Myristoylation, a Protruding Loop, and Structural Plasticity Are Essential Features of a Nonenveloped Virus Fusion Peptide Motif*

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Members of the fusion-associated small transmembrane (FAST) protein family are a distinct class of membrane fusion proteins encoded by nonenveloped fowl-pox virus. The 125-residue p14 FAST protein of reptilian reovirus has an ~38-residue myristoylated N-terminal ectodomain containing a moderately apolar N-proximal region, termed the hydrophobic patch. Mutagenic analysis indicated sequence-specific elements in the N-proximal portion of the p14 hydrophobic patch affected cell-cell fusion activity, independent of overall effects on the relative hydrophobicity of the motif. Circular dichroism (CD) of a myristoylated peptide representing the majority of the p14 ectodomain suggested this region is mostly disordered in solution but assumes increased structure in an apolar environment. From NMR spectroscopic data and simulated annealing, the soluble nonmyristoylated p14 ectodomain peptide consists of an N-proximal extended loop flanked by two proline hinges. The remaining two-thirds of the ectodomain peptide structure is disordered, consistent with predictions based on CD spectra of the myristoylated peptide. The myristoylated p14 ectodomain peptide, but not a nonmyristoylated version of the same peptide nor a myristoylated scrambled peptide, mediated extensive lipid mixing in a liposome fusion assay. Based on the lipid mixing activity, structural plasticity, environmentally induced conformational changes, and kinked structures predicted for the p14 ectodomain and hydrophobic patch (all features associated with fusion peptides), we propose that the majority of the p14 ectodomain is composed of a fusion peptide motif, the first such motif dependent on myristoylation for membrane fusion activity.

Complex, multimeric viral fusion proteins mediate the fusion of viral envelopes to target cell membranes during virus entry into cells (1). Membrane destabilization during the fusion process is dependent on a fusion peptide motif contained within these enveloped virus fusion proteins (2–4). Fusion peptides are moderately hydrophobic stretches of ~20 amino acids, frequently rich in glycine and alanine residues (3, 5, 6). In the case of the class I fusion proteins typified by influenza hemagglutinin (HA),1 human immunodeficiency virus gp41, and the F proteins of paramyxoviruses, the fusion peptide motifs are located at the N terminus of the fusion polypeptide (4). Conversely, these motifs are embedded internally within the amino acid sequence of the class II fusion proteins (e.g. alphaviruses and flaviviruses) and the G protein of vesicular stomatitis virus (VSV) (7, 8). Structural predictions for fusion peptides based on CD or infrared spectroscopy have yielded conflicting results (9–12) and are influenced by the different methods used for preparation of the water-insoluble, flexible fusion peptide (13). The properties of conformational flexibility and environmentally induced structural changes may represent essential features of fusion peptides, intimately linked to their function in the fusion process (4, 14, 15).

To deal with the issues of peptide solubility and environmental effects on peptide structure, Han et al. (16) used a host-guest peptide design to render the influenza virus HA fusion peptide water-soluble. This approach facilitated structural determination of the fusion peptide by NMR spectroscopy in the context of a hydrophobic environment (i.e. detergent micelles). The structure is characterized by an N-terminal α-helix (residues 2–10) and a turn (residues 11–13) at both neutral pH and the fusion-triggering low pH. Although the C-terminal arm of the fusion peptide does not form a regular structure at pH 7, it forms a short 310-helix (residues 14–18) at pH 5 (4, 16, 17). At low pH, both helices are amphipathic and place all bulky hydrophobic residues on the inner side of the V-shaped structure to create a hydrophobic pocket. The outer side of the N-terminal α-helix comprises a ridge of glycine residues that has been hypothesized to be an essential component of HA-induced fusion pore formation (4).

Aside from influenza HA, the only other defined atomic-resolution structures of fusion peptides are the “fusion loops” of the class II fusion proteins, determined in the context of the entire ectodomain of these proteins (18–20). Unlike the helical class I fusion peptides, the internal class II fusion loops appear to be extended, flexible structures in both the solvent-exposed and membrane-bound forms (19, 20). The hydrophobic pocket created by the helix-hinge-helix structure of the influenza HA fusion peptide is replaced in the class II fusion peptides by a hydrophobic bowl flanked by β-strands at the tip of the loop.

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1 The abbreviations used are: HA, hemagglutinin; FAST, fusion-associated small transmembrane; MD, molecular dynamics; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; E-COSY, exclusive correlated spectroscopy; HP, hydrophobic patch; RRV, reptilian reovirus; LUV, large unilamellar vesicle; DOPC, 1,2-dioleoyl-sn-glycerol-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine.

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The loop structure may assume the same basic conformation in different environments, contrary to the significant pH- and membrane-dependent changes in the structure of the class I HA fusion peptides (4, 19, 20).

The reovirus fusion-associated small transmembrane (FAST) proteins are a new family of membrane fusion proteins, the only fusion proteins encoded by nonenveloped viruses (21–23). The FAST proteins represent a distinct class of membrane fusion proteins that are easily distinguished from the enveloped virus class I and class II fusion proteins by their exceedingly small ectodomains (~20–40 residues) (21–23). Furthermore, as nonstructural viral proteins, the FAST proteins do not play a role in virus-cell fusion but instead are dedicated to promoting cell-cell fusion and multinucleated syncytium formation following their expression in transfected or virus-infected cells (21, 22).

The reptilian reovirus (RRV) p14 FAST protein is a 14-kDa protein with an ~38-residue ectodomain that is myristoylated at its N terminus (23). We now demonstrate that the p14 ectodomain displays myristoylation- and sequence-specific lipid mixing and cell-cell fusion activity. Structural analyses of ectodomain peptides by NMR and CD spectroscopy revealed that the p14 ectodomain possesses structural plasticity, and a moderately apolar region near the N terminus of the p14 ectodomain contains an extended loop structure flanked by proline hinges. We suggest that over half of the small p14 ectodomain comprises an N-terminally myristoylated fusion peptide motif with a protruding loop that is intimately involved in the membrane fusion process.

EXPERIMENTAL PROCEDURES

Cells, Virus, and Antibody—Reptilian reovirus was isolated from the kidney of a python (Python regius) (24) and obtained from W. Ahne (University of Munich, Munich, Germany). Vero and Q5M cells were maintained in medium 199 as previously described (25). The production of p14 polyclonal antiserum was described previously (23). All constructs were confirmed by cycle sequencing (Thermo-Biosciences/ml leucine-free medium (Invitrogen Select-Amine kit). Radiolabeled cell lysates were prepared using radiotracer precipitation buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Igepal Sigma), 0.5% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, and protease inhibitors (200 mM aprotinin, 1 mM leupeptin, and 1 mM pepstatin) as described previously (23). Cell lysates were immunoprecipitated using rabbit polyclonal anti-p14 serum, and precipitates were analyzed by SDS-15% PAGE and Me2SO-PPO fluorography as described previously (23).

Fluorescent Cell Staining—Q5M cells seeded on coverslips were transfected with p14 expression plasmids, and 6 h post transfection, cell monolayers were fixed with ice-cold methanol and stained using rabbit polyclonal anti-p14 and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson Immunochemicals) as described previously (23). Stained cells were visualized and photographed using a Zeiss LSM510 scanning argon laser confocal microscope and the >10 objective.

Circular Dichroism—The CD spectra were recorded using an Aviv Model 215 circular dichroism spectrometer (Proterion Corp., Piscataway, NJ). The peptide sample at concentrations of 10–80 μM in water was contained in a 1-mm path-length quartz cell that was maintained at 25 °C in a thermostated cell holder. For samples containing lipid, the lipid was first made into a dry film from a solution of chloroform and methanol. The film was hydrated by vortexing and freeze-thawing, and the suspension was then denatured to clarify small unilamellar vesicles. Data were analyzed with the Self Consistent Method for CD Analysis, version 3 (Selcon 3) (25). The CD data are expressed as the mean residue ellipticity. Secondary structure was estimated with the program Selcon3.

Lipid Mixing Assay for Membrane Fusion—The ability of p14 peptides to promote lipid mixing was assessed using the resonance energy transfer (RET) assay of Street et al. (26) with large (0.1–0.2) unilamellar vesicles (LUVs) composed of 1,2-dioleyl-sn-glycerol-3-phosphocholine (DOPC), 1,2-dioleyl-sn-glycerol-3-phosphoethanolamine (DOPE), and cholesterol (1:1:1 molar ratio) (Avanti Polar Lipids) as described previously (27). One population of LUVs was labeled with 2 mol % each of N-(N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine and 6-nitro-2,1,3-benzo[d]1,3-benzoxadiazol-4-yl)phosphatidylethanolamine, and a 9:1 mixture of unlabeled liposomes was used. Fluorescence was recorded in an SLM Aminco Bowman Series II spectrofluorimeter (Fig. 7, A and B) or a Varian Cary Eclipse spectrofluorimeter (Fig. 7, C), with the excitation and emission wavelengths set at 465 and 530 nm, respectively. A freshly prepared solution of the peptide in water (the p14 peptide precipitated in the presence of salt) or Me2SO was added to 2 ml of water in the cuvette containing 25, 50, or 100 μM LUVs for several minutes, and then the fluorescence was recorded. Initial fluorescence intensity prior to addition of p14 peptide (or using Me2SO alone) was set to 100 fluorescence units.

Solution NMR Spectroscopy—Initially, two samples were prepared by dissolving 5 mg of lyophilized acetyl-p14 peptide or 0.5 mg of myristoylated p14 peptide in a 500-μl solution of 90% H2O and 10% D2O buffered at pH 7.2 with 50 mM sodium phosphate. 1H NMR data sets (one-dimensional, one-dimensional WATERGATE (28), TOCSY (29), 100- and 250-ms WATERTAGE NOESY (30), and WATERTAGE E-COSY (31)) were collected at 5 °C on a Bruker AVANCE 500 spectrometer. A further six one-dimensional NMR data sets were recorded between the pH range of 3 and 10 for each of the two samples for 0 and 450 mM NaCl. The pH values for one-dimensional 1H NMR data were determined from a standardized pH electrode designed for NMR tubes. All data sets were processed using Bruker XWIN software on a Silicon Graphics workstation.

NMR Structure Calculations—For the acetyl-p14 peptide, the majority of the distance constraints were obtained from the 250-ms NOESY spectra. Cross-peak intensities were classified as very strong, strong, medium, or weak and roughly corresponded to interproton distance ranges of <2.0, 2.0–2.75, 2.75–3.5, and 3.5–5.0 Å. In cases where stereospecific assignments were not available, distances were adjusted according to the pseudoequator position. A total of 148 unique inter-residue constraints (114 sequential, 27 intermediate range (i + 2, i + 4), and 10 long range) were obtained with 185 unique inter-residue NOEYs that aided with the confirmation of some side chains. The E-COSY spectrum was used to determine the NH-HN and Hα-Hα 3JHNH' couplings, which are necessary for determining NH-Hα and Hα-Hα dihedral angles. All structural calculations were performed based on previous procedures (32, 33) using the XPLOR (34–36) soft-
defined, acceptable regions of the Ramachandran plot. With the exceptions of the random coil overall quality of these refined structures was examined with the pro-
tations, peptide dihedral angles (61) were forced to a trans-configuration
were scaled to their full values. In the last five steps, the temperature
conjugate gradient before proceeding to the restrained simulated an-
generated a total of 100 embedded structures. To remove close con-
ware package. Briefly, an initial extended structure was built and used
to generate a total of 100 embedded structures. To remove close con-
contacts, the embedded structures were minimized with 1000 steps of
couple gradient before proceeding to the restrained simulated an-
nealing molecular dynamics (MD) calculations. During MD calculations,
peptide dihedral angles (61) were forced to a trans-configuration for all residues. A nonbonded cut-off distance of 4.5 Å was used. A total
of eight steps with a total simulation time of 120 ps was used for the MD simulations. At the beginning, all of the force constants for bonded, NOE, dihedral angle, and nonbonded interactions were scaled down from their full values. By the end of the third step, the force constants were scaled to their full values. In the last five steps, the temperature was decreased uniformly from 1000 to 300 K. The calculated structures were minimized with 2000 steps of conjugate gradient methods. A total of 14 structures were retained that had no violations of NOE constraints >0.4 Å or dihedral angle constraints >30° (Fig. 5A). The overall quality of these refined structures was examined with the program PROCHECK (37–39). With the exception of the random coil (Gly14-Thr15) all of the backbone dihedral angles reside in the well defined, acceptable regions of the Ramachandran plot.

RESULTS
Features of a Potential Fusion Peptide Motif in the Small Ectodomain of p14—The only region of the RRV p14 FAST protein that possesses any hydrophobic character, aside from the transmembrane domain, is the N-terminal 21 residues of the ectodomain (Fig. 1), a region we termed the hydrophobic patch (HP). This region also contains a high percentage of glycine and alanine residues (6 of 20 = 30%) typical of fusion peptides (1). In addition, insertion of an HA epitope tag between residues seven and eight, or substitution of Val9 with serine, eliminated p14-induced syncytium formation (23) indi-
cating this region is sensitive to mutation and important for membrane fusion activity. These results lead us to suspect that the p14 HP might serve as a fusion peptide. However, the p14 HP is considerably less hydrophobic than what is typically observed for fusion peptides of enveloped viruses (1). Furthermore, analysis using the whole-residue hydrophobicity scale of Wimley and White (40), which predicts the preference of a region of a peptide chain to partition into the hydrated inter-
facing of a lipid bilayer, also led to the question of whether the p14 HP has inherent membrane interaction potential. Therefore, we conducted a more comprehensive structural and functional analysis of the p14 ectodomain to more clearly define the properties and role of the p14 HP in membrane fusion.

The p14 Ectodomain Hydrophobic Patch Exerts a Sequence-specific Effect on Membrane Fusion—Although there is little direct sequence conservation in the fusion peptides of different enveloped viruses, these motifs are frequently sensitive to even conservative substitutions (5, 41). To more fully investigate the importance of the p14 HP in the mechanism of p14-mediated fusion, amino acids within this region were altered in the context of the whole p14 protein. All 12 of the altered p14 constructs were expressed to similar levels in transfected cells, as determined by radiolabeling and immunoprecipitation with polyclonal anti-p14 antiserum (Fig. 2B). Furthermore, a qualitative assessment of surface localization by immunofluorescence also revealed that all of the mutant p14 constructs were trafficked to the plasma membrane of transfected cells, as demonstrated for a subset of p14 constructs in Fig. 2D. Therefore, decreased fusion activity due to any of the substitutions was unlikely to be the result of altered upstream steps in p14-induced syncytium formation (e.g. protein expression or trafficking to the plasma membrane).

The ability of each construct to mediate membrane fusion was assessed by quantifying the formation of multineucleated syncytia in transfected Vero cells, and the results were expressed as a percent of authentic p14 fusion ability (Fig. 2C). Several observations were noted from this mutagenic analysis. First, the fusion ability of each construct did not always correlate with the overall hydrophobicity of the hydrophobic patch (residues 2–21); fusion did not necessarily increase with a more hydrophobic motif or decrease with a more hydrophilic one. For example, the N10A and H11A constructs were both more hydrophobic than authentic p14 and yet were fusion-impaired (Fig. 2C). These results suggest that the p14 ectodomain HP exerts a sequence-specific role(s) in the fusion process.

Second, although certain residues were tolerant to substitution, residues clustered within the N-proximal portion of the p14 HP were particularly sensitive to substitution. For example, the P13A, G14A, and E15A substitutions all displayed normal levels of fusion activity (Fig. 2C). Conversely, the H11A substitution was only partially tolerated; although the rate of fusion mediated by H11A was significantly decreased (Fig. 2C), syncytium formation progressed to completion (e.g. fusion of the entire cell monolayer) at later times (data not shown). Substitution of His11 with arginine or glutamic acid eliminated fusion activity, further underscoring the importance of this histidine to p14 function. Asn10 and Gly2 were also sensitive to substitution, with the N10A and G2A p14 constructs displaying no fusion activity (Fig. 2C). The role of Gly2 in p14 fusion activity reflects its status as the target for myristoylation, a post-translational modification essential for p14-induced cell-cell fusion (23). The relevance of Asn10 to p14 function is currently unknown and is the subject of ongoing NMR structural characterization studies.

The third notable observation was the involvement of an aliphatic residue near the N terminus of the p14 HP. As re-
ported recently (23), the V9T substitution abolished fusion activity (Fig. 2C) while creating an N-linked glycosylation site in the p14 ectodomain that is functional (Fig. 2B). Previous results demonstrated that tunicamycin effectively eliminated glycosylation of the V9T construct (23), however, treatment with tunicamycin did not restore fusion activity (Fig. 2A), suggesting it is the loss of the valine residue, and not the additional carbohydrate moiety, that contributes to the loss of fusion activity. The preference for a bulky aliphatic residue at this location was supported by the robust fusion activity of the V9I construct (Fig. 2C). In contrast, there appeared to be less of a requirement for the cluster of three aliphatic residues near the C terminus of the HP. The I17V and I17T constructs behaved like the H11A construct, displaying a reduced rate of syncytium formation (Fig. 2C) that still progressed to completion (data not shown).

Based on the mutagenic studies, we inferred that the N-terminal myristoylation site and several residues clustered within the N-proximal portion of the HP (Val9, Asn10, and His11) all play an important role in either the formation of a fusion-competent structure or the fusion process itself. The mutagenic analysis was not easily interpretable based on simple disruption of amphipathic secondary structures or a glycine/alanine ridge, features that have been reported as essential to the activity of the fusion peptide of HA (4). Modeling the p14 HP as α-helix revealed no clear periodicity in the distribution of small apolar residues or bulkier hydrophobic residues that would generate an amphipathic structure or a glycine/alanine ridge (Fig. 3A). An amphipathic structure was more evident when the HP was modeled as a β-strand, with clustering of glycine/alanine on one face and branched hydrophobic or charged/polar residues on the other face (Fig. 3A). However, the key substitutions (V9T, N10A, and H11A, H11E, and H11R) did not dramatically alter this possible sided structure, suggesting these substitution-sensitive residues exert a sequence-specific effect near the center of the p14 HP.

**Figs. 2. p14 constructs with site-specific substitutions in the hydrophobic patch alter fusion.** A, Vero cells were transfected with authentic p14 (a) or the fusion-minus p14-V9T construct (b) (Val-Thr substitution creates an N-linked glycosylation site at residue 7). Cells were immunostained at 20 h post-transfection using anti-p14 antisera to reveal the presence of antigen-positive syncytia (a) or single antigen-positive transfected cells (b). Cells in panel b were incubated in the presence of the glycosylation inhibitor, tunicamycin, to demonstrate that the loss of fusion activity of the V9T construct reflected the amino acid substitution and not the presence of the carbohydrate moiety. Scale bar = 100 μm. B, p14 constructs containing site-specific substitutions within the HP were transfected into QM5 cells. At 12 h post-transfection, cells were radiolabeled with 3H-leucine, immunoprecipitated with polyclonal anti-p14 antisera, and resolved by SDS-PAGE, and p14 was detected by fluorography. The slower migrating band in the V9T lane represents the glycosylated form of this p14 construct. C, The cell-cell fusion abilities of substituted constructs of p14 were quantified by a syncytial indexing assay. The average number of nuclei present in syncytia was determined by microscopic examination of five random fields; the results are expressed as the percent of authentic p14 fusion activity (left axis) ± the standard error. The horizontal line denotes the relative hydrophobicity of the HP of each construct as indicated on a normalized hydrophobicity scale (right axis). D, QM5 cells transfected with p14 constructs were assessed for plasma membrane localization by immunofluorescent microscopy using polyclonal anti-p14 and fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody. Mutant p14 proteins are identified by the nature and location of the substitution. The left-hand panels show fluorescent images, and the right-hand panels show the fluorescent images overlaid with the differential interference contrast image. Scale bar = 10 μm.
was examined. This peptide represents the majority of the p14 ectodomain (residues 2-31), including the p14 HP (residues 2-21) plus the 10 adjacent predominantly polar C-proximal residues to aid in peptide solubility and folding. The peptide was also myristoylated at the N-terminal glycine residue, as is the case for p14. CD spectra obtained from the myr-30HP peptide dissolved in water demonstrated a notable absence of the case for p14. CD spectra obtained from the myr-30HP peptide (10 μM) was performed using various concentrations of the peptide dissolved in water. C, CD spectroscopy of the myr-30HP (10 μM) was performed dissolved in water or 100 μM egg phosphotidylcholine (PC) liposomes (lipid/peptide ratio = 10). D, CD spectroscopy of the myr-30HP (10 μM) was performed in water or 56% trifluoroethanol (TFE).

FIG. 3. Secondary structure predictions of the p14 hydrophobic patch. A, the p14 hydrophobic patch (residues 4-21) modeled as an α-helix or β-strand. B, CD spectroscopy of the myr-30HP peptide was performed using various concentrations of the peptide dissolved in water. C, CD spectroscopy of the myr-30HP peptide (10 μM) was performed dissolved in water or 100 μM egg phosphotidylcholine (PC) liposomes (lipid/peptide ratio = 10). D, CD spectroscopy of the myr-30HP peptide (10 μM) was performed in water or 56% trifluoroethanol (TFE).

TABLE I

| Assay condition | % α-Helix | % β-Sheet | % Turn | % Random |
|-----------------|----------|----------|-------|---------|
| 10 μM in water  | 5        | 8        | 5     | 90      |
| 40 μM in water  | 7        | 37       | 23    | 33      |
| 80 μM in water  | 7        | 37       | 23    | 33      |
| 10 μM in egg PC | 24       | 23       | 30    |         |
| 80 μM in egg PC | 22       | 30       | 24    |         |
| 10 μM in 56% TFE| 33       | 22       | 15    | 30      |

All spin systems were identified through their characteristic chemical shifts (44–47) and TOCSY cross-peak patterns (Fig. 4A). Residues Phe^8, His^13, Ile^17, Leu^21, and Asp^26 were easily assigned, as each residue occurs only once in the sequence. TOCSY-detected aromatic ring spin systems exhibited NOEY cross-peaks to weakly coupled three-spin system, and residues were thus assigned to Phe^8 and His^13 (Fig. 4B). Starting from these five residues, determination of the proton intra-residue NOEY connections (through Nᵢ-Nᵢ₋₁, α-Nᵢ₋₁, and/or β-Nᵢ₋₁) lead to the sequence-specific assignment of the backbone and side chain residues 3–31. Gly^2 is the only residue that could not be uniquely identified. No portion of the peptide could be identified as α-helical through typical α-Nᵢ₋₁ or β-Nᵢ₋₁ connections, confirming our CD analysis. There were a number of dipolar contacts between distant residues (Ser^6 and Pro^13, Asn^7 and Pro^13, Phe^8 and Pro^13, Val^10 and His^11, and Glu^15 and His^13) that were observed in duplicate NOEY experiments (Fig. 4B), indicative of a “more defined” structure in this region. In contrast, no long range connections were observed among residues 14–31.

NMR Analysis Suggests the p14 HP Contains an Extended Loop—Solution NMR spectroscopy was used to provide additional insight into why residues clustered near the central portion of the p14 HP exert a sequence-specific effect on p14 fusion activity, while at the same time CD analysis predicts the p14 ectodomain is mostly disordered. A nonmyristoylated version of the 30-residue N-terminal peptide was used for NMR structural characterization, the myristoylated myr-30HP version of this peptide formed micelles that hindered structural analysis.
There are two distinct features of the calculated structure (Fig. 5). First, the lack of dipolar contacts for residues Gly14–Thr31 led to calculated structures for this region that were essentially random coils (Fig. 5A). The second feature is the likely presence of a "loop structure" that incorporates residues Pro5–Pro13 (Fig. 5B). Examination of the calculated loop structures suggested the extended loop might be stabilized by hydrogen bonds between the side chain carbonyl and imidazole NH groups of Asn7 and His11 and between the backbone carbonyls and amides of Val9 and His11 (Fig. 5B). This structural prediction was in line with results obtained from bioinformatic and CD studies that predicted extensive flexibility in the p14 ectodomain and with the mutagenic results, in the context of the whole protein, that implicated Val9 and His11 in p14 structure and/or function. Regardless of salt concentration or pH, the one-dimensional NMR spectra of the acetylated or myristoylated p14 peptides were virtually identical. 1H chemical shifts are diagnostic of structural elements, and the lack of change in the chemical shifts is indicative of the lack of structural change within the peptide on changing pH or salt concentration (representative spectra shown in Fig. 6).

**FIG. 4. Two-dimensional NMR spectra.** A, low field region of the 500-MHz 1H NMR clean TOCSY spectrum (80 ms) mixing time recorded at 5 °C of the acetyl-p14 peptide in a solution of 90% H2O and 10% D2O buffered at pH 7.2 with 50 mM potassium phosphate. B, low field portion of the 500-MHz NOESY spectrum (mixing time 250 ms) illustrating assignments and cross-peaks for the long range connections within the loop structure. Similar long range NOESY connections were observed in the 100-ms NOESY spectrum.

**FIG. 5. NMR structural predictions of the p14 ectodomain.** The overlaid 14 nonviolating structures from MD simulations with dipolar and dihedral constraints determined from 250-ms NOESY and E-COSY NMR spectroscopy experiments. A, an overlay for the entire peptide. B, an expansion of the boxed region from A, which focuses on the loop structure (Pro5–Pro13) as shown from behind. The possible hydrogen bonds (dotted lines) that may help stabilize this structure are indicated.

The p14 Hydrophobic Patch Mediates Lipid Mixing—The sensitivity of the p14 HP to amino acid substitutions, the predicted presence of a protruding loop structure, and indications that the p14 HP and/or flanking region are flexible are all observations consistent with this region of p14 serving an essential role in the membrane fusion process. To determine whether the p14 HP exerts membrane destabilization activity, another generally accepted feature of fusion peptides (3), versions of the myr-30HP peptide were tested for their ability to mediate lipid mixing in an in vitro assay. The myr-30HP peptide was added to large unilamellar vesicles (DOPC:DOPE:cholesterol ratio 1:1:1), and lipid mixing was quantified by a resonance energy transfer assay (26). When dissolved in either water (Fig. 7, A and B) or Me2SO (Fig. 7C), the myr-30HP peptide mediated efficient lipid mixing in a dose-dependent manner. No lipid mixing occurred between vesicles in the absence of peptide (Fig. 7A) or when Me2SO alone was added to the assay (Fig. 7C). A myristoylated scrambled version of the myr-30HP peptide exhibited no lipid mixing activity (Fig. 7C), indicating that the lipid mixing observed with myr-30HP was dependent on the specific sequence context of the ectodomain peptide and was not solely due to the hydrophobic nature of the myristate moiety. The amino acid residues of the hydrophobic patch were not, however, sufficient to induce lipid mixing on their own, as the nonmyristoylated version of the myr-30HP peptide did not mediate lipid mixing (Fig. 7C). Based on these results, we propose that the p14 HP may function in an analogous manner as the fusion peptides of enveloped virus fusion protein, inducing the lipid rearrangements required for membrane fusion. However, the combined properties of an atypical amino acid sequence, an unusual loop structure, and the requirement for acylation distinguish the RRV p14 HP from all previously described fusion peptide motifs.

**DISCUSSION**

**Features of a Predicted p14 Fusion Peptide—**Recent structural studies of enveloped virus fusion peptides have contributed to the concept that membrane destabilization reflects the
need for structural plasticity in the fusion peptide. Inherent flexibility may be required to facilitate dynamic interactions between “protruding” peptide structures and lipid bilayers or other hydrophobic peptide motifs (e.g. the transmembrane domain) (3, 4). These properties of enveloped virus fusion peptides are shared, to various degrees, with the p14 HP. Mutagenic studies indicated the p14 HP is an essential component of the mechanism of p14-mediated fusion (Fig. 2), lipid-mixing assays revealed the p14 ectodomain has membrane-destabilizing activity (Fig. 7), NMR spectroscopy indicated the presence of a loop between Pro5 and Pro13 (Fig. 5), and CD and NMR spectroscopy suggested the ectodomain is flexible and/or structurally dynamic (Figs. 3 and 5). We therefore propose that the small, N-terminal ectodomain of the p14 FAST protein comprises an N-terminal fusion peptide motif linked via a short flexible or structurally dynamic region to the transmembrane domain and the remainder of the protein.

The p14 HP, however, possesses several features that distinguish it from the typical enveloped virus fusion peptides, most notably, an essential requirement for myristoylation and an atypical amino acid content. In contrast to the class I and II enveloped virus fusion peptides, the p14 HP is only moderately hydrophobic and is predicted to have a thermodynamically unfavorable likelihood of inserting into the lipid bilayer or bilayer interface using the whole-residue hydrophobicity scale of Wimley and White (40). The concept that p14 HP membrane interactions are dependent on more than just the hydrophobic nature of the amino acid residues is supported by the lack of a direct correlation between overall hydrophobicity of the p14 HP and cell-cell fusion activity (Fig. 2), as well as by the inability of the nonmyristoylated versions of the p14 protein or ectodomain peptide to induce cell-cell membrane fusion (23) or lipid mixing (Fig. 7), respectively. We propose that the myristate moiety and/or the predicted protruding loop may compensate for the low overall hydrophobicity of the p14 HP and function to promote membrane interaction. As far as we are aware, the p14 HP is the first example of a naturally existing fusion peptide-like motif that is dependent on both sequence-specific residues and a myristate moiety for efficient membrane interaction and destabilization.
**Relationship of the p14 HP to Enveloped Virus Fusion Peptides**—The p14 HP shares features with both the class I and class II fusion peptides of enveloped viruses. Similar to class I fusion peptides (4), the p14 HP is N-terminal and is joined to the remainder of the protein by a flexible polar region that is disordered in an aqueous environment, potentially allowing the fusion peptide to fold as an independent domain. However, whereas class I fusion peptides adopt a helix-hinge-helix structure, the p14 HP is predicted to form a fusion loop like the class II fusion peptides (19, 20). The exposed Phe<sup>8</sup> and Val<sup>9</sup> residues near the apex of the p14 HP loop may partially insert into the lipid bilayer, similar to the surface-exposed aromatic side chains in the extended loops of the class II fusion peptides which are proposed to form an “aromatic anchor” by embedding in only the outer leaflet of the lipid bilayer (20). Hydrogen bonds between exposed backbone carbonyls and amides (His<sup>11</sup> and Val<sup>9</sup>) or between polar side chains (His<sup>11</sup> and Asn<sup>7</sup>) are predicted to stabilize the p14 loop and may also be required to mask these polar groups, facilitating insertion of the loop into the membrane.

**Structural Plasticity of the p14 Fusion Peptide**—NMR and CD spectroscopy both predicted that much of the p14 ectodomain is disordered in solution but may assume increased structure, dependent on environmental effects, similar to the situation with other membrane fusion peptides (13, 15). The inherent structural plasticity of many fusion peptides is likely essential for their role as membrane destabilizing modules (14, 15). We propose that the p14 HP is no exception, requiring a dynamic structure in addition to a potential membrane-embedding loop in order to achieve maximal function as a membrane-destabilizing module.

Structural plasticity of the p14 ectodomain could serve one or more roles in the fusion process. The C-terminal disordered region (residues 15–31) may act as a flexible linker, facilitating conformational changes in either the structure or spatial arrangement of the p14 HP, similar to the proposed role of the linker region that connects the influenza HA fusion peptide to the remainder of the HA2 polypeptide (4). Based on the CD results, it is also possible that the C-terminal portion of the p14 HP (residues 15–21), which was disordered in the predicted NMR structure, assumes increased structure when placed in an apolar environment. Once again, a similar situation exists for the HA fusion peptide, in which the C-terminal portion undergoes a transition from a disordered structure to a short 3<sub>10</sub>-helix at the fusion-activating low pH (4, 16, 17). However, in contrast to the HA fusion peptide, the one-dimensional NMR spectra suggest that low pH does not serve as a trigger to induce essential conformational changes in the structure of the p14 HP, an observation consistent with the fact that p14-induced cell-fusion and p14 ectodomain peptide-induced lipid mixing occur at physiological pH. We are currently using NMR spectroscopy of membrane-embedded p14 to determine whether the disordered region of the p14 ectodomain serves as a flexible linker and/or assumes an altered structure in a membrane environment.

**Myristoylation and p14-induced Membrane Fusion**—The requirement for an N-terminal myristate on p14 was recently shown to be essential for p14-induced cell-cell fusion (23). The loss of myristoylation did not affect p14 membrane topology in the plasma membrane, suggesting the fatty acid residue may be intimately involved in the fusion process. This speculation is supported by the inability of the nonmyristoylated p14 ectodomain peptide to induce lipid mixing (Fig. 7). Although evidence defining the role of myristate in p14-induced membrane fusion is currently lacking, the nature of myristate interactions with polypeptides or membranes suggest the myristate could contribute to changes in either p14 ectodomain structure or to localized membrane structure during the fusion reaction. Myristoylation is known to contribute to the structural stability of many proteins (48–50). Although we know of no examples of myristoylated integral membrane proteins that utilize the modification for structural stability, it is conceivable that interactions between the myristate and apolar residues might serve to stabilize the structure of the p14 ectodomain. Structural analyses of other myristoylated peptides or proteins suggest that such amino acid-myristate interactions are more likely to stabilize an existing structure rather than generate an entirely new conformation (50–52). Myristate could also stabilize the p14 ectodomain structure via interactions with the outer leaflet of the membrane in which p14 is embedded, in a manner analogous to the association of N-terminal signal peptides with the luminal leaflet of the endoplasmic reticulum prior to their removal from the nascent protein by signal peptidase (53, 54). It is not difficult to envision how simultaneous anchoring of both the C and the N termini of the p14 ectodomain in the same membrane, mediated by the transmembrane domain and myristate, respectively, could contribute to stabilization of the p14 ectodomain structure. Furthermore, because the C<sub>14</sub> acyl chain of myristic acid does not stably associate with membranes (55), reversible myristate-membrane interactions could also promote altered conformations of the p14 ectodomain that may exist in aqueous versus membrane environments.

Aside from possible influences of the myristate moiety on p14 structure, the myristate could also alter membrane structure. Assuming that multiple p14 molecules cluster at the fusion site, as is the case with influenza HA (56), insertion of the single acyl chain of several myristates would be expected to contribute to localized perturbations in lipid packing. This theory is supported by the increased membrane permeabilization effected by myristoylated peptides and by the ability of other acyl groups to tilt the orientation of a membrane-embedded peptide relative to the plane of the bilayer (57, 58). Although further structural analysis of soluble p14 ectodomain peptides might be informative, a clear indication of the role of myristate in p14 structure will likely require determination of the structure of myristoylated and nonmyristoylated versions of the membrane-embedded p14 protein. Such studies are currently under way using NMR analysis of isotopically labeled p14 in model membranes.

**Implications on the Mechanism of p14-mediated Membrane Fusion**—Our present results suggest that the majority of the p14 ectodomain is composed of a fusion peptide motif linked to the remainder of the protein via a flexible linker. This is in stark contrast to the fusion peptides of enveloped viruses, which are a minor component of a much larger ectodomain. Consequently, it is highly unlikely that the mechanism of p14-mediated membrane fusion is dependent on extensive energy-releasing conformational changes, which are believed to be essential for enveloped virus-mediated membrane fusion. In the case of enveloped viruses, extensive structural remodeling of large ectodomains is clearly involved in regulating the exposure of the buried fusion peptide and in drawing the viral envelope and target membrane into close proximity prior to membrane fusion (2, 4). Because p14 is a nonstructural viral protein, it is not involved in virus-cell fusion but functions, in effect, as a “cellular” fusion protein to mediate only cell-cell fusion. We therefore propose that energy-releasing conformational changes within the small p14 ectodomain are unlikely to be involved in promoting close apposition of donor and target membranes, which may instead reflect the activity of cellular proteins involved in mediating close cell-cell contact. The p14
ectodomain, therefore, may have evolved to contain only those features required to effect merger of the closely apposed membranes. This fusion "module" appears to comprise three essential components: a protruding loop, a terminal myristate moiety, and a flexible or conformationally dynamic structure. We suggest that the N-proximal loop and/or the myristate may mediate the initial interactions of the p14 ectodomain with the membrane. Exposure of these apolar motifs could also be largely responsible for dehydrating the membrane interface, thereby removing one of the predominant forces in stabilizing the membrane bilayer structure. Subsequent interactions of bulky hydrophobic or hydrophilic residues would alter lipid packing and/or electrostatic repulsion of lipid head-groups, possibly aided by dynamic changes in the spatial orientation or structure of the p14 ectodomain. Whether such interactions drive the formation of specific fusion intermediates, as envisioned in the predominant stalk-pore model of membrane fusion (described in Ref. 2), or induce fusion intermediates that are dynamic and less ordered, as recently proposed in alternate models of the fusion process (4, 59, 60), remains to be determined.

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