MEMBRANE FUSION INDUCED BY VESICULAR STOMATITIS VIRUS
DEPENDS ON HISTIDINE PROTONATION

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Running title: Role of His on VSV-induced membrane fusion

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SUMMARY

Entry of enveloped animal viruses into their host cells always depends on a step of 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 at the acidic environment of the endosomal compartment. VSV-induced membrane fusion occurs at a very narrow pH range, between 6.2 and 5.8, suggesting that His protonation is required to this process. To investigate the role of His in VSV fusion, we chemically modified these residues using diethylpyrocarbonate (DEPC). We found that DEPC treatment inhibited membrane fusion mediated by VSV in a concentration dependent manner, and that the complete inhibition of fusion was fully reversed by incubation of modified virus with hydroxylamine. Fluorescence measurements showed that VSV modification with DEPC abolished pH-induced conformational changes in G protein, suggesting that His protonation drives G protein interaction with the target membrane at acidic pH. Mass spectrometry analysis of tryptic fragments of modified G protein allowed the identification of the putative active His residues. Using synthetic peptides, we showed that the modification of His148 and His149 by DEPC as well as the substitution of these residues by Ala completely inhibited peptide-induced fusion, suggesting the direct participation of these His in VSV fusion.
INTRODUCTION

Membrane fusion is an essential step in the entry of enveloped viruses into their host cells (1-3). Virus-induced fusion is always mediated by viral surface glycoprotein, and may occur through two different general mechanisms: (i) surface fusion between viral envelope and host cell plasma membrane after virus interaction with its cellular receptor, and (ii) fusion of endosomal membrane with viral envelope, after virus particle internalization by receptor-mediated endocytosis. In the latter case, fusion is triggered by conformational changes in viral glycoproteins induced by the decrease in the pH of the endosomal medium.

Vesicular stomatitis virus (VSV)\(^1\) is a member of *Rhabdoviridae* family, genus *Vesiculovirus*. Rhabdoviruses contain helically wound ribonucleocapsid surrounded by a lipid bilayer through which spikes project. These spikes are formed by trimers of a single type of glycoprotein, named G protein. VSV enters into the cell by endocytosis followed by low-pH-induced membrane fusion in the endosome (4,5), which is catalyzed by VSV G protein (6). A common feature of viral fusion protein is that they bear a highly conserved hydrophobic fusion domain, which is most often located at the N-terminal of the polypeptide chain (7). However, VSV G protein does not contain an apolar amino acid sequence similar to the fusion peptides found in other viruses, suggesting alternative mechanisms involved in VSV-induced membrane fusion.

\(^1\) The abbreviations used are:

VSV, vesicular stomatitis virus; VHSV, viral hemorrhagic fever virus; DEPC, diethylpyrocarbonate; PS, phosphatidylserine; PC, phosphatidylcholine; PMSF, phenylmethylsulfonyl fluoride 10-PyPC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine; MALDI-TOF, matrix-assisted desorption ionization-time of flight; HA, Influenza virus hemagglutinin; HIV-1, human immunodeficiency virus type 1; gp, glycoprotein; SIV, simian immunodeficiency virus.
We have recently shown that VSV-induced fusion depends on a dramatic structure reorganization of G protein, which occurs within a very narrow pH range, close to 6.0 (8). In addition, we have found that VSV binding to membranes as well as the fusion reaction were highly dependent on electrostatic interactions between negative charges on membrane surface and positively charged amino acids in G protein at the fusion pH (9). These results suggest the involvement of histidyl residue(s) in G protein conformational changes required for fusion, since the protonation of imidazole ring occurs at the fusion pH range (pK = 6.0).

Hydrophobic photolabeling experiments allowed the identification of a G protein segment comprising amino acids 59 to 221, which interacts with membranes at low pH (10). Furthermore, studies using site-direct mutagenesis in the region spanning amino acids 117 to 137 have shown a reduction of G protein-induced fusion efficiency (11-13). However, there is no conclusive evidence that this sequence participates directly in the fusion reaction. Another region of rhabdovirus G protein has been implicated in its interaction with anionic phospholipids. This segment was better characterized for viral hemorrhagic septicemia virus (VHSV), a rhabdovirus of salmonids, and it was named p2 peptide (14,15). VHSV p2 peptide mediates phospholipid vesicle fusion, lipid mixing, and leakage of liposome contents and inserts itself into liposome membranes by adopting a β-sheet conformation (16). p2-like peptides was found among all rhabdoviruses and contains two histidyl residues in VSV G protein (17).

In order to evaluate the role of G protein His residues in VSV-induced membrane fusion we modified these residues using diethylpyrocarbonate (DEPC). We showed that His protonation was essential both for low-pH-induced conformational changes of VSV G protein and for the fusion reaction itself. Mass spectrometry analysis of G protein fragments
obtained by limited proteolysis allowed the identification of the putative active His residues. Using synthetic peptides, we found that VSV p2-like peptide (sequence between amino acids 145 and 168) was as efficient as the virus in catalyzing membrane fusion at pH 6.0, and that the modification of His148 and His149 by DEPC completely abolished fusion activity. Substitution of the His by Ala residues inhibits peptide-mediated fusion, confirming the requirement of His protonation in VSV-induced membrane fusion.
MATERIALS AND METHODS

Chemicals

Diethylpyrocarbonate (DEPC), phosphatidylserine (PS) and phosphatidylcholine (PC) from bovine brain, trypsin from bovine pancreas and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co., St. Louis, MO, USA. 1-hexadecanoyl-2-(1-pyrenedicoyl)-sn-glycero-3-phosphocholine (10-PyPC) was purchase from Molecular Probes Inc., Eugene, OR, 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 roller bottles containing 150 mL of Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 100 µg/mL ampicilin, 5 µg/mL gentamicin. When the cells reached confluence, the medium was removed, and the cell monolayer was infected with VSV at a multiplicity of 0.1 pfu/mL. The cultures were kept at 37°C for 16-20 h and the virus were harvested and purified by differential centrifugation followed by equilibrium sedimentation in a sucrose gradient as described elsewhere (18). Purified virions were stored at –70°C.

Preparation of liposomes

Phospholipids were dissolved in chloroform and evaporated under nitrogen. The lipid film formed was resuspended in 20 mM MES, 30 mM Tris buffer (pH 7.5 or 6.0) at a final 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) 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 10 cycles). The vesicles used in this study were composed of phosphatidylcholine (PC) and phosphatidylserine (PS) at 1:3 ratio. For fusion assays, 1% 10-PyPC was incorporated in PC:PS vesicles by vortexing for 10 min.

**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 (19). For modification with DEPC, VSV was diluted in 20 mM MES, 30 mM Tris buffer, pH 7.5. At fixed time intervals, aliquots of DEPC were added to the mixture and the reaction was monitored by the increase of absorbance at 240 nm due to the formation of N-carbethoxyhistidine using a Hitachi U-2001 Spectrophotometer. The final concentration of DEPC ranged from 0.005 to 0.05 mM.

To study the kinetics of modification, VSV was diluted in 20 mM MES, 30 mM Tris buffer, pH 7.5, and the reaction was initiated by the addition of 0.02 mM DEPC at 25°C. The time course of the reaction was monitored by an increase of absorbance at 240 nm.
**Reversal of DEPC inactivation**

VSV was reacted with 0.02 mM DEPC at 25°C. After 3 min, the mixture was incubated with 400 mM hydroxylamine (from a 3 M stock solution of hydroxylamine in 20 mM MES, 30 mM Tris buffer, adjusted to pH 7.5) for 15 min at 25°C. For demodification of peptides, the experiment was carried out at the same conditions except that the concentrations of DEPC and hydroxylamine used were 0.2 mM and 500 mM respectively. For the control, a solution contained the same concentration of hydroxylamine without DEPC was used.

**Liposome fusion assay**

A suspension of liposomes of different phospholipid composition containing equal amounts of unlabeled vesicles and vesicles labeled with 10-PyPC were prepared in 20 mM MES, 30 mM Tris buffer, pH 6.0 or 7.5, with a final phospholipid concentration of 0.1 mM. The emission spectrum of pyrene-labeled vesicles exhibited a broad excimer fluorescence peak with maximal intensity at 480 nm, and two sharp peaks at 376 and 385 nm due to monomer fluorescence emission (not shown). The fusion reaction was initiated by addition of purified VSV preincubated with different concentrations of DEPC for 3 min at 25°C, ranging from 0.005 to 0.02 mM. Fusion was followed by the decrease in the 10-PyPC excimer/monomer fluorescence intensity ratio, which was measured by exciting the sample at 340 nm, and collecting the fluorescence intensities of excimer and monomer at 480 and 376 nm, respectively. A control experiment using equivalent volumes of ethanol (without DEPC) was performed under comparable conditions. For peptide-induced fusion, the concentration of DEPC used was 0.02 and 0.2 mM.
**Intrinsic fluorescence measurements**

G protein conformational changes during VSV interaction with membranes of different phospholipid composition were monitored by the changes in virus intrinsic fluorescence. VSV (final protein concentration of 15 µg/mL) was incubated with a liposome suspension containing 1 mM phospholipid in 20 mM MES, 30 mM Tris buffer, pH 6.0. Intrinsic fluorescence data were recorded using a Hitachi F-4500 Fluorescence Spectrometer, exciting the samples at 280 nm and collecting emission between 300 and 420 nm.

**MALDI-TOF mass spectrometry of modified VSV G protein**

VSV (0.3 mg/mL) was reacted with 0.02 mM DEPC for 15 min at 25°C. After modification, G protein was denatured by virus incubation with 8 M urea for 1 h. Then, the sample was diluted 4 fold in 10 mM Tris buffer, pH 7.4 and incubated with trypsin (final concentration of 11 mg/mL) for 4 hs at 37°C. The reaction was stopped by addition of 0.1 mM PMSF. The tryptic peptides were separated from the remaining virus by filtration. For mass spectrometry analysis, aliquots of 1 µL of the digested sample mixed with 1 µL of the matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid) were applied on the plate and allowed to dry at room temperature. Mass profiles of digested G protein were obtained on a Voyager-DE PRO (Applied Biosystems) equipped with a nitrogen laser (λ = 337 nm). Fifty laser shots were summed per sample spectrum and the average of 5 spectra was used. The masses obtained were searched against a protein database containing the sequence of bovine trypsin and

Searches were also done with DEPC modification option turned on.

**Peptides synthesis**

All peptides 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 x 250 mm) and a two-solvent system: (A) trifluoroacetic acid/H₂O (1:1000, v/v) and (B) trifluoroacetic acid/acetonitrile/H₂O (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 x 150 mm), which was eluted with solvent systems A1 (H₃PO₄/H₂O, 1:1000, v/v) and B1 (acetonitrile/H₂O/H₃PO₄, 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).
RESULTS

Role of G protein His residues in VSV-induced membrane fusion

VSV was incubated with increasing concentrations of DEPC, which reacts with His forming N-carbethoxyhistidyl derivatives (19) and the virus-mediated membrane fusion was quantified by measuring the decrease in pyrene phospholipid excimer/monomer fluorescence ratio (9,20) (Fig.1). Incubation of 0.02 mM DEPC with VSV (15 µg/ml) completely abolished virus ability to mediate membrane fusion, while lower concentrations of DEPC partially inhibits it. The formation of N-carbethoxyhistidine was followed spectrophotometrically by the absorbance increase in 240 nm (19). The major changes observed in absorbance occurred when the virus was incubated with DEPC in final concentrations up to 0.03 mM (Fig. 2A). Kinetics of VSV modification with 0.02 mM DEPC revealed that the reaction was completed after 3 min (Fig. 2B). To further test whether modification of His residues was responsible for inhibition of virus fusion activity, hydroxylamine, which removes the carbethoxy group from imidazole group (19), was added 3 min after VSV incubation with 0.02 mM DEPC. Virus incubation with hydroxylamine after modification with 0.02 mM DEPC completely restored its ability to catalyze membrane fusion (Fig. 3). This set of results indicates that His protonation is required for membrane fusion catalyzed by VSV, suggesting a central role of His in pH-induced conformational changes in VSV G protein.

His protonation is involved in pH-induced conformational changes on G protein
We have recently shown that G protein interaction with liposomes at pH 6.0 resulted in dramatic protein conformational changes, which can be followed by intrinsic fluorescence (8). In the presence of vesicles composed of PC and PS, a great increase in tryptophan fluorescence of G protein occurred upon acidification of the medium, while pH decrease led to intrinsic fluorescence quenching in the absence of liposomes (8). VSV incubation with DEPC inhibited intrinsic fluorescence quenching during acidification, suggesting the involvement of His protonation in G protein conformational changes (Fig. 4A). Time course of fluorescence increase after VSV incubation with liposomes, at pH 6.0, is shown in Figure 4B. The increase in fluorescence was completely inhibited when the virus was incubated with 0.02 mM DEPC. These results indicate that the G protein conformational changes that take place during protein-lipid interaction are mediated by His protonation at pH 6.0.

**Mass spectrometry analysis of modified G protein**

VSV G protein contains a total of 16 His residues. Previous investigations have revealed that a specific domain spanning residues 59 to 221, which contains 6 His, interacted with the target membrane at low pH (10). To determine whether DEPC treatment modified the His residues within this sequence, the peptides obtained after limited proteolysis of modified G protein were analyzed by MALDI-TOF mass spectrometry. Seven fragments could be identified as VSV G protein peptides (Table I). These peptides cover 64% of G protein (329/511 amino acids). We also analyzed the data considering the increase in mass due to DEPC modification, and four modified peptides could be identified (Table II). Two of these peptides are included in the membrane-interacting domain (32-87 and 110-168), suggesting that the active His are located within this segment.
Role of p2-like peptides in VSV-induced membrane fusion

The putative fusion peptide (region 117-137) and the p2-like peptide (region 145-168) are located within one of the modified segment of G protein identified by mass spectrometry. To evaluate the ability of both the p2-like peptide and the putative fusion peptide in catalyzing fusion in vitro, we synthesized a number of peptides (Fig. 5). Besides the putative fusion peptide and the p2-like peptide, we synthesized three other His-containing sequences to be used as controls. The peptides corresponding to the sequences between amino acids 65-85 and 170-190 contain two His residues and are located within the sequence that was identified as the membrane-interacting segment by photolabeling experiments (10). The peptide between amino acids 395-418 was also chosen since it was found to be modified by DEPC treatment by mass spectrometry analysis (Table II).

Figure 6 shows that the p2-like peptide was as efficient as the whole virus to catalyze fusion of PC:PS vesicles. Using p2-like peptide in a 50-fold lower concentration, which gives a peptide molar concentration similar to G protein concentration used in virus-induced fusion, we obtained a very similar profile (Fig. 6B). In addition, peptide-induced fusion presented the same requirements of VSV-mediated fusion. It occurs at pH 6.0 but not at pH 7.5, and depends on the presence of PS on the target membrane (Fig. 6B). These data suggest a direct participation of p2-like peptide in VSV-induced membrane fusion. On the other hand, when the synthetic peptide corresponding to the VSV putative fusion peptide was assayed for liposome fusion, it failed to induce a decrease in pyrene excimer/monomer fluorescence ratio (Fig. 6C). This result shows that this sequence alone is not able to catalyze fusion reaction, and reinforce the involvement of p2-like peptide in VSV fusion.
The pH-dependence of membrane fusion mediated by p2-like peptide suggests the participation of His in the process. In order to evaluate whether His protonation was also necessary for peptide-induced fusion, as observed for the virus, the effect of peptide incubation with DEPC on the membrane fusion was analyzed. As shown on the Fig. 7A, His modification by DEPC abolished peptide activity, suggesting that His residues are crucial for membrane recognition and fusion. Hydroxylamine treatment reversed fusion inhibition by DEPC modification (Fig. 7A). In addition, substitution of both His148 and His149 for Ala residues on the peptide sequence completely abolished fusion, whereas removing one of the His residues led to a less efficient fusion (Fig. 7B). All other G protein amino acid sequences containing two His residues used as controls did not present fusion activity (Fig. 7C). These results together suggest that VSV p2-like peptide directly participates in membrane fusion mediated by G protein and that protonation of His is necessary for peptide fusion activity.
DISCUSSION

In this work, we describe two main findings concerning VSV-induced membrane fusion. First, we showed that fusion is driven by His protonation at the pH range of endosomal medium. Although several residues have already been implicated in G protein fusion ability, to our knowledge His has never been considered. Second, we found that VSV p2-like peptide was as efficient as the whole virus in catalyzing fusion, whereas the putative fusion peptide failed to induce fusion. VSV p2-like peptide contains two His residues, whose protonation is required for its fusion activity.

The identification of the amino acid residues essential for membrane fusion mediated by viral glycoproteins might contribute to the elucidation of the molecular mechanisms underlying the fusion event. In the case of VSV, mutational analysis have shown that substitution of conserved Gly, Pro, or Asp present in the region between amino acids 117 and 137 either abolished fusion ability of G protein or shifted the optimum pH of fusion (11-13). Based on these results, the authors proposed that this segment were the putative fusion domain of VSV G protein. However, direct evidence that this particular region interacts with the target membrane is still lacking. VSV-induced membrane fusion occurs in a very narrow pH range, between 5.8 and 6.2 (4,8). This indicates that the protonation of a small number of ionizable groups is required for G protein structural changes. His is the only amino acid whose ionization pKₐ is in the range of VSV fusion, suggesting that fusion is driven by His protonation. Using DEPC, we showed that His modification abolished pH-induced conformational changes on G protein and the fusion reaction catalyzed by the virus. VSV putative fusion peptide contains no His, and thus it cannot be modified by DEPC. In addition, we found that a synthetic peptide corresponding
to the VSV putative fusion sequence failed to induce phospholipid vesicle fusion, although several studies have reported that synthetic fusion peptides of different viruses promote fusion independent of the remainder protein (21-25). Further investigation will be necessary to answer whether the segment between amino acids 117 and 137 of 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-interacting sequence in G protein.

Another question to be answered is how general is the requirement of His protonation for pH-dependent viral membrane fusion. In the case of Influenza virus, for example, the participation of HA N-terminal peptide in fusion is very well established, although this peptide does not contain His residues. In this case, however, the fusion occurs at pH 5.0, in which protonation of acidic amino acids could take place. Another possibility that could not be discarded so far is that the protonation of His residues in other regions of the fusion protein could affect the overall protein structure leading to the exposure of the fusion peptide.

We have recently shown that G protein-membrane interaction is highly dependent on the presence of PS, a negatively charged phospholipid, in the target membrane (9). In addition, we have found that G protein conformational changes as well as VSV-mediated fusion are driven by electrostatic interactions. Based on the results showed here, we believe that the protonation of His residues could generate positive charges on G protein, which might contribute to the electrostatic interactions required for protein insertion in membrane during fusion.

Heptad repeats play an important role in many viral membrane fusion processes. Three-dimensional structure of fragments from several viral fusion proteins, including
Influenza HA, Moloney leukemia virus TM subunit, HIV-1 gp41, Ebola virus GP2 and SIV gp41, have been determined (26-30). The results obtained revealed that these proteins adopt a post-fusion hairpin structure formed by the interaction of N-terminal and C-terminal heptad-repeat segments, which generates a trimeric coiled-coil (31). For Sendai virus, heptad repeats were shown to bind phospholipid membranes with high affinity, probably assisting in bringing viral and cellular membranes closer (32,33). Indeed, studies using synthetic peptides supported a direct role of the N-terminal heptad repeat in Sendai virus fusion event (34). The G protein from all rhabdoviruses also presents heptad-repeats (14), which were mapped as the PS-binding domain of this protein (17). We showed here that a synthetic peptide corresponding to VSV G protein heptad repeat, the p2-like peptide, was very efficient in mediating pH-dependent fusion of PS-containing vesicles, which, as found for the whole virus, was inhibited by treatment of the peptide with DEPC. p2-peptide from VHSV, another rhabdovirus, was also able to induce membrane fusion (16). These results together suggest that p2-peptides play an active role in rhabdoviral fusogenic process. Whether they can be considered the actual rhabdovirus fusion peptide or not depends on further investigation.

A common feature of several viral fusion glycoproteins is that they are synthesized as a fusion-incompetent precursor that is cleaved to generate the fusogenic protein. The fusion machinery from rhabdovirus is completely different. The fusion occurs through reversible conformational changes that do not require activation by proteolytic cleavage (35,36). Our previous results showed that VSV G protein underwent a dramatic loss of secondary structure at the fusogenic pH, which was shown to be necessary for fusion (8). The loss of secondary structure during fusion seems to be another particular feature of rhabdovirus fusion, since most of viral fusion peptides adopt an \( \alpha \)-helical structure when
inserted in the lipid bilayer, which is necessary for their fusogenic activity (21-24, 37). In the case of HIV-1, however, it is hypothesized that the fusion peptide underwent conformational transitions from $\alpha$-helix to $\beta$-structures when bound to the target membrane (38-40), suggesting that fusion may require conformational flexibility of the fusion peptide itself. The results described here suggests that, at least in the case of VSV, the structural transitions that drive fusion reaction depend on His protonation.
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REFERENCES


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**Table II:** MALDI-TOF mass spectrometry analysis of DEPC-modified peptides from VSV G protein

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H: possible sites of modification
FIGURE LEGENDS

Figure 1: *His modification by DEPC impairs VSV-induced membrane fusion.* Equal amounts of unlabeled vesicles and vesicles labeled with 10-PyPC were incubated with purified VSV (●), or VSV pre-incubated with 0.005 (∆), 0.01 (□) and 0.02 (○) mM DEPC. The vesicles were composed of PC:PS (1:3) and were prepared in 20 mM MES, 30 mM Tris buffer, pH 6.0, in a final phospholipid concentration of 0.1 mM. VSV-induced membrane fusion was measured by the decrease in the 10-PyPC excimer/monomer fluorescence ratio. 10-PyPC was excited at 340 nm, and the intensities were collected at 376 nm and 480 nm for monomer and excimer, respectively. The final protein concentration was 15 µg/ml.

Figure 2: *VSV modification with DEPC.* (A) Purified VSV was diluted in 20 mM MES, 30 mM Tris buffer, pH 7.5 and incubated with different concentrations of DEPC. The formation of carbethoxyhistidyl residues was followed by the increase in the absorbance at 240 nm. The final protein concentration was 35 µg/ml. (B) Kinetics of VSV His modification with 0.02 mM DEPC. The final protein concentration was 140 µg/ml.

Figure 3: *Reversal of DEPC-induced modification in VSV G protein His residues with hydroxylamine.* Equal amounts of unlabeled vesicles and vesicles labeled with 10-PyPC were incubated with purified VSV (●), VSV pre-incubated with DEPC 0.02 mM (○), or VSV pre-incubated with DEPC 0.02 mM for 3 min and then incubated with hydroxylamine...
400 mM for 15 min (▲). The final protein concentration was 15 µg/ml. Other experimental conditions as in Fig.1.

**Figure 4:** *pH-induced conformational changes on VSV G protein involve His protonation.*

(A) Purified VSV (●), or VSV pre-incubated with 0.002 mM DEPC (Δ) or 0.02 mM DEPC (○) were diluted in 20 mM MES, 30 mM Tris buffer, pH 7.5, to a final protein concentration of 25 µg/ml. Tryptophan fluorescence emission at 334 nm was recorded while pH was gradually acidified by addition of HCl. The excitation wavelength was 280 nm. (B) Kinetics of G protein interaction with liposomes at low pH was measured by intrinsic fluorescence of purified VSV (●), or VSV pre-incubated with 0.02 mM DEPC (○). Vesicles composed of PC:PS (1:3) were prepared in 20 mM MES, 30 mM Tris buffer, pH 6.0, in a final phospholipid concentration of 0.1 mM. The excitation wavelength was 280 nm and the emission was collected at 334 nm. The final protein concentration was 25 µg/ml.

**Figure 5:** *Amino acid sequences of the peptides used in this study.* The putative VSV fusion peptide corresponds to the G protein sequence between residues 117 and 137. VSV p2-like peptides used in this study correspond to VSV G protein residues between 145 and 168. His 148 or 149, or both were substituted for Ala residues. Dots represent wt residues. Peptides corresponding to other G protein sequences between residues 65 and 85; 170 and 190; and 395 and 418 were used as control peptides containing two His.
**Figure 6:** Role of p2-like peptides in VSV-induced membrane fusion. Equal amounts of unlabeled vesicles and vesicles labeled with 10-PyPC were incubated with purified VSV (A), VSV p2-like peptide (B), or VSV peptide 117-137 (C). The vesicles were prepared in 20 mM MES-30 mM Tris buffer in a final phospholipid concentration of 0.1 mM. Membrane fusion was measured by the decrease in the 10-PyPC excimer/monomer fluorescence ratio. 10-PyPC was excited at 340 nm, and the intensities were collected at 376 nm and 480 nm for monomer and excimer, respectively. The vesicles used were composed of PC:PS (1:3) at pH 6.0 (●); PC:PS (1:3) at pH 7.5 (○); and PC only at pH 6.0 (▲). The final viral protein concentration was 15 µg/ml and peptide concentration was 10 µg/ml (●,○,▲) or 0.2 µg/ml (■).

**Figure 7:** His 148 and His 149 are important for peptide-induced membrane fusion. (A) Membrane fusion induced by p2-like peptide (●), peptide pre-incubated with 0.02 mM DEPC ( ), or 0.2 mM DEPC (○), or peptide pre-incubated with 0.2 mM DEPC for 3 min and then incubated with hydroxylamine 500 mM for 15 min (▲). (B) Effect of His substitution on p2-like peptide-induced fusion. Membrane fusion activity was evaluated for wt p2-like peptide (●), H148A (▲), H149A (■), and H148,149A double-mutant peptide (▼). (C) Membrane fusion activity of VSV peptide 65-85 (○), 170-190 (□) and 395-418 (▲). The final peptide concentrations was 10 µg/ml. Other experimental conditions as in Fig. 1.
Figure 1

Time (min)

Excimer/monomer fluorescence ratio
Figure 2
Figure 3
Figure 4

(A) pH vs. Fluorescence Intensity (A.U.)

(B) Time (min) vs. Fluorescence Intensity (A.U.)
Figure 5
Figure 6

Eximer/monomer fluorescence ratio vs. time (min) for different samples labeled A, B, and C.
Figure 7

Time (min)

Excimer/monomer fluorescence ratio

1.45

1.55

1.65

1.75

1.85

A

B

C

Time (min)

Time (min)

Time (min)
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