The VP4 peptide of Hepatitis A Virus (HAV) Ruptures Membranes
Through Formation of Discrete Pores

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Abstract
Membrane active peptides, components of capsid structural proteins, assist viruses in overcoming the host membrane barrier in the initial stages of infection. Several such peptides have been identified and their roles in membrane fusion or disruption have been characterized through biophysical studies. In several members of the picornaviridae family, the role of the VP4 structural peptide in cellular membrane penetration is well established. However, there is not much information on the membrane penetrating capsid components of Hepatitis A Virus (HAV), an unusual member of this family. The VP4 peptide of HAV differs from its analogues in other picornaviruses by being significantly shorter in length, and also by lacking a signal for myristoylation, thought to be a critical requisite for VP4-mediated membrane penetration. Here we report, for the first time, that the atypical VP4 in HAV contains significant membrane-penetrating activity. Using a combination of biophysical assays and molecular dynamics simulation studies, we show that VP4 integrates into membrane vesicles through its N-terminal region, to finally form discrete pores of 5-9 nm diameter, which induces leakage in the vesicles without altering their overall size or shape. We further demonstrate that the membrane activity of VP4 is specific towards vesicles mimicking the lipid content of late endosomes, at acidic pH. Taken together, our data indicates that VP4 might be essential for the penetration of host endosomal membranes and release of genome during HAV entry. (231 words)

Importance
Hepatitis A Virus (HAV) causes acute hepatitis in humans through the faecal-oral route, and is particularly prevalent in underdeveloped regions with poor hygienic conditions. Although a vaccine for HAV exists, its high cost makes it unsuitable for universal application in developing countries. Studies on host-virus interaction for HAV have been hampered due to lack of starting material, since the virus is extremely slow growing in culture. Among the unknown aspects of the HAV life cycle is its manner of host membrane penetration, which is one of the most important initial steps in viral infection. Here, we...
present data to suggest that a small peptide VP4, a component of the HAV structural polyprotein, might be essential in helping the viral genome cross cell membranes during entry. It is hoped that this work might help in elucidating the manner of initial host cell interaction by HAV. (144 words)

Introduction

Traversing the host membrane barrier is an essential step in the establishment of a viral infection, and viruses typically contain sequestered, hydrophobic or amphipathic components to execute this step. These components, which are elements of viral glycoproteins or capsid proteins, are instrumental either in negotiating fusion of the viral lipid envelopes with host membranes, or in causing disruption of plasma or endosomal membranes (1-3). While membrane fusion proceeds through a mechanistically similar pathway, the process of cellular membrane disruption appears to vary from virus-to-virus, rather than display a unified mechanism. Formation of voltage-gated channels, creation of size-selective pores leading to osmolysis and induction of positive curvature on membranes, have been proposed as possible mechanisms for the latter process (4-6).

Although virus-mediated membrane penetration has hitherto been neatly classified based on the presence or absence of a lipid envelope, the discovery of viruses at the borderline of enveloped and non-enveloped species has complicated our understanding of this seemingly familiar process (7, 8). The first established example of this phenomenon is Hepatitis A Virus (HAV), an unusual member of the picornaviridae family. Recent studies have shown that HAV exists in two forms – a regular, non-enveloped form, and an enveloped form (eHAV), which is predominant in the serum of infected individuals (7). Although both forms are equally infectious, their means of cellular membrane penetration – specifically, whether the two forms of HAV have separate pathways of entry into host cells, or whether a unified pathway exists for this purpose - is unclear. While it is possible that eHAV has evolved its own separate mechanism for membrane fusion-based entry, however, no protein
component analogous to glycoproteins of enveloped viruses has been detected in the eHAV lipid envelope so far. Another plausible entry mechanism involves the conversion of eHAV into its non-enveloped counterpart during the initial encounter with host cells. The shedding of the lipid component will allow the viral capsid proteins to interact with the receptor HAVCR-1 (9, 10), and to disrupt host cell membranes, but there is no experimental support for this pathway of entry either. A study carried out prior to the discovery of eHAV suggest an endosomal route for entry (11), however, the role played by the capsid proteins of HAV in this process has not been addressed.

The HAV capsid, like that of other members of the picornavirus family, is expected to be a pseudo T=3 icosahedral particle, constructed from 60 copies each of four structural proteins VP1, VP2, VP3 and VP4. Whether the small VP4 protein is a component of the HAV capsid is still not clearly known (12). The exact size of VP4 is also ambiguous, since it may be composed of 21 or 23 amino acids based on which one of two alternative start codons are utilized to initiate HAV polyprotein synthesis (12). The absence of a high resolution structure of the HAV capsid has also precluded an accurate understanding of the positioning of capsid proteins in the capsid shell so far (13). A recently determined, unpublished structure of the HAV capsid could be extremely useful in elucidating the structural details of individual capsid proteins in future.

For several well-studied picornaviruses, capsid proteins VP1 and VP4 have been implicated in cellular membrane penetration during virus entry. For poliovirus, membrane disruption during virus entry is negotiated by the N-terminal 31 residues of VP1, and VP4, a myristoylated, hydrophobic peptide, which remains in the interior of the capsid prior to entry (5, 14). The release of poliovirus genome in the host cell cytosol requires concerted membrane interaction by these components - while VP4 forms voltage-gated channels in the plasma membrane (5), thus allowing the RNA genome to pass through, the amphipathic N-terminus of VP1 anchors the virus particle safely onto the membrane during this process, through the formation of long extended networks or channels (15). The VP1 N-termini of other picornaviruses like rhinovirus (HRV2), have a more active role in cellular entry, since they
probably participate directly in cellular membrane penetration, as indicated by their ability to disrupt liposomes in vitro (16, 17). The VP4 peptide, which is often myristoylated at the N-terminus, has also been found to be directly involved in membrane disruption by picornaviruses other than poliovirus (18). Thus, the necessity of VP4 and the N-terminus of VP1 in the entry pathway of picornaviruses has been adequately demonstrated by experimental evidence, whereby mutation in these regions has severely affected virus infectivity.

Since it is conceivable that the mechanism of membrane penetration by the non-enveloped form of HAV is similar to that of other picornaviruses, we attempted to identify amphipathic or hydrophobic regions in VP1 N-terminus or VP4 of HAV, which could potentially be involved in virus entry. Using hydrophobicity plots, two hydrophobic stretches (residues 1-23 and 43-60) were detected at the N-terminus of VP1, while residues 6-23 of the short, 23 amino acid polypeptide VP4 was found to be almost perfectly amphipathic. Interestingly, it is known that unlike the VP4 peptide in most other members of the picornaviruses, the VP4 peptide of HAV is not myristoylated (19). We tested the ability of synthetic peptides corresponding to either un-myristoylated VP4, or the N-terminal regions of VP1, to interact with fluorescent-dye filled liposomes in vitro, and found that VP4 was extremely efficient in causing disruption of membrane vesicles, although no membrane penetrating activity was displayed by the N-terminal region of VP1. We also found that the activity of VP4 is highly specific for vesicles mimicking the lipid composition of late endosomal compartments, at low pH conditions. Using a combination of biochemical methods and molecular dynamics simulation studies, we propose a mechanism for VP4-orchestrated membrane penetration, whereby initial penetration of the N-terminus of VP4 into the outer leaflet of membranes eventually results in induction of small pores in lipid vesicles. Our studies indicate that VP4 might be involved in allowing the viral genome to escape endosomal compartments during entry of the envelope-less form of HAV in host cells.

Materials and methods
**Peptides:** Peptides corresponding to amino acids 1-23 and 43-60 of HAV VP1, 1-23 of HAV VP4, and 364-381 of the capsid protein of an insect virus, Flock House Virus, were obtained from GenPro Biotech. The latter is designated the $\gamma$ 1 peptide.

**Preparation of liposomes:** Liposomes composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids), and encapsulating the fluorescent dye Sulforhodamine B (Sigma- Aldrich), were prepared using the protocol described in (20). Briefly, DOPC was dried under N$_2$ gas, and rehydrated to a final concentration of 2 mM, in 10 mM sodium phosphate buffer (pH 7.0) containing 50 mM SulforhodamineB. After multiple freeze-thaw cycles, the lipid suspension was extruded through a mini-extruder (Avanti Polar Lipids) equipped with a polycarbonate filter containing 100 nm size pores. The liposomes were purified by passing them through a PD-10 column (GE Healthcare), and utilized for assays within 3 hours of preparation. DOPC-liposomes containing 8-Aminonaphthalene -1,2,3-trisulfonic acid (ANTS) and p-Xylenebis(pyridinium) bromide (DPX) were similarly prepared, with the rehydration being carried out in phosphate buffer containing 100 mM ANTS, 200 mM DPX, or a combination of 50 mM ANTS and 100 mM DPX.

Liposomes mimicking cellular membranes/compartment, composed of various molar ratios of Cholesterol, POPC, phosphatidyl ethanolamine, Sphingomyelin, phosphatidyl serine and bis (monoacylglycero) phosphate were also prepared similarly, based on the molar ratios mentioned in (21, Supplementary Table 1). The total lipid concentration in the rehydration buffer was maintained at 10 mM.

Liposomes incorporating dextran molecules were prepared similarly, but with minor modifications. Three different types of fluorescent dye-labeled dextrans were utilized - fluorescein isothiocyanate (FITC)-conjugated, 3000-5000 Da (FD 3-5), FITC-conjugated 10,000 Da (FD 10), and tetramethylrhodamine-conjugated 40,000 Da (FD 40) (Sigma Aldrich). Total concentration of lipid or lipid mixture in rehydration buffer (10 mM sodium phosphate, pH 7.0) was maintained at 10 mM. The
rehydration buffer also contained 100 mg/ml of FD3-5, FD10 or FD40. After several freeze-thaw cycles, the mixture was extruded through a polycarbonate filter containing 200 nm size pores. To separate liposomes from unincorporated dextrans, the mixtures were subjected to size exclusion chromatography on a Superdex S200 column (GE Helathcare). The fractions in the void volume of the column were collected, and the extent of fluorescence dequenching upon addition of 1% Triton X-100 to individual fractions was utilized to confirm the presence of liposomes. The liposomes were utilized for assays within 3 hours of preparation.

**Liposome disruption assay:** For the assay, 1μl of purified liposomes was incubated, at room temperature, with HAV VP4, VP1 or Flock House Virus (FHV) γ 1 peptide at a concentration ranging from 300 nM to 50 μM for a time period of 20 minutes. Disruption of SulforhodamineB containing liposomes was measured with fluorescence spectroscopy (Perkin Elmer), based on dequenching of SulfoB fluorescence at 585 nm. The percentage of SulfoB released was calculated as 100 \( \frac{(F1 - F0)}{(Ftx100 - F0)} \), where F1 - fluorescence intensity measured in the presence of peptide, F0 - fluorescence intensity of liposome only, Ftx100 - fluorescence intensity in the presence of 1% Triton X-100. Similarly, disruption of liposomes incorporating fluorescent dye-labeled dextrans was measured by dequenching of FITC (for FD 3-5 and FD10) and Tetramethylrhodamine (for FD40) at emission wavelengths of 520 nm and 575 nm respectively.

**Dynamic Light scattering:** 1μl of purified liposomes, untreated and in buffer or treated with either 50 μM HAV VP4 or FHV γ 1 peptide, or 1% TX-100, at room temperature, were subjected to DLS in a Zetasizer (Malvern). Data corresponding to each sample was collected in triplicate. The mean hydrodynamic radius, \( R_h(\text{mean}) \), of representative samples is shown.

**Circular Dichroism:** CD spectra of the HAV VP4 and FHV γ 1 peptide were measured in the far UV range (190-250nm) using a J-815 CD spectrophotometer (JASCO) with a 1-mm path length cuvette. The peptides were at a concentration of 50 μM in the presence of 10 mM sodium phosphate buffer or 50%
TFE, and at 100 μM in the presence of lipid vesicles. Representative data from three separate studies are shown.

**ANTS/DPX assay:** ANTS/DPX liposome leakage/fusion assays were carried out with HAV VP4 and γ 1 peptides at a concentration of 50 μM. Peptides were incubated with DOPC liposomes loaded separately with ANTS and DPX, for 20 minutes at room temperature, and leakage of ANTS was assayed by fluorescence spectroscopy (Perkin Elmer) (λ<sub>ex</sub> = 355 nm; λ<sub>em</sub> = 520 nm).

**Electron Microscopy:** 5 μl of diluted DOPC liposomes, either untreated or incubated with 50 μM HAV VP4 peptide or FHV γ peptide at pH 5.5, were applied to 300 square mesh carbon-coated copper grids (Electron Microscopy Sciences). The excess material was removed by filter paper, followed by addition of 5μl of 1% (w/v) aqueous solution of uranyl acetate as a negative staining agent. After washing with distilled water, the grid was dried at room temperature and the morphology of the liposomes was examined using a TECNAI TF20 Transmission Electron Microscope (FEI Company) at an acceleration voltage of 200 KV.

**Molecular Dynamics Simulation:**

**Structure prediction of HAV VP4:** The 3D structure of HAV-VP4 (1MNMSRQGIFQTVGSGLDHILSLA<sup>23</sup>) was predicted using Bhageerath-H (a homology ab-intio hybrid web server for protein tertiary structure prediction) (22-26). Out of the five candidate predicted structures (Supplementary Figure 1), the lowest energy structure was used as the starting point for further studies. The predicted structure displayed a helix-turn-helix conformation, which correlated well with structures predicted from other prediction servers.

**System setup:** Three different starting orientations of the HAV VP4 peptide, with respect to the membrane surface, were used for the simulations and analyses. The peptide was placed on top of an equilibrated lipid bilayer comprising 238 POPC (16:0–18:1 Diester PC, 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine) molecules (a CHARMM36 force field compatible POPC molecule was
made available by Dr. A. Ghosh, Centre for Development of Advanced Computing, India). The POPC bilayer system had 238 lipid molecules in total, \textit{i.e.}, 119 lipid molecules in each leaflet. The orientations of the HAV VP4 peptide with respect to the membrane were denoted as (a) the N-terminal residue, Met1, oriented and placed towards the membrane surface (b) the C-terminal residue, Ala23, oriented and placed towards the membrane surface, (c) a parallel orientation of the peptide on the membrane surface.

After setups, three independent, all-atom explicit solvent molecular dynamics (MD) simulations were carried out with these structures. All the HAV VP4-bilayer systems were fully solvated in water. Counterions (\(\text{Cl}^-\) or \(\text{Na}^+\)) were added to neutralize each system.

**Molecular Dynamics Simulations:** Simulations were initiated using HAV VP4 in three different orientations with respect to the membrane. All MD simulations were performed for 50 ns in the isothermal-isobaric (NPT) ensemble using the GROMACS 4.6.1 software package (27, 28). The simple point charge (SPC) model was used for water molecules in the simulations (29). All simulations were performed according to the standard protocol, consisting of energy minimization, followed by gradual heating of the system. Each of the systems was initially minimized employing 20000 steps of steepest descent followed by conjugate gradient minimization. Topology and parameter files were generated using the CHARMM36 force field (30, 31). The equilibration of each system was achieved by performing a 5 ns MD run. Positional restraints were applied to each system during equilibration, while the system was slowly heated up from 0 to 300 K, at every 2 ps. After equilibration was reached, three independent 50 ns duration MD simulations were carried out with periodic boundary conditions at a temperature of 300 K. The Berendsen thermostat with a coupling constant of 0.1 ps was employed to keep the temperature constant (300 K) (32). A pressure coupling was applied semi-isotropically, with a coupling constant of 1.0 ps, using the Berendsen algorithm, to keep the pressure constant (1 bar). Lennard-Jones interactions were used as a cut-off at 1.2 nm. The long-range electrostatics was handled by the particle mesh Ewald (PME) method (33), and Van der Waals (vdW) interactions were calculated using a cutoff of 1.4 nm. The LINCS algorithm was used to constrain all bond lengths (34). Analyses
were performed with GROMACS analysis tools and visual molecular dynamics (VMD) software (version 1.9) (35). All MD simulations were performed on a single graphics processing unit (GPU) card, installed at the Supercomputing Facility (http://www.scfbiotech.res.in/) of Indian Institute of Technology Delhi.

Graphics: All figures were generated using VMD (35), UCSF CHIMERA package (36) and the PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC (http://www.pymol.org), as required.

Results:

**HAV VP4, and not the N-terminus of VP1, disrupts membranes in vitro:** Peptides corresponding to amino acids 1-23 and 43-60 of VP1, and 1-23 of VP4 (Figure 1A, B) were synthesized and their ability to interact with liposomes was tested. A peptide corresponding to amino acids 364-381 (designated the $\gamma_1$ region) of the capsid protein of Flock House Virus (FHV) was utilized as a positive control for these assays (37). The $\gamma_1$ region of FHV is well established as being competent for membrane penetration in vitro (37, 38), and required for endosomal membrane penetration by FHV during entry into host cells (20, 39). The ability of the peptides corresponding to HAV VP4 and VP1 N-terminus to disrupt Sulforhodamine B containing DOPC-liposomes was compared with that of the $\gamma_1$ peptide at concentrations ranging from 300 nM to 50 $\mu$M (Figure 2A). Although the peptides corresponding to the N-terminus of HAV VP1 displayed little ability to disrupt liposomes, the peptide corresponding to VP4 was able to disrupt liposomes to almost the same extent as $\gamma_1$ at a concentration of 50 $\mu$M (Figure 2A).

Both $\gamma_1$ and VP4 were able to cause ~ 70% de-quenching of Sulforhodamine B fluorescence, compared to the extent of de-quenching (considered 100%) caused by a detergent, Triton X-100. Thus, HAV VP4 has the ability to disrupt liposomes to the same level as a recognized cellular membrane penetrating peptide from a model non-enveloped virus (39).
To check the conformation of VP4 in hydrophobic environment, CD spectroscopy was carried out and the percentage of secondary structure elements computed. While in phosphate buffer, VP4 mostly existed as a random coil, the structure of the peptide altered to a significantly $\alpha$-helical (47.2%) conformation in the presence of 50% TFE (Tetrafluoroethylene) (Figure 2B). This indicates the possibility of a major structural alteration in VP4 in the hydrophobic environment of cellular membranes. Several tertiary structure prediction softwares indicated that the preferred, energy-minimized conformation for VP4 is predominantly helical, with an elongated, N-terminal helix separated from a diminutive, C-terminal one by a loop region (Supplementary Figure 1). The $\gamma$ 1 peptide also demonstrated a significantly helical (~ 58%) secondary structure in 50% TFE (40) (Figure 2B).

**Liposomes mimicking endosomal vesicles are specifically disrupted by HAV VP4:** The optimal activity of viral membrane active peptides is expected to depend on the nature of the limiting membrane. The limiting membrane for HAV entry is not clearly known, with support existing in literature for both entry through plasma membrane at neutral pH, and low-pH dependent entry through the endosomal route (9-11). It is also possible that different forms of HAV might utilize disparate modes of entry (7). In order to check whether there is any lipid composition-specific variation in the membrane penetration activity of HAV VP4, separate batches of liposomes mimicking various cellular organelles were produced. The liposomes were generated from a combination of phosphatidyl choline (POPC), phosphatidyl ethanolamine (POPE), phosphatidyl serine (POPS), sphingomyelin, cholesterol and bis (monoacrylglycerol) phosphate (BMP), in order to obtain the closest working approximation of the mammalian plasma membrane, as well as the membranes of organelles such as endoplasmic reticulum, golgi bodies and late endosomal compartments (21, Supplementary Table 1). The extent of disruption of these liposomes by HAV VP4 was quantified at pH 7.0, and additionally at pH 5.5 for liposomes.
mimicking endosomal compartments. The last condition generated the maximum membrane disruption by HAV VP4, while approximately half the disruption (~ 40%) was detected with the same compartments at neutral pH (Figure 3). This indicates that HAV VP4 is equipped to cause maximum membrane disruption at late endosomal conditions. The two-fold increase in membrane disruption ability of HAV VP4 at a lower pH condition may be due to a) alterations in the conformational state of the peptide, or, b) due to increased association between peptides and lipid, or between multiple peptides, as a function of pH change. In order to check the former possibility, the secondary structure of HAV VP4, in lipid vesicles mimicking late endosomal conditions, was computed. The α-helical content of VP4 was found to be similar at neutral or low pH conditions (Supplementary Figure 2), which indicated that low pH probably promotes association between peptides in a hydrophobic environment, rather than increase the helical content of individual peptides.

Interestingly, although the FHV γ 1 peptide also caused maximum membrane disruption with liposomes mimicking the lipid content of late endosomal vesicles, it did not demonstrate any pH-specific activity. This is somewhat expected since the role of low endosomal pH during FHV entry is merely to expose the γ peptide from the capsid interior, and not to cause conformational changes in γ (20, 40).

Of the rest of the organelle-specific liposomes tested, the ones mimicking the lipid content of golgi bodies and endoplasmic reticulum membranes were somewhat disrupted by HAV VP4, although there was minimal effect of the peptide on plasma membrane specific liposomes (Figure 3). This indicates the possibility of VP4-mediated entry of HAV into mammalian cells through the endocytic pathway.

HAV VP4 does not cause complete collapse of liposomes: Unlike the “trimer-of-hairpins” fusion pathway executed by hydrophobic fusion proteins of enveloped viruses (2), there is no unifying
mechanism for the functionality of membrane active peptides of non-enveloped viruses, which damage membranes (1). The suggested modes of action for the latter vary widely - with poliovirus VP4 creating voltage-gated channels in membranes (5), reovirus $\mu 1$ generating size-selective pores (4, 41), and protein VI of adenovirus collapsing membranes through induction of positive curvature (6). In order to gain an understanding of the mechanism of HAV VP4 mediated disruption of liposomes, DOPC-liposomes were subjected to dynamic light scattering, before and after treatment with VP4 peptide, and the mean hydrodynamic radius ($R_h$) of the liposomes was obtained in each case (Figure 4A). While addition of 1% Triton X100 reduced the $R_h$ (mean) of a liposome population from 52.8 nm to 7.4 nm, indicating complete collapse, the addition of 50 $\mu$M of VP4 hardly altered the mean $R_h$ of liposomes in the observed 20 minutes. This result indicates that unlike detergents, or some non-enveloped viruses like adenovirus, which cause complete collapse of membrane vesicles, HAV VP4 causes membrane damage more discretely, probably through the introduction of small pores or channels, reminiscent of the early interaction of reovirus $\mu 1$ with model membranes (4). This observation is supported by electron microscopy, which was utilized to visualize liposomes before or after addition of HAV VP4 (Figure 4B). In both cases, liposomes were visualized as circular, unilamellar vesicles of approximately 100 nm diameter, which indicated that VP4-mediated membrane damage did not alter the essential shape or size of liposomes within 20 minutes, in spite of causing enough membrane damage within that time period to allow the encapsulated fluorescent dye to escape (Figure 2A). This manner of membrane disruption is somewhat distinct from that exhibited by the $\gamma 1$ peptide, which appears to cause substantial disruption and clumping of the vesicles (Figure 4B). Dynamic light scattering shows that incubation of liposomes with $\gamma 1$ increases the $R_h$ (mean) of liposomes from 52.8 nm to 72.1 nm, which possibly indicates coalescence of the vesicles (Figure 4A), in addition to induction of leakage. Similar results were obtained upon testing the effect of HAV VP4 and FHV $\gamma 1$ on liposomes mimicking the lipid composition of late endosomal vesicles (Supplementary Figure 3).
Discrete pores of size 5-9 nm are formed in endosome-specific vesicles through the action of HAV VP4: Further studies were carried out to understand the nature of the damage inflicted on host membranes by HAV VP4. DOPC liposomes as well as liposomes mimicking late endosomal compartments, each incorporating fluorescently labeled dextran molecules of various sizes (3-5 KD corresponding to an average diameter of 2.8 nm; and 10 and 40 KD, corresponding to diameters of 4.6 and 9 nm respectively) were generated (41). The ability of HAV VP4 to release each type of dextran from encapsulating vesicles was separately determined from measuring the dequenching of dextran-associated fluorescence (Figure 5). While HAV VP4 was able to cause almost equivalent release of all three sizes of dextran molecules tested from DOPC-liposomes; the release of 40 KD dextrans from late endosome mimicking liposomes was significantly reduced, although there was no change in the extent of release of 3-5 KD and 10 KD dextrans. Thus, although morphological and DLS-based studies of VP4-incubated liposomes indicated formation of discrete pores in the membranes, the size of pores formed was somewhat dependent on the lipid composition of the membrane. While pores generated on DOPC membranes by HAV VP4 probably had a diameter close to or larger than 9 nm, the pores formed on late endosome-specific membrane vesicles essentially were of smaller diameters (5-9 nm), which did not allow 40 KD dextrans to pass through (Figure 5). It is possible that the presence of a combination of lipids and cholesterol effectively decreased the pore diameters.

HAV VP4 does not induce fusion in membranes: The discovery that HAV exists in both enveloped (eHAV) and non-enveloped forms, essentially dictates either – a) two separate modes of entry by the virus, and the existence of viable mechanisms for both lipid fusion and membrane disruption, or, b) Conversion of one form into the predominant “entry-specific” form. There is no example so far in literature for the occurrence of both types of entry mechanisms in the same virus capsid. The fusion peptides of enveloped viruses, and the membrane penetration peptides of non-enveloped viruses, typically have a specific and unique way of interacting with lipid membranes, in spite of occasional
structural similarities (40). The HAV VP4 peptide was nonetheless tested for its ability to carry out mixing of aqueous vesicle content by the ANTS/DPX assay, which is traditionally utilized for detecting vesicle fusion. Separate DOPC liposomes, containing either 100 mM of the polyanionic fluorescent dye ANTS, or 200 mM of the cationic quencher DPX, or a mixture of 50 mM ANTS and 100 mM DPX, were prepared, and ANTS fluorescence in the presence or absence of 50 μM HAV VP4 was monitored over time (Figure 6). In the event of liposome fusion by the action of VP4, the fluorescence of ANTS would be quenched by DPX. However, addition of HAV VP4 or FHV γ 1 peptide caused an instantaneous increase in ANTS fluorescence, which remained steady over a time period of 20 minutes, indicating that the peptides cause vesicle leakage instead of fusion (Figure 6). Similar results were obtained for 0.5% Triton-X100, which is known to cause leakage in liposomes. Thus, HAV VP4 was not a multifunctional peptide capable of inducing membrane fusion, and its functionality appeared limited to causing membrane damage.

Molecular dynamics simulations reveal the initial contact of VP4 with membranes: The objective of the simulations of HAV VP4 with pre-equilibrated POPC membrane was to determine a plausible mode of interaction and penetration of HAV VP4 into the membrane surface. Three independent 50 nanosecond MD simulations were carried out in order to model the possible modes of interaction of VP4 residues with membrane (Figure 7A-C). The simulations were carried out with a tertiary structure for VP4 predicted by Bhageerath-H (22-26). Out of the five predicted, possible structures, the lowest energy structure, comprised of a helix-turn-helix conformation, was selected as the starting structure for MD simulations (Supplementary Figure 1). Three different starting orientations of the peptide with respect to the membrane surface was used for the simulations - (a) N-terminus of VP4 (Met1) oriented and placed towards the membrane (b) C-terminus oriented and placed towards the membrane, and (c) a parallel orientation of the peptide placed on the POPC membrane surface. In the simulation study corresponding
to the first orientation of VP4 (Figure 7A), the Met\textsuperscript{1} residue rapidly approached the hydrophilic lipid slab of the membrane surface during the initial 5 ns of the simulation. The peptide further attained a parallel orientation with respect to the membrane surface and remained unaltered in this conformation up to ~ 25 ns. During this time, the C-terminal helical region remained stable while the loop region at the N-terminus appeared to form a hinge-like structure (Figure 7A). After the 25 ns run, the N-terminal region embedded itself into the upper leaflet of the membrane bilayer and continued in this conformation till the end of the simulation (50 ns) (Figure 7A). During this period, several hydrogen bonding interactions among N-terminus residues of VP4, such as Met\textsuperscript{1}, Asn\textsuperscript{2}, Ser\textsuperscript{4}, and Arg\textsuperscript{5}, with the head group regions of the POPC membrane were detected (Supplementary Table 2), which were probably instrumental in allowing the peptide to penetrate into the membrane. The backbone root mean-square deviation (RMSD) of VP4 remained stable during the simulation, suggesting that the overall structure of the system remained unaltered and stable during the MD run (Supplementary Figure 4A).

With the second orientation, consisting of the C-terminal residue (Ala\textsuperscript{23}) placed towards the membrane (Figure 7B), the peptide appeared unstable on the membrane surface till 40 ns. The peptide also exhibited loss of secondary structure during this time. From 40 ns onwards, the peptide managed to position itself in an inclined orientation with respect to the membrane surface, so that the N-terminus pointed towards the membrane surface (Figure 7B). Further, within a span of 2 ns, the peptide acquired an upright position, allowing the N-terminal residues to face the membrane. Eventually, the N-terminus region formed a hinge shaped structure and penetrated the hydrophilic head groups of the upper leaflet of membrane surface (Figure 7B). This event was accompanied by H-bonding interactions forming between Ser\textsuperscript{4}, Arg\textsuperscript{5} and Ile\textsuperscript{8} of the peptide, and POPC molecules (Supplementary Table 2). The backbone RMSD of VP4 in this orientation appeared to be less stable (Supplementary Figure 4B), indicating that the peptide was not in a favorable orientation to interact with the membrane.

In the third and final orientation, the peptide was placed parallel to the membrane surface (Figure 7C). During the first 2 ns, the N-terminus interacted with the membrane, through mainly H-bonding...
contacts (Supplementary Table 2) mediated through Met\(^1\), Asn\(^2\), and Arg\(^5\) of the peptide, till the end of simulation (50 ns). The backbone RMSD of VP4 showed that the peptide and its interactions with the membrane surface was stable throughout the simulation (Supplementary Figure 4C).

The observation that during the course of the simulations, only the N-terminus of HAV VP4 was interacting and penetrating the membrane bilayer, irrespective of the initial orientation, indicates that this is the perhaps the preferred mode of interaction of VP4 with lipid membranes (Figure 7A-C). The initial penetration is probably mediated by formation of H-bonding interaction between Met\(^1\), Asn\(^2\), and Arg\(^5\) residues, situated at the N-terminus of VP4, with the hydrophilic head groups of the outer leaflet of the lipid bilayer. Also, at several instances during the first and third simulations, a circular ring-like conformation was acquired by the C-terminal region of VP4 (Supplementary Figure 5). It is possible that this structural unit observed during simulations might eventually become stable through interaction with lipid molecules, and participate in creating a pore through the membrane.

**Discussion**

HAV is responsible for ~40% of all cases of viral hepatitis worldwide, and is prevalent among populations in developing countries with poor hygienic and sanitary conditions (12). Recent socio-economic improvement in some countries has created an incipient danger of large-scale outbreaks of HAV due to the collocation of susceptible pockets with generally resistant populations. China has experienced several outbreaks over the last couple of decades, and it has been reported recently that the level of anti-HAV antibodies in Indian newborns has been depleted to 50-60% within a span of 15 years (42). Further, economic progress in specific areas has coincided with an epidemiological shift of the disease from usually asymptomatic early childhood infections to symptomatic infections in the 2\(^{nd}\) and 3\(^{rd}\) decades of life, which carry an increased risk of liver failure (43). Thus, it is essential to develop and implement more effective preventive measures and therapies against HAV, which necessitates a clear understanding of the manner of host cell interaction by HAV. HAV is arguably the least well studied of
all picornaviruses, since the slow-growing nature of the virus precludes production of large quantities of purified material for biochemical and structural studies (13). This has hampered studies on, among other areas, the role of specific capsid proteins in establishment of infection.

We have identified the capsid structural peptide VP4 of HAV as a potential component for host membrane penetration during entry. The *in vitro* membrane disruption ability of HAV VP4 is comparable to well-established, well-studied membrane penetration peptides, such as the γ 1 peptide of the model non-enveloped virus FHV (39). The involvement of VP4 in mediating cellular entry for members of the *picornaviridae* family is also well established in literature. Poliovirus VP4 is known to form ion channels in membranes to allow genome escape (5), and a GST-tagged recombinant VP4 from human rhinovirus (HRV16) can cause disruption of model membranes *in vitro* (18). Although there is no direct evidence for the involvement of the VP4 polypeptides of coxsackievirus, echovirus and enterovirus in membrane penetration, they are comparable to the VP4 polypeptides of poliovirus and HRV16 in length, degree of hydrophobicity and presence of a myristoylation signal (N-terminal G-x-x-x-T/S). These striking similarities suggest possible parallel functionalities during cellular entry. The presence of a hydrophobic myristate has been linked to the efficiency of membrane penetration as well as assembly and maturation of viruses in the *picornaviridae* and *reoviridae* family (44-49). Interestingly, HAV VP4 differs from its analogues in other picornaviruses in a number of fundamental points – it is about a third of the length of other VP4 polypeptides, displays significantly elevated hydrophobicity and does not contain a myristoylation signal at the N-terminus. There is a putative myristoylation signal 5 residues downstream of VP4 N-terminus, however, experimental evidence proves that VP4 does not undergo any modification at this region (19). Thus, the membrane disruption activity displayed by HAV VP4 is entirely a function of its own hydrophobic character and in no way dependent on myristate-lipid interactions.

By analogy with other members of the *picornaviridae* family, we had hypothesized that both...
VP4 and VP1 might be involved in membrane penetration during HAV entry. The N-terminal region of VP1 in picornaviruses displays a varying degree of hydrophobicity, and is expected to have essential, but somewhat diverse roles during virus entry. VP1 N-terminus from human rhinovirus HRV2 has been shown to contain membrane-disrupting activity in vitro, and is probably instrumental in allowing the viral genome to escape from endosomes (16, 17). In contrast, the N-terminal residues of poliovirus VP1 are capable of associating with liposomes (14), although there is no experimental evidence for vesicle disruption. Latest data indicates that VP1 probably functions to anchor poliovirus to cellular membranes, and to protect the genomic RNA during disassembly, while VP4 primarily creates pores in cellular membranes (5, 15). The two hydrophobic regions identified at the N-terminal domain of HAV VP1 are unable to cause disruption of membrane vesicles in vitro. It is, however, possible that these regions can associate with membranes and assist in virus entry in some fashion.

The membrane activity of HAV VP4 is significantly more pronounced towards liposomes with a lipid profile corresponding to that of the late endosomal compartments; while minimal preference is exhibited for double membrane vesicles mimicking the mammalian plasma membrane. While early endosomes are generated from plasma membranes and contain a similar lipid composition, upon maturation, the membranes of these vesicles become enriched in the anionic lipid, bis (monoacylglycero) phosphate (BMP) (21, 50). It has been shown that the presence of BMP is essential for membrane penetration and delivery of cargo by several cell penetrating peptides (CPPs). CPPs can disrupt late endosomal vesicles containing BMP, but are ineffective against the plasma membrane, which lacks BMP while containing a similar proportion of sterols and other lipids components (21, 50). Thus, HAV VP4 might be similar to CPPs in its requirement for the presence of BMP for optimal membrane penetration. VP4 has an additional requirement for low pH conditions for optimal activity, since only half as much disruption of vesicles mimicking late endosomal compartments is achieved by 50 μM of the peptide at pH 7.0. However, we have confirmed that low pH conditions do not serve to
alter the secondary structure of the peptide in any way.

The membrane activity of HAV VP4 does not cause any dramatic change in the structure or physical appearance of either DOPC-liposomes or liposomes mimicking late endosomes. Our data shows that the overall size and shape of liposomes remains constant up to 20 minutes after incubation with the peptide, while pores of 5-9 nm diameter are induced in the membranes, allowing fluorescent dye-associated dextrans of various sizes to pass through. Thus the membrane penetrating mechanism of HAV VP4 appears similar to that of poliovirus and HRV2, which both form pores in cellular membranes (5, 16, 17). Interestingly, the pores induced by HAV VP4 in DOPC–liposomes are somewhat larger in diameter (> 9 nm) compared to those formed in late endosome specific liposomes. This slight difference in the end result might reflect a somewhat altered degree or manner of association of VP4 with vesicles containing different lipid compositions. This mode of membrane damage is similar to that carried out by the μ1N peptide of orthoreovirus, which is required for endosomal membrane disruption during virus entry. μ1N has been shown to induce the formation of size-specific pores in membrane vesicles, which eventually rupture due to osmolysis (4, 41).

We utilized all atom Molecular Dynamics (MD) simulation studies with a predicted tertiary structure of HAV VP4 in order to understand the mode of interaction of the peptide with model membranes. Although MD simulation is a powerful tool, and has been utilized effectively for mapping the manner of membrane interaction by peptides of bacterial and mammalian origin, as well as fusion peptides from enveloped viruses like Influenza virus and HIV (51-54), there are very few examples for utilization of this technique for understanding the mechanism of action of viral membrane disrupting or associating peptides (51). Among the latter group, only the membrane interaction of the N-terminal region of poliovirus VP1, which is responsible for the association of the virus with the host plasma membrane during genome release, has been mapped using MD simulation (51). Our studies with various orientations of HAV VP4 with respect to a model membrane clearly indicate that the initial encounter...
and penetration into the leaflet of cellular membranes is mediated through the N-terminal end of the peptide. Indeed, the relative instability of the backbone RMSD of the system, when the C-terminal end of the peptide is placed close to the model membrane, suggests that this is an unfavorable orientation. Optimal membrane disruption may also require an oligomeric form of VP4. It is possible that VP4 peptides are released from the HAV capsid in an oligomeric form, or association between peptides is promoted during membrane interaction. Significantly longer simulations incorporating multiple copies of VP4, coupled with corroborating functional evidence, is required to confirm whether oligomerization is a functional necessity for VP4-mediated membrane disruption.

The recent discovery of an enveloped version of HAV has made understanding the cellular entry process of this virus a uniquely interesting challenge. Interestingly, both eHAV and non-enveloped HAV are thought to use the same receptor, HAVCr-1, for cellular entry (7). Thus, it is possible that the envelope of eHAV is removed upon initial encounter with host cells, allowing the same set of capsid proteins to mediate receptor interaction and membrane disruption for both the enveloped and the non-enveloped version. It is difficult to speculate on the possibility of eHAV utilizing membrane fusion to enter host cells, since protein components analogous to lipid-embedded glycoproteins have not yet been detected in eHAV. A fusion-competent peptide was previously discovered in capsid protein VP3 (55, 56), however, utilization of this region for fusion of the eHAV envelope with host cell membranes will necessitate its exposure on the surface of the eHAV lipid coat.

The preferential activity of VP4 towards late endosome-specific vesicles suggests that this peptide might be involved in endosomal escape of HAV genome in the initial steps of virus entry. HAV VP4 has previously been shown to have an essential role in virion morphogenesis (57), although detection of this peptide in HAV capsid preparations has been difficult. This is probably due to a combination of limited starting material and the small size of VP4. Additionally, VP4 does not display any fusion activity, which rules out the scenario that it might be a multifunctional membrane-active peptide, able to assist in both membrane fusion and disruption activities by the enveloped and non-
enveloped versions of the virus respectively. Thus, it is possible that although HAV spends a major part of its life cycle as an enveloped virus, its mode of entry into host cells is through the traditional route of membrane disruption employed by non-enveloped viruses in general. VP4 may be required for membrane disruption by eHAV after its lipid envelope has been shed during entry.

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References


Figure Legends

Figure 1: A) Hydrophobicity plots of the N-terminal of VP1 (left panel) and VP4 (right panel) B) Helical wheel representations, from left to right, of VP1 (1-23 residues), VP1 (43-60 residues), and VP4 (6-23 residues). The sequences corresponding to these regions are provided.
**Figure 2:** A) Disruption of DOPC-liposomes by VP1 and VP4 peptides at concentrations ranging from 0.3 to 50 μM. Degree of disruption is represented by the extent of de-quenching of the fluorescent dye Sulforhodamine B (Sulfo B), with the de-quenching obtained by addition of 1% Triton-X 100 considered to be 100%. Data are represented as the mean of triplicate independent samples ± SD (**P < 0.01; * P < 0.05; NS (Non Significant) P >0.05, Student’s t- test in comparison with FHV γ1 peptide). B) Circular Dichroism spectroscopy of HAV VP4 and FHV γ 1, in phosphate buffer, pH 7.0, and in the same buffer containing 50% TFE. The concentration of each peptide used was 50 μM.

**Figure 3:** Degree of disruption of liposomes mimicking different cellular organelles, by VP4 and VP1 peptides at concentrations ranging from 0.3 to 50 μM. Liposomes mimicking late endosomal vesicles were tested at two pH conditions – 7.0 and 5.5. FHV γ1 (in black) was utilized as a control for all assays. Data are represented as the mean of triplicate independent samples ± SD (**P < 0.01; * P < 0.05; NS (Non Significant) P >0.05, Student’s t- test in comparison with FHV γ1 peptide).

**Figure 4:** A) Dynamic light scattering studies, showing the mean hydrodynamic radius or Rh(mean) of DOPC-liposomes in buffer, or after 20 minutes of incubation with 1% Triton X-100, or 50 μM of HAV VP4 or FHV γ 1, at pH 7.0 and 5.5. B) Images obtained upon negative staining and electron microscopy of DOPC-liposomes, either untreated (top panel - left), or after 20 minutes of incubation with 50 μM HAV VP4 (top panel - right) at pH 5.5, FHV γ1 (bottom panel - left) at pH 5.5 and 1% Triton X-100 (bottom panel - right).

**Figure 5:** Extent of release of fluorescent-dye labeled dextrans of various sizes from A) DOPC-liposomes, and B) Liposomes mimicking late endosomal vesicles, by HAV VP4 at concentrations...
ranging from 0.3 to 50 μM, at pH 5.5. The extent of de-quenching, upon release, of free or dextran-associated fluorescent dyes rhodamine, FITC and tetramethyl rhodamine was determined by measuring increase in fluorescence at emission wavelengths of 585 nm, 520 nm and 575 nm respectively, with the de-quenching obtained by addition of 1% Triton-X 100 considered to be 100% in each case. The incubation time of peptide with liposome was maintained at 20 minutes for each assay. Data are represented as the mean of triplicate independent samples ± SD (**P < 0.01; * P < 0.05; NS (Non Significant) P >0.05, Student’s t- test in comparison with rhodamine dye release )

**Figure 6:** ANTS/DPX fusion assay with HAV VP4 and FHV γ1. A mixture of liposomes containing 100 mM fluorescent dye ANTS, and 200 mM of the quencher DPX, was either left untreated or were treated with 50 μM VP4, FHV γ1, or 0.5% Triton X-100, and the fluorescence of ANTS was monitored continuously for 20 minutes, at an emission wavelength of 520 nm. No decrease in ANTS fluorescence was detected at any point, suggested leakage, but not fusion, of vesicles.

**Figure 7:** Snapshots from Molecular Dynamics simulation studies of HAV VP4 placed with A) N-terminus facing model membrane, B) C-terminus facing model membrane, and C) an orientation parallel to the model membrane. Images shown are from the beginning (left panel), after 20-40 nanoseconds (middle panel) and 50 ns (right panel) of simulations.
Liposome containing 100 mM (ANTS)

Liposome containing 100 mM (ANTS) + Liposome containing 200 mM DPX

Liposome containing 100 mM (ANTS) + Liposome containing 200 mM DPX + HAV VP4 (50 μM)

Liposome containing 100 mM (ANTS) + Liposome containing 200 mM DPX + FHV γ1 (50 μM)

Liposome containing 100 mM (ANTS) + Liposome containing 200 mM DPX + 0.5% Triton X-100