistroviruses were initially referred as picorna-like viruses due to their similarities with the *Picornaviridae* family: the non-enveloped icosahedral capsid structure, the +ssRNA genome and the capsid protein composition. However, dicistroviruses are characterized by the possession of two different and separated open reading frames (ORFs), which was the main reason for the establishment of this new family (7). The first ORF codifies for the non-structural proteins (helicase, protease and RNA-dependent RNA polymerase). On the other hand, the second ORF codifies for the four structural proteins which build the capsid in the order N-terminus-VP2-VP4-VP3-VP1-C-terminus as a unique protein precursor called P1. Once the P1 precursor has been cleaved into VP0 (VP2+VP4), VP3 and VP1 by the encoded viral protease, and after particle assembly, VP0 undergoes autoproteolytic cleavage into its components by an unknown mechanism that takes place only in RNA-encapsulating TrV particles (8, 9).
Contrary to rhinoviruses and enteroviruses from the related *Picornaviridae* family, in which the structural proteins are ordered as N-terminus-VP4-VP2-VP3-VP1-non-structural protein-C-terminus, the order in dicistroviruses, in which VP4 N-terminus is bound to VP2, prevents the attachment of the highly hydrophobic saturated fatty acid myristoyl to the α amino group of the N-terminus of VP4. Furthermore the N-myristoylation process occurs at the N-terminal glycine residue and this reaction is unlikely in *TrV* VP4 because its first residue is an alanine. This observation was experimentally verified by mass spectrometry (8) and illustrates a significant difference between the picornavirus and dicistrovirus VP4 proteins.

The knowledge regarding VP4 in dicistroviruses is still very poor and therefore its characteristics and functionality have been assumed to be similar to picornaviruses VP4s, which are the most closely related studied proteins. However, the structural studies carried out with *TrV* show significant differences between the dicistrovirus and picornavirus VP4 proteins. In addition to not being myristoylated, the solution of the crystallographic structure of *TrV* uncovered another intriguing difference. *TrV* capsid structure was solved at high resolution (10) and it was shown to be composed of 60 copies of the three major structural proteins (VP1, VP2 and VP3), all folded in a jelly-roll core. Surprisingly the small protein VP4 (5.5 kDa) was not observed, either in the X-ray crystallographic structure or in cryo-electron microscopy (cryo-EM) three-dimensional reconstructions (10, 11), despite it is known to be present in infectious *TrV* particles (8). These data showed that *TrV* VP4 protein is disordered within the capsid, contrary to picornaviruses.
VP4 that is largely ordered inside the particle matching the icosahedral capsid
symmetry (12, 13).

In picornaviruses, the small hydrophobic protein VP4 (5-7 kDa) was
proposed to be involved in the membrane alterations required for genome
transfer to the cytoplasm of cells during infection (14, 15). Moreover, a recent
study (16) has shown that human rhinovirus VP4 interacts with model
membranes inducing permeability by the formation of multimeric
proteinaceous pores. The permeabilization activity was enhanced by the
myristoylation of VP4 and was higher at acidic pH values in concordance with
the genome delivery mechanisms in acidic endosomes proposed for some
picornaviruses like poliovirus and rhinoviruses (17–21).

Oppositely, in TrV the low endosomal pH was excluded as a genome
release trigger because it has been shown not to be acid-labile (22).
Moreover, emptied-capsids (after genome delivery) do not display any
conformational changes similar to those in picornaviruses (11). Therefore, a
different mechanism of uncoating was proposed by characterizing the capsid
disassembly and genome release using atomic force microscopy (AFM) and
native-mass spectrometry techniques (22). The increase of the pH to 8 - 8.5
destabilizes the genome-capsid interactions ultimately resulting in a capsid
unable to resist the outwards forces of the genome that trigger the
disassembly of the capsid into pentons and the release of the genome along
with VP4. Despite this alkaline mediated disassembly seems unlikely to be the
natural trigger for uncoating, this model was supported by the fact that some
areas of the intestinal tract of the insect have pH values of 8.9 (21).
Considering all of these differences with previously studied VP4 proteins and with the aim to address the function of the *Triatoma virus* VP4 protein, in this study we have cloned, expressed and purified VP4 as a Maltose Binding Protein (MBP) fusion to characterize its ability to promote membrane permeabilization. Here, we show for the first time that VP4 induce membrane permeability in a pH- and membrane composition-dependent manner through the formation of structurally heterogeneous proteo-lipidic pores.
MATERIALS AND METHODS

Materials. The fluorescent probes 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and p-xylene-bis-pyridinium bromide (DPX) were purchased from Molecular Probes while fluoroisothiocyanate-dextran 4 (FD4) and fluoroisothiocyanate-dextran 40 (FD40) were from Sigma Aldrich. Sodium hydrosulfite (dithionite) was purchased from Merck. And all the lipids (L-α-phosphatidic acid (PA), L-α-phosphatidylcholine (PC), L-α-phosphatidylserine (PS), L-α-phosphatidylinositol (PI), L-α-lysophosphatidylcholine (Lyso-PC), sphingomyelin (SM), 1-palmitoyl-2-oleoyl-sn-glycerol (DAG), L-α-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (PE-Rh) and L-α-phosphatidylethanolamine-N-(4-nitrobenzo-2-oxa-1,3-diazole) (PE-NBD)) were purchased from Avanti Polar Lipids.

Sequence alignment and transmembrane regions prediction. A sequence comparative analysis was performed by aligning the amino-acid sequences of the following viruses: TrV, Cricket paralysis virus (CrPV), Drosophila C virus (DCV), Poliovirus (Polio) and Human rhinovirus 16 (HRV16). The alignments were performed using the software package T-Coffee (23). The presence of possible transmembrane helices in TrV VP4 protein was predicted using the hydrophobicity-based online available TopPred software (24, 25).

Cloning and expression of VP4. The cDNA fragment corresponding to VP4 protein of TrV was obtained by RT-PCR using the TrV extracted RNA and the following primers: sense 5'-GGAATTCCATATGGCAGGTAAAGAACAGTTAGGTCC-3' containing an Ndel restriction site...
Cloning and purification of recombinant MBP-VP4. The cDNA fragment corresponding to VP4 protein of TrV was obtained as described above. The resulting PCR product was cloned after the Tobacco Etch Virus protease site (TEV) into pET28-HMT plasmid (a modified pET28a plasmid that encodes for a His-tagged Maltose Binding Protein with a C-terminal TEV site). After confirming the correctness of the cloned sequence by DNA-sequencing, pET28-HMT-VP4 recombinant plasmid was transformed in *E.Coli* BL21 (DE3) strain. A 50 mL overnight growth culture was used to inoculate 1 L of LB medium supplemented with 50 μM kanamycin. When this culture, incubated at 37 °C, reached a value of $A_{600\text{nm}}$ of 0.6-0.8, protein expression was induced by adding 0.5 mM IPTG for 3 h at 37 °C. Cells were harvested by centrifugation at 5000 g for 15 min at 4 °C.
Cell pellet was resuspended in Binding Buffer (50 mM Tris pH 7.5, 0.5 M NaCl, 30 mM imidazole) supplemented with Complete Protease Inhibitor Cocktail (Roche) and disrupted by a high-pressure homogenizer at 4 °C. The lysate was incubated for 20 min with 25 U/mL Benzonase (Invitrogen) and cell debris and protein precipitates were removed by centrifugation at 30,000 g for 30 min at 4 °C. The supernatant was applied to a His-Trap (GE) affinity column and MBP-VP4 was eluted by a 30-500 mM linear gradient of imidazole. Fractions containing the protein were checked by SDS-PAGE and Western Blot and pure protein fractions were dialyzed overnight against the Assay Buffer (50 mM Tris pH 8.0, 200 mM NaCl).

Preparation of liposomes. Large Unilamellar Vesicles (LUVs) were prepared by the extrusion method (26) with some modifications. Briefly, lipids dissolved in a chloroform:methanol 2:1 (v/v) mixture were dried using a nitrogen stream, and the residual solvent was eliminated under vacuum. Lipid dry film was hydrated by adding the assay buffer followed by 10 freeze-thaw cycles at 50 °C. Multilamellar Vesicles (MLVs) obtained were turned onto LUVs of 400 nm diameter by extruding the suspension through polycarbonate filters of the respective size. The size was confirmed by dynamic light scattering using a Zetasizer (Malvern) instrument. Lipid concentration in the liposome solution was calculated by determination of the phosphorus concentration (27).

Membrane binding assay. LUVs made of phosphatidic acid (PA) and 0.5% (mol/mol) PE-Rhodamine in 50 mM Tris pH 8.0, 200 mM NaCl, as described above, were incubated with either MBP-VP4 or with MBP in the same buffer.
at a molar lipid:protein ratio of 500:1 (lipid concentration 1.5 mM and protein concentration 3 µM) for 30 min at 25 ºC. To assess the protein-membrane binding, a liposome flotation assay was performed as previously described (28). Briefly, the protein-liposomes mixture was adjusted to a sucrose concentration of 1.4 M and loaded on the bottom of a sucrose gradient containing two more fractions (0.8 and 0.5 M sucrose from bottom to top, respectively). Gradients were centrifuged at 400,000 g for 3 h at 4 ºC and then were fractionated in four different fractions. The top first fraction corresponds to the liposomes-containing fraction, whereas the bottom fraction contains non-bonded protein. Protein location in the gradient was analyzed by SDS-PAGE.

**Leakage assays.** LUVs were prepared at 100 µM as described above but in the presence of 25 mM 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and 90 mM of its quencher p-xylene-bis-pyridinium bromide (DPX) in the assay buffer. Non-encapsulated dyes were removed by passing the sample through a Sephadex G-25 PD-10 column (GE).

Leakage of vesicle contents was measured in 50 mM Tris pH 8.0, 200 mM NaCl by a modified ANTS/DPX assay (29). In this case, ANTS fluorescence was recorded continuously (excitation at 355 nm, emission at 520 nm) after MBP-VP4 addition in a Fluoromax spectrometer at 25 ºC with continuous stirring. When fluorescence intensity reached equilibrium, Triton X-100 was added to induce 100% release. The absence of leakage (0%) was assigned to the fluorescence of the vesicles before protein addition and the maximum leakage (100%) corresponds to the fluorescence obtained after
vesicle lysis by 0.5% (v/v) Triton X-100. The leakage was calculated using the equation 1:

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

(Eq. 1)

where \( F \) is the fluorescence after protein addition, \( F_0 \) is the fluorescence of the intact vesicles and \( F_{100} \) is the fluorescence after addition of Triton X-100.

For the pore-sizing experiments, leakage assays were complementary performed using two probes of different sizes: fluoroisothiocyanate-dextran 4 (FD4) and fluoroisothiocyanate-dextran 40 (FD40) (30). Both LUVs suspensions were prepared as above in the presence of 100 mg/mL of each dye and fluorescence emission was measured at 530 nm upon excitation at 465 nm.

**Entry assays.** Entry of solutes into vesicles was measured by selective reduction of the NBD-labeled lipids in the inner leaflet of the vesicle membranes by an extravesicular solute as previously described (31). 100 µM LUVs containing 0.6% (mol/mol) NBD-PE in 50 mM Tris pH 8.0, 200 mM NaCl were incubated with the protein in the same buffer for 2 h at 25 °C. Freshly prepared 0.6 M sodium hydrosulfite (dithionite) in 1 M Tris pH 10 was then added to the mixture to reach a dithionite final concentration of 12 mM. The reduction of the NBD group by dithionite can be followed as a fluorescence emission decrease (excitation at 460 nm, emission at 540 nm). The degree of permeabilization was calculated from the percentage of inner NBD reduced by the equation 4:

\[
\%\text{Entry} = \left( \frac{F_0-F}{F_0-F_{100}} \right) \times 100
\]  

(Eq. 4)
where $F$ is the measured fluorescence for the sample, $F_0$ is the fluorescence after the addition of dithionite to intact vesicles (outer leaflet NBD reduced) and $F_{100}$ is the fluorescence after addition of 0.5% (v/v) Triton X-100.

**Cryo-Electron Microscopy.** For the cryo-EM 2D image analysis of membrane disruption due to the effect of VP4 protein, vitrified grids of LUVs, incubated with the protein in 50 mM Tris pH 8.0, 200 mM NaCl, were prepared at different protein:lipid ratios. As control, vitrified grids of LUVs without the presence of VP4 protein, were also prepared, following standard procedures. 3 µl of sample were applied to glow-discharged 200-mesh Quantifoil holey EM grids and vitrified in liquid ethane, cooled with liquid nitrogen, using a Vitrobot (FEI).

Different vitrified grids were observed on a JEM-2200FS/CR (JEOL, Ltd.) field emission gun (FEG) transmission electron microscope, operated at 200 kV at liquid nitrogen temperature. An in-column omega energy filter helped to record images with improved signal to noise ratio by zero-loss filtering, with the energy slit width set at 15 eV. Zero tilted two-dimensional digital micrographs were recorded on a 4K x 4K Ultrascan4000T CCD camera (Gatan Inc.), under low-dose conditions (on the order of 10-20 electrons/Å² per exposure) with an underfocus range from 1.5 to 4.0 µm, at nominal magnifications from 40000X to 80000X, producing a final pixel size from 2.6 Å/pixel to 1.2 Å/pixel. Digital images were collected using DigitalMicrograph (Gatan Inc.) software and the 2D image analysis was performed with ImageJ software.
Statistics of membrane discontinuities were performed by counting ~60 different unilamellar liposomes at each protein concentration.

Hemolysis Assay. The hemolysis assay was conducted as previously described (32). Briefly, different protein solutions (MBP-VP4, MBP or hemolytic control Adenylate cyclase) were mixed with a human erythrocyte cell suspension in 50 mM Tris pH 8.0, 200 mM NaCl (prepared in such a way that a 80-fold dilution gave an absorbance of 0.6 at 412 nm). Cells and protein mixtures were incubated at room temperature for 3 h and centrifuged at 4000 g for 10 min at 4 °C. The absorbance of the supernatants was read at 412 nm. The blank (zero hemolysis) consisted of a mixture of protein buffer and erythrocytes.
RESULTS AND DISCUSSION

Sequence analysis of VP4. As described in the Introduction, VP4 proteins from Picornavirales order viruses are small hydrophobic peptides coded by the structural gene. Specifically, TrV VP4 is a small 5.5 kDa peptide composed of 57 amino acids. Hydrophobicity-based analysis (Fig. 1A) predicts that this protein may contain two transmembrane regions of 20 residues structured as helices separated by a loop formed by 12 amino acids. These transmembrane helices could be involved in the membrane-related activity of VP4 (described in the following subsections).

Sequence alignment of TrV VP4 protein against dicistroviral and picornaviral proteins is shown in Fig. 1B and Fig. 1C respectively. As expected, similarity between VP4 protein from TrV and dicistroviruses is higher (T-Coffee scores: 837 for TrV-CrPV, 799 for TrV-DCV) than similarity between TrV VP4 and that from picornaviruses (544 for TrV-HRV16, 759 for TrV-Polio). Moreover, the few conserved amino acids between TrV and picornaviruses seem not to be significant, since they are mostly not conserved in dicistroviruses. The high similarity between dicistroviruses VP4, in addition to being related to their function, may also be related to a common mechanism of action. By the contrary, the diminished similarity when comparing TrV VP4 to that of picornaviruses, especially to that of rhinovirus, may imply a different functionality.

VP4 can be successfully expressed as an MBP fusion. As described in the Introduction, VP4 proteins from Picornavirales order viruses are small hydrophobic peptides coded by the structural gene. To characterize TrV VP4
protein, it must be expressed as a soluble and stable protein. This effort was initiated by direct cloning of VP4 into different plasmids and expressing it in *E. Coli* Rosetta 2 (DE3) plyS strain. These attempts resulted in an interesting phenomenon: 20 min after the induction of VP4 expression, the cloudy culture turned into a clear solution, suggesting that a cell death process was occurring.

In order to monitor cell death, culture turbidity (absorbance at 600 nm) was measured as a function of time (Fig. 2). As observed in this figure, both the lack of induction and the induction of other viral control protein (VP1) allowed a normal growth of the culture. However, when the induced protein was VP4, culture turbidity dropped approximately an 80%. This drop in turbidity can be interpreted not only as a cell death process, but also as a drastic reduction of the number of structured microorganisms. The presence of VP4 was checked by SDS-PAGE and by anti-histidine Western Blot but these attempts failed. The low amount of synthesized protein prior to cell death and the internal cell contents leakage to the external media may explain the absence of detectable amounts of protein. The presence of VP4 in the external media was also checked through ammonium sulfate precipitation but this attempt did not yield results. The lack of significant amounts of protein prevented further biochemical and biophysical analyses of VP4 protein.

In order to solve this problem, different expression approaches were assayed. From these approaches VP4 expression as a MBP fusion protein was selected due to the high yield of soluble protein and because this approach has been used in other cases showing no interferences with membrane-related assays (33, 34). The MBP-VP4 (His-tag, MBP, TEV
protease site and VP4 from N- to C-terminus) expressed and purified protein was used to test the viability of VP4 cleavage after TEV protease digestion in a variety of different conditions (data not shown). However, VP4 yield and solubility after cleavage were drastically reduced, preventing the use of this strategy. Thus, the fusion protein MBP-VP4 was selected to characterize the membrane binding and lytic activity of VP4. For this reason, all experiments were accompanied with MBP (His-tag, MBP and TEV protease site) controls in the same conditions.

**VP4 interacts with lipid membranes.** To assess the interaction of VP4 with membranes, pre-incubated PA:PC liposomes and MBP-VP4 (or MBP) were loaded in a sucrose flotation gradient as described in Materials and Methods section. The fractions of the gradient were analyzed by SDS-PAGE for the presence of the protein and by rhodamine fluorescence for the presence of liposomes. As shown in Fig. 3, MBP-VP4 is found in fraction 1 (F1) comigrating with the liposomes. In contrast, MBP control is found mainly in fraction 4 (F4), the bottom fraction, thus showing no such lipid association. Therefore, we can conclude that the interaction of MBP-VP4 with model membranes is driven by VP4.

**MBP-VP4 induces the release of liposome contents in a lipid-dependent manner.** Having proved the interaction between liposomes and MBP-VP4, we examined whether this interaction was able to lead liposome permeabilization. Vesicles encapsulating ANTS and DPX can be used to measure permeability because upon leakage, ANTS fluorescence is not longer quenched by DPX.
due to the high dilution of both molecules (29). Therefore, if permeabilization occurs, fluorescence emission should increase. In Fig. 4A leakage curves of 100 μM PA:PC (1:1 molar) liposomes after addition of different amounts of MBP-VP4 are plotted. These curves show that the protein is able to permeabilize vesicles. To further characterize the leakage activity, titration curves (Fig. 4B), where saturated leakage values (obtained as in the Fig. 4A) are plotted as a function of protein concentration, were represented. These curves show that leakage is protein concentration dependent and that it reaches a plateau (approximately 100 % leakage) at high MBP-VP4 concentrations (2 μM). It can also be seen that leakage remains significant even at very low protein doses (e.g., MBP-VP4 at 10 nM with a protein:lipid ratio of 1:10000 induces a leakage of 24%). This result indicates that vesicle lysis is not a simple effect of membrane destabilization due to huge amounts of membrane-adsorbed protein (like happens with the control MBP), but it is mediated by a specific mechanism.

Control experiments using MBP showed that there was not significant leakage for protein concentration bellow 200 nM. Therefore, unless otherwise indicated, 100 nM MBP-VP4 concentration was selected to perform all experiments to avoid MBP interference.

As shown above, MBP-VP4 elicits a huge permeabilization activity compared to rhinovirus VP4 (16) even though in our case VP4 is not myristoylated. This modification, which consists of the addition of a 14-carbon saturated fatty acid, is found in a variety of proteins and enables them to anchor to membranes (35, 36). Although myristoylation is not needed by a protein to induce membrane permeability, as shown by a huge variety of
proteins, e.g. the antimicrobial peptides (37, 38) and bacteriophage holins (39, 40), the permeabilization activity of our natively non-myristoylated VP4 allows us to propose that the membrane insertion mechanism may be different from the myristoyl-driven mechanism of picornaviruses. Moreover the low sequence identity of TrV VP4 and that from rhinovirus (Fig.1C) emphasizes the possible existence of a different protein-membrane interaction mode.

To determine if the permeabilization activity is lipid-dependent, similar leakage assays were performed using different lipid compositions. As shown in Table 1, MBP-VP4 requires anionic lipids to elicit its leakage activity. Liposomes composed of neutral lipids, such as PC and SM, were not efficiently permeabilized. In contrast, internal contents of vesicles made of anionic lipids (PI and PS) were spilled to a greater extent by the action of the protein. Among them, PI was the phospholipid that induced the highest leakage. PA liposomes were not used because they were refractory to ANTS/DPX encapsulation. This specificity for anionic lipids may explain the incapability shown by MBP-VP4 to lyse erythrocyte membranes in hemolysis assays (data not shown). The reason of this behavior is because the membrane outer leaflet of these cells is composed of 44.8% of PC and 42.1% of SM (41), both neutral lipids.

MBP-VP4 permeabilization activity is pH-dependent. The activity of VP4 may be linked to its externalization and as the pH has been shown as a key factor in VP4 externalization and genome release in both picornaviruses and dicistroviruses, here we investigate the influence of the pH on the function of MBP-VP4. PA:PC (1:1 molar) liposomes encapsulating ANTS/DPX were
prepared in different buffers, containing the same ionic strength but at different pH values: 5.5, 7.5 and 8.0. The possible influence of the pH on the fluorescence properties of ANTS was discarded because all the samples were normalized by liposome solubilization with Triton X-100. As shown in Table 2, the leakage due to MBP-VP4 is significantly more efficient at high pH.

This behavior, that is opposite of that observed in picornaviruses (16), is consistent with the disassembly and genome release model proposed for TrV (22). In that model, the alkalization of the solution to approximately pH 8 destabilizes the genome-capsid interactions triggering the disassembly of the capsid into pentons accompanied by the release of the genome along with VP4. Although alkaline conditions are not typical during viral infections, this model was supported by the fact that the intestinal tract of the insect (where TrV infects) can reach pH values of 8.9 (42). The high pH conditions that favor TrV VP4 permeabilization activity are consistent with the above described model, suggesting that its activity may be optimized for the alkaline conditions encountered during infection.

**Liposome contents are leaked via a true pore.** Membrane breaching is a key step in the transfer of the genome of naked viruses. This can be accomplished using different mechanisms, either by the formation of discrete pores or by direct membrane disruption (43). To characterize the mechanism involved in MBP-VP4 vesicle contents leakage, previously shown leakage assays were combined with solute entry experiments. These assays were performed using PA:PC (1:1 molar) liposomes containing 0.6% (mol/mol) PE-NBD. Two hours after the addition of the protein, dithionite was added and the
entry of contents was calculated from the percentage of internal NBD reduced by this externally added salt (see Materials and Methods section). Since dithionite is membrane impermeable, it can only reduce internal fluorophores if discrete pores allow dithionite to enter through the bilayer. Therefore, this assay allows discriminating if the permeabilization phenomenon occurs due to discrete pores or due to membrane integrity disruption (33, 37). As both sides of the membrane are equally labeled with PE-NBD, when dithionite is added the external NBD (approximately 55%) is reduced. This value was assigned to the 0% of solute entry. As shown in Fig. 5A, after reaching equilibrium, liposomes allowed solutes to enter. Noteworthy, the extent of solutes leakage and entry was almost equal. These results suggest that the diffusion of solutes across the membrane occurs through discrete pores.

MBP-VP4 pores are of variable size and structurally heterogeneous.

Previous results allowed us to propose a permeabilization mechanism through a discrete pore, but the results did not distinguish between fixed protein pores such as ion channels, described by the barrel-stave model (44), and dynamic proteo-lipidic pores, described by the toroidal model (45). To further characterize the nature of these pores, sizing experiments were conducted. Although initially these assays were performed to calculate the size cut-off of the solutes diffusing through the MBP-VP4 pore, finally the data obtained (see below) were used to distinguish whether MBP-VP4 forms stable protein pores or dynamic proteo-lipidic pores. To perform sizing experiments, leakage from vesicles encapsulating ANTS/DPX (Stokes radius 0.5 nm), FD4 (Stokes
radius 1.8 nm) and FD40 (Stokes radius 3.0 nm) was measured at different protein concentrations.

As shown in Fig. 5B, at the lowest protein concentration, ANTS/DPX and FD4 were released, while FD40 remained inside the vesicles. Moreover, ANTS/DPX leakage was a little higher than that from FD4. As the protein concentration increased, the leakage of the smaller solutes increased while the release of the largest compound (FD40) began. Therefore, it can be concluded that the leakage of the different sized solutes depends on the protein:lipid ratio. On the other hand, the mechanism of LUVs disruption by MBP-VP4 was analyzed by cryo-EM imaging. The pores induced by MBP-VP4 were counted, their size was estimated from the micrographs and they were clustered in three different groups (Fig 6A): 6-12 nm (Fig. 6B), 13-20 nm (Fig. 6C) and larger than 20 nm (Fig. 6D) (pores smaller than 6 nm are very difficult to observe due to technical limitations). This two-dimensional image analysis shows the structural heterogeneity of the pores formed by MBP-VP4. The diameter of the pores was not uniform and increases as protein concentration increases. Moreover these pores seemed to be membrane discontinuities. Structure-fixed proteinaceous pores are expected to be of invariable size, structurally homogeneous and not affected by the protein concentration. In this type of pores, all compounds that fit the pore size are released to the same extent at each protein concentration, and the solutes larger than the pore size are never released regardless of the protein:lipid ratio. However, in our case, the pores are heterogeneous and their size seems likely to increase as a function of the protein concentration, arguing against a proteinaceous pore model and being consistent with the dynamic
proteo-lipidic pores. This pore model has already been proposed for a variety of different peptides and proteins (45–50).

In the proteo-lipidic pore model or toroidal pore model the proteins affect the local curvature of the membrane bending back the bilayer such a way that a proteo-lipidic toroid is formed in the membrane. This membrane deformation depends on the membrane intrinsic curvature that is in turn lipid composition dependent (51, 52). Therefore, to provide further evidence for the proteo-lipidic nature of the pores formed by MBP-VP4, the leakage activity of the protein was measured in the presence of lipids known to change the intrinsic curvature of the membrane. This approach has been previously used to test the validity of the proteo-lipidic model (51).

Lyso-PC, a positive intrinsic curvature inducer, was introduced in PA:PC (1:1 mol) liposomes at different molar percentages by exchanging with the PC. As shown in Fig. 7, the incorporation of Lyso-PC to the liposomes induced a linear increase of the leakage activity of MBP-VP4. Otherwise, the incorporation of DAG, an inducer of intrinsic negative curvature, induced a linear decrease of its activity. The membrane curvature on Lyso-PC or DAG containing vesicles is linearly dependent on the concentration of these lipids (51, 52), which is consistent with the observed linear correlation between MBP-VP4 activity and Lyso-PC/DAG contents (Fig. 7). Therefore these results show that the leakage activity of MBP-VP4 protein is affected by membrane intrinsic curvature changes induced by Lyso-PC and DAG arguing in favor of the proteo-lipidic pore model.

The likely toroidal pores formed by TrV recombinant VP4 protein emphasize the differences observed with the picornaviral VP4 proteins in their
sequence, myristoylation, membrane permeation pH dependence, etc. The only fully characterized picornaviral VP4 protein is the one from *Human rhinovirus 16* (16). This protein elicits its permeabilization activity through the formation of multimeric pores that can be interpreted with the barrel-stave model (44), in opposition to the toroidal model (45) used to explain our case.
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RS-E was supported by a predoctoral fellowship from the Basque Government (BG). JG was recipient of a student summer grant from the Fundación Biofísica Bizkaia (FBB), Spain. LS-M holds a JAE-Doc fellowship from CSIC, Spain. This work was partially supported by grants from the BG (MV-2012-2-41; AE-2012-1-44, S-PE12FB001), UPV/EHU (IT849-13, OTRI code 2013.0666) and by MICINN (BFU2012-36241), Spain.

We thank Ganeko Bernardo for his interest and for the provision of the pET28-HMT plasmid as well as Dr. Cesar Martin and Dr. Helena Ostolaza who provided the red blood cells for the hemolysis assay. We are also grateful to Dr. Aritz Durana for his interest, technical support and critical comments and to Professor Félix M. Goñi who read and provided critical comments regarding the manuscript.
REFERENCES


41. Virtanen J a, Cheng KH, Somerharju P. 1998. Phospholipid composition of the mammalian red cell membrane can be rationalized


TABLE 1

Effect of lipid composition on MBP-VP4-induced membrane permeability.

<table>
<thead>
<tr>
<th>LUV composition</th>
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<th>% Leakage&lt;sup&gt;b&lt;/sup&gt; MBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA:PC</td>
<td>-:+/-</td>
<td>51</td>
<td>6</td>
</tr>
<tr>
<td>PC</td>
<td>+/-</td>
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<tr>
<td>PI</td>
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</tr>
</tbody>
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<sup>a</sup> stands for anionic groups and +/- stands for zwitteronic groups.

<sup>b</sup> Leakage activity calculated as described in Material and Methods for a protein concentration of 100 nM and a lipid concentration of 100 μM. Mean values of three measurements are indicated.

TABLE 2

Effect of the pH on MBP-VP4 leakage in PA:PC (1:1) liposomes.

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FIGURE LEGENDS

Figure 1. VP4 sequence analysis. A) Transmembrane helix prediction performed using TopPred software as described in Material and Methods. Two helices are predicted, the first one from residue 5 to 25 and the second one from residue 37 to 57. B) Sequence alignment of VP4 protein from TrV and other dicistroviruses: Cricket paralysis Virus (CrPV) and Drosophila C virus (DCV). C) Sequence alignment of VP4 protein from TrV and picornaviruses Human rhinovirus 16 (HRV16) and Poliovirus (Polio). B), C), Conserved residues are in white font on a black background and similar residues are framed.

Figure 2. Protein expression influence on culture turbidity. The turbidity of the cultures expressing VP4 (squares) and a control protein (circles) was measured after induction by monitoring the absorbance at 600 nm. A bacterial growth control without heterologous protein expression was also measured (empty circles).

Figure 3. Membrane binding assay. Proteins (MBP-VP4 and control MBP) were incubated with liposomes preparations and loaded on the bottom of a sucrose gradient. After ultracentrifugation, gradients were fractionated in four different fractions (F1-F4, from top to bottom) and analyzed by SDS-PAGE. Liposomes banded at fraction 1. Molecular weight markers are indicated in kDa.
Figure 4. Membrane permeabilization induced by MBP-VP4. A) Time-course leakage curves after addition of MBP-VP4 or MBP at the indicated concentrations. The proteins were added at second 0 and after 1000 s liposomes were disrupted by addition of Triton X-100. B) Dose-dependent response of MBP-VP4 leakage activity and control MBP at the same concentrations. Data points correspond to mean values of three independent measurements and error bars represent the standard deviations.

Figure 5. Characterization of the leakage activity of MBP-VP4 and pore type determination. A) Solutes entry experiment. Extent of solutes entry is compared with solutes leakage from 100 μM PA:PC (1:1 molar) liposomes using 25, 50, 100 and 200 nM MBP-VP4. B) Sizing experiment. Release of different sized compounds from 100 μM PA:PC (1:1 molar) liposomes was measured as a function of protein concentration. Figures data points correspond to mean values of three independent measurements and error bars represent the standard deviations.

Figure 6. Cryo-EM imaging. A) Statistics of pore sizes as observed by Cryo-EM. PA:PC (1:1, mol) liposomes at 500 μM were incubated with different protein concentrations. Membrane interruptions observed in digital micrographs, acquired at different nominal magnifications from 40,000X to 80,000X, were clustered in three different groups: 6-12 nm, 13-20 nm and >20 nm. B), C) D), Cryo-EM micrographs of membrane disruption. Interruptions in the membrane, marked with black arrows, correspond to the 6-12 nm, 13-20 nm and >20 nm groups respectively for B, C and D.
Figure 7. Effect induced by different concentrations of Lyso-PC and DAG on the leakage activity of MBP-VP4. The protein:lipid ratio was 1:1000 (molar). Data points correspond to mean values of three independent measurements and error bars represent the standard deviations.
A) 1 10 20 30 40 50 57
AGKEQLGPISGLTNTVTGVGGVADAVKGFFPSIGKYADPLVGIIGNGLTGLLSALGF

5-25 TMH prediction

37-57 TMH prediction

B) TrV-VP4  AGKEQLGPISGLTNTVTGVGGVADAVK--GFFPSIGKYADPLVGIIGNGLTGLLSALGF
CrPV-VP4  AS-DL-QKL-ETNNS-PCTALGCESEGLTTLSHPVGNGIFSTPAMTSAKAADKLKLFGF
DCV-VP4  AS-DL-TQL-KESGTLSEGTHIQVEGLSTMSKTPILGMFTKPAWISAQVSNIKMLGF

C) TrV-VP4  AGKEQLGPISGLTNTVTGVGG--VADAVKGFFPSIGKYADPLVGIIGNGLTGLLSALGF
HRV16-VP4  GAVWSSQRVGHSTQHNSSNMGSSSLSNYFHNQYFDASSASGGASRILDESDPSKFTDPKVPDLVLEKGIPTLD----
Polio-VP4  GAVWSSQRVGAHENSRRAYGDSSTNYTTINYRDSASNNAKQDPSQDPSKTPIKDVLKTIKTAPMLN----
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