Membrane Fusion Induced by Vesicular Stomatitis Virus Depends on Histidine Protonation*

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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 for 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 His-148 and His-149 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.

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 particle interaction with its cellular receptor, and (ii) fusion of viral envelope and host cell plasma membrane after virus interaction 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 His-148 and His-149 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.

Vesicular stomatitis virus (VSV) is a member of the 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 proteins is that they bear a highly conserved hydrophobic fusion domain, which is most often located at the N terminus 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.

We have shown recently (8) 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. 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, because 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-directed 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, a rhabdovirus of salmonids, and it was named p2 peptide (14, 15). Viral hemorrhagic septicemia virus 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 peptide was found among all rhabdoviruses and contains two histidyl residues in VSV G protein (17).

To evaluate the role of G protein His residues in VSV-in-

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duced 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 His-148 and His-149 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—DEPC, phosphatidylethanolamine (PS) and phosphatidylcholine (PC) from bovine brain, trypsin from bovine pancreas, and phenylmethylsulfonyl fluoride were purchased from Sigma. 1-Hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (10-PyPC) was purchased from Molecular Probes Inc., Eugene, OR. 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 containers containing 150 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml ampicillin, 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 plaque-forming unit/ml. The cultures were kept at 37 °C for 16–20 h, and the virus was harvested and purified by differential centrifugation followed by equilibrium sedimentation in a sucrose gradient as described elsewhere (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 sonication of 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 (~10 cycles). The vesicles used in this study were composed of PC and PS at a 1:3 ratio. For fusion assays, 1% 10-PyPC was incorporated in PC:PS vesicles by vortexing for 10 min.

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 because of the formation of dienine using a Hitachi U-2001 spectrophotometer. The final concentration of DEPC ranged from 0.005 to 0.02 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 6.5) 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 and 500 mM, respectively. For the control, a solution that 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 radiolabeled 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 because of 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 h at 37 °C. The reaction was stopped by addition of 0.1 mM phenylmethylsulfonyl fluoride. The tryptic peptides were separated from the remaining virus by filtration. For mass spectrometry analysis, aliquots of 10 μl of the digested sample were mixed with 1 μl of the matrix solution (a saturated solution of a-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 an average of five spectra was used. The masses obtained were searched against a protein data base containing the sequence of bovine trypsin and VSV G protein using the ExPaSy Molecular Biology Server (www.exasy.org). Searches were also done with the DEPC modification option turned on.

Peptides Synthesis—All peptides were synthesized by solid phase using the Fmoc UV-6-(N-methylmethoxycarbonyl) methodology, and all protected amino acids were purchased from Calbiochem-Novabiochem 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 semi-preparative HPLC using an Econosil C-18 column (30 μm, 22.5 × 250 mm) and a two-solvent system, Solvent A (trifluoroacetic acid/H2O) (1:1000) (v/v) and Solvent B (trifluoroacetic acid/acetonitrile/H2O) (1:900:100) (v/v/v). The column was eluted at a flow rate of 5 ml/min 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-visible detector coupled to an Ultrastard C-18 column (5 μm, 4.8 × 150 mm), which was eluted with a two-solvent system, Solvent A1 (H3PO4/H2O) (1:1000) (v/v) and Solvent B1 (acetonitrile/H2O) (900:100) (v/v) at a flow rate of 1.7 ml/min 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 (ToSpect-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-carbethoxyhistidinyl 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, whereas 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 carboxyhydrox 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 shown recently (8) that G protein interaction with liposomes at pH 6.0 resulted in dramatic protein conformational changes, which can be followed by intrinsic fluorescence. 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, whereas 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 Fig. 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 residues, 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 because of DEPC modification, and four modified peptides could be identified (Table II). Two of these peptides are in-
Role of His on VSV-induced Membrane Fusion

Table I

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Expected mass</th>
<th>Measured mass</th>
<th>Δ mass</th>
<th>Sequence</th>
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<td>7375.351</td>
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</tr>
<tr>
<td>32–87</td>
<td>6460.004</td>
<td>6459.685</td>
<td>0.319</td>
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</tr>
<tr>
<td>110–168</td>
<td>6588.162</td>
<td>6588.522</td>
<td>-0.360</td>
<td>CHASKWVTTCDFR</td>
</tr>
<tr>
<td>392–417</td>
<td>3028.443</td>
<td>3028.409</td>
<td>0.033</td>
<td>TSSGYKFPFLYMIHGMDSDLHLSSK</td>
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Table II

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<th>Measured mass</th>
<th>Δ mass</th>
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<th>Sequence</th>
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<tr>
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<td>6459.685</td>
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<td>110–168</td>
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<td>0.033</td>
<td>2</td>
<td>TSSGYKFPFLYMIHGMDSDLHLSSK</td>
</tr>
</tbody>
</table>

* Underlined letters indicate possible sites of modifications.

Inclusion of the membrane-interacting domain (32–87 and 110–168), suggesting that the active His are located within this segment.

Role of p2-like Peptide 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 segments of G protein identified by mass spectrometry. To evaluate the ability of both the p2-like 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, because it was found to be modified by DEPC treatment by mass spectrometry analysis (Table II).

Fig. 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 that 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 sequence alone is not able to catalyze fusion reaction and reinforces 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. 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 His-148 and His-149 for Ala residues abolished peptide activity, suggesting that His residues are 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 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.
putative fusion peptide failed to induce fusion. VSV p2-like peptide contains two His residues, whose protonation are 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 was 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 \( pK_a \) 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 induced 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 sequences 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 hemagglutinin 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 shown recently (9) that G protein-membrane interaction is highly dependent on the presence of PS, a negatively charged phospholipid, in the target membrane. 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 structures of frag-
ments from several viral fusion proteins, including influenza hemagglutinin, Moloney leukemia virus transmembrane (TM) subunit, HIV-1 glycoprotein 41, Ebola virus GP2, and simian immunodeficiency virus glycoprotein 41, have been identified (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 generate a trimeric coiled-coil (31). For Sendai virus, heptad repeat helices 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-like peptide from viral hemorraghic septicemia virus, another rhabdovirus, was also able to induce membrane fusion (16). These results together suggest that p2-like peptides play an active role in the rhabdoviral fusogenic process. Whether they can be considered the actual rhabdovirus fusion peptides 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, because most of viral fusion peptides adopt an α-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 α-helix to β-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 suggest 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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