Cell-Cell Membrane Fusion Induced by p15 Fusion-associated Small Transmembrane (FAST) Protein Requires a Novel Fusion Peptide Motif Containing a Myristoylated Polyproline Type II Helix*  

Received for publication, September 20, 2011, and in revised form, October 24, 2011 Published, JBC Papers in Press, December 24, 2011, DOI 10.1074/jbc.M111.305268

Deniz Top‡1,2, Jolene A. Read‡1,3, Sandra J. Dawe‡1,4, Raymond T. Syvitski$, and Roy Duncan‡5,‡5

From the Departments of 1Microbiology and Immunology, 2Biochemistry and Molecular Biology, and 3Pediatrics, Dalhousie University, Halifax, Nova Scotia B3H 4R2 and the 4National Research Council Institute for Marine Biosciences, Halifax, Nova Scotia B3H3Z1, Canada

Background: The p15 FAST protein mediates cell-cell fusion using a 19-residue ectodomain.

Results: The p15 ectodomain requires both an N-terminal myristate and a polyproline type II helix for membrane fusion activity.

Conclusion: The p15 ectodomain functions as a novel fusion peptide motif.

Significance: This novel fusion peptide provides new insights into the essential biological process of protein-mediated membrane fusion.

The p15 fusion-associated small transmembrane (FAST) protein is a nonstructural viral protein that induces cell-cell fusion and syncytium formation. The exceptionally small, myristoylated N-terminal ectodomain of p15 lacks any of the defining features of a typical viral fusion protein. NMR and CD spectroscopy indicate this small fusion module comprises a left-handed polyproline type II (PPII) helix flanked by small, unstructured N and C termini. Individual prolines in the 6-residue proline-rich motif are highly tolerant of alanine substitutions, but multiple substitutions that disrupt the PPII helix eliminate cell-cell fusion activity. A synthetic p15 ectodomain peptide induces lipid mixing between liposomes, but with unusual kinetics that involve a long lag phase before the onset of rapid lipid mixing, and the length of the lag phase correlates with the kinetics of peptide-induced liposome aggregation. Lipid mixing, liposome aggregation, and stable peptide-membrane interactions are all dependent on both the N-terminal myristate and the presence of the PPII helix. We present a model for the mechanism of action of this novel viral fusion peptide, whereby the N-terminal myristate mediates initial, reversible peptide-membrane binding that is stabilized by subsequent amino acid-membrane interactions. These interactions induce a biphase membrane fusion reaction, with peptide-induced liposome aggregation representing a distinct, rate-limiting event that precedes membrane merger. Although the prolines in the proline-rich motif do not directly interact with membranes, the PPII helix may function to force solvent exposure of hydrophobic amino acid side chains in the regions flanking the helix to promote membrane binding, apposition, and fusion.

The genus Orthoreovirus contains five recognized species, four of which induce cell-cell fusion and multinucleated syncytium formation (1). Additional fusogenic orthoreoviruses continue to be discovered (2), and the related genus Aquareovirus also contains members that induce syncytium formation (3). Nonenveloped viruses lack a lipid membrane and virus entry into cells does not involve a membrane fusion event. Thus, nonenveloped viruses generally do not encode membrane fusion proteins and as a result do not induce syncytium formation. The fusogenic orthoreoviruses are a rare exception to this rule. Studies over the past few years have identified the orthoreovirus and aquareovirus proteins responsible for syncytigenesis (2, 4–7). These fusion-associated small transmembrane (FAST) proteins define a new family of viral fusogens whose structural and functional features distinguish them from the well characterized fusion proteins of enveloped viruses, a para-

*This work was supported in part by grants from the Canadian Institutes of Health Research (CIHR).

†This article contains supplemental "Experimental Procedures" and Figs. S1–S4.

The atomic coordinates and structure factors (code 2LKW) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 These authors contributed equally to this study.

2 Supported by scholarships from the Nova Scotia Health Research Foundation (NSHRF) and the Cancer Research Training Program with funding from the Dalhousie Cancer Research Program.

3 Supported by scholarships from the CHIR and NSHRF and an Eliza Ritchie scholarship.

4 Supported by scholarships from the Natural Sciences and Engineering Research Council (NSERC) of Canada, the Killam Foundation, and the NSHRF.

5 To whom correspondence should be addressed: Dept. Microbiology and Immunology, Dalhousie University, 5850 College St., P.O. Box 15000, Halifax, Nova Scotia B3H 4R2, Canada. Tel.: 902-494-6770; Fax: 902-494-5125; E-mail: roy.duncan@dal.ca.

6 The abbreviations used are: FAST, fusion-associated small transmembrane; PRM, proline-rich motif; PPII, polyproline type II helix; FP, fusion peptide; HP, hydrophobic patch; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; NBD-DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-ylyl); Rho-DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl); DPC, dodecyl phosphocholine; DMSO, dimethyl sulfoxide; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; CD, circular dichroism; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine.
p15 Polyproline Helix Fusion Peptide
digm for the general process of protein-mediated membrane fusion (8). The unique features of the FAST proteins indicate their mechanism of action is unlikely to adhere to the tenets of the enveloped virus membrane fusion model, suggesting there are alternate mechanisms of protein-mediated membrane fusion.

The three different classes of enveloped virus fusion proteins have dramatically different structures but remarkably conserved structural changes occur during the fusion reaction. These virus structural proteins contain large, multimeric ectodomains that undergo complex conformational changes to drive virus-cell membrane fusion (9–11). An intermediate structure positions a fusion peptide (FP) at one end of the structure for partial insertion into the target membrane, whereas the opposite end is anchored in the viral membrane by the transmembrane domain. Collapse of the extended structure into a compact trimeric hairpin is presumed to pull the two membranes together and positions the fusion peptide and transmembrane domain at the same end of the structure, thereby driving membrane merger (8, 12). The helical N-terminal fusion peptides present in Class I viral fusion proteins, such as influenza hemagglutinin, form an amphipathic kinked helix or helical hairpin that exposes a hydrophobic face for membrane insertion and fusion (13–15). Some of the Class I fusogens, and all of the Class II and III fusogens, have internal FPs at the tips of elongated β-strands. These fusion loops position hydrophobic and aromatic residues at the tips of the loops for shallow insertion into the outer leaflet of the target membrane (16–21). Whether FPs serve merely as membrane anchors or function to destabilize the lamellar structure of the outer leaflet of the bilayer to induce the first stage of membrane fusion is still unclear (22, 23).

In contrast to the enveloped virus fusion proteins, the FAST proteins are all small (95–198 amino acids), single pass membrane proteins with very small N-terminal ecto- and C-terminal endodomains that function from both sides of the membrane to drive the fusion process (24). Unlike the enveloped virus fusogens, the FAST proteins are nonstructural viral proteins that are expressed inside virus-infected cells where they traffic to the plasma membrane to induce fusion of virus-infected cells with neighboring uninfected cells (5, 7). The FAST proteins have therefore specifically evolved to induce cell-cell, rather than virus-cell, membrane fusion. The structural limitations of the FAST protein ectodomains (only 19–43 residues) means they cannot induce membrane merger using complex structural rearrangements to pull membranes into close proximity. Instead, mutational studies indicate the FAST protein ectodomains, endodomains, and transmembrane domains are all directly involved in the fusion process (25–27). Specific functional motifs have been identified in each of these fusion modules, although each FAST protein fusion module has its own unique repertoire of these motifs. Chimeric studies demonstrate that various combinations of the fusion modules from the different FAST proteins can be assembled to generate functional chimeras, but not all combinations are tolerated (24, 26, 28). The FAST proteins are therefore modular fusogens, but specific combinations of functional motifs must be present in the different fusion modules on either side of the membrane to generate an active cell-cell fusogen. Defining the roles of these functional motifs is the focus of ongoing research efforts.

One such functional motif is the hydrophobic patches (HPs) present in each of the FAST proteins. These motifs are the only regions of the FAST proteins that possess an overall hydrophobic character (aside from the transmembrane domain), although they are actually more amphiphilic than hydrophobic (2, 4–7). The HP motifs are located in the ectodomains of the p10 and p14 FAST proteins, but in the cytosolic endodomains of the p13, p15 and p22 FAST proteins. In the case of p10 and p14, the ectodomain HPs possess functional features of FPs. Synthetic peptides of both motifs induce liposome-liposome lipid mixing indicating they have membrane destabilizing properties, and amino acid substitutions in these motifs have adverse effects on cell-cell fusion activity of the full-length FAST protein (29–31). The p14 HP contains a myristoylated, proline-hinged loop, whereas the p10 HP contains a cystine loop, and the apices of both of these loops contain a Phe-Val dipeptide, similar to the residues located at the tips of the enveloped virus fusion loops. The p10 and p14 FPs are not sequenced within a complex protein tertiary structure, nor are they likely to serve as membrane anchors because the FAST proteins cannot use mechanical energy provided by complex ectodomain structural rearrangements to promote close membrane apposition. Instead, the Phe and Val residues in the p10 and p14 FPs may be positioned to directly interact with a closely apposed target membrane, thereby inducing curvature changes or other alterations to the interfacial region of the membrane required for merger of the outer leaflets.

The p15 FAST protein of baboon reovirus contains the hallmark features of a FAST protein, including a single transmembrane domain that functions as a reverse signal anchor to direct an $N_{\text{external}}/C_{\text{internal}}$ membrane topology, an essential fatty acid modification (i.e. $N$-terminal myristoylation), a membrane-proximal polyanionic motif of no known function, and a HP (Fig. 1A). The p15 FAST protein has the smallest ectodomain (19 residues), and this fusion module has a proline-rich motif (PRM) and an overall hydrophilic nature with no apparent FP motif (5, 32). It is not at all apparent how this fusion module could function to promote cell-cell membrane fusion. To examine this issue, we used a combination of NMR and CD spectroscopy, synctium formation, peptide-induced lipid mixing assays, and peptide-liposome binding studies. Results indicate the p15 ectodomain functions as a novel FP motif to induce lipid mixing, dependent on both the N-terminal myristate and the presence of a polyproline type II (PPII) helix. Although residues in the PPII helix do not directly associate with membranes, this structure appears to be required to force exposure of flanking hydrophobic residues that do interact with membranes, stabilizing the weak myristate-membrane interactions and driving the fusion reaction.

**EXPERIMENTAL PROCEDURES**

**Cells, Transfections, and Antibodies**—QM5 and Vero cells were maintained as previously described (4). Cells growing in 12-well plates were transfected with Lipofectamine using the manufacturer’s protocol (Invitrogen), and supplemented with serum-containing medium 5 h post-transfection and incubated

---

**VOLUME 287 • NUMBER 5 • JANUARY 27, 2012**
for the indicated periods of time. The generation and specificity of the rabbit polyclonal p15-specific antisera has been previously described (32).

Cloning—The Stratagene QuikChange mutagenesis method was used to replace individual residues within the proline-rich motif (PPAPPP; residues 10–15). Authentic p15 was used as a template to make the following substitutions: Q9A, A12P, P13A, P15A, P11A/A12P, P10A/P14A, p15pro1 (AAAAA), and p15pro2 (AAAAAPA). The sequence of all constructs was confirmed.

Circular Dichroism Spectroscopy—The p15 ectodomain proline-rich motif (VQPPAPPNN) and a mutant version of this peptide (VQPPAAPANA) were obtained from Dalton Chemical Laboratories, Inc. at 95% purity. A poly-L-proline peptide was purchased from Sigma. All CD spectra were acquired using a Jasco J-810 spectropolarimeter with a 0.1-cm optical path. The p15 peptides were dissolved in phosphate buffer (10 mM, pH 7.0) to a final concentration of 0.2 mg/ml, unless otherwise noted. Spectra were obtained between 250 and 200 nm, and 10 scans were acquired and summed for each sample and the resulting spectrum was smoothed to remove residual noise. Measurements requiring different buffers were modified to include either 3 M urea or 4 M NaCl. Because of the absorbance and light scattering of the buffer components in these solutions at short wavelengths, measurements were taken to 210 nm.

Nuclei Staining and Syncytial Indexing—Transfected monolayers were fixed and stained with Wright-Giemsa stain (Diff-Quik), and the syncytigenic activity of various p15 mutants was quantified using a syncytial index assay as previously described (4), based on microscopic quantification of the numbers of syncytial nuclei present in five random fields of view.

Immunostaining and Immunoblotting—Transfected monolayers were methanol-fixed and immunostained using primary rabbit polyclonal anti-p15 antiserum as previously described (32). For immunoblot analysis, cells were grown in 10-cm dishes and lysed in RIPA buffer 8 h post-transfection. Equivalent protein loads of sample (as determined by a Lowry assay) were separated by SDS-PAGE and p15 was detected by immunoblotting as described previously (5).

Nuclear Magnetic Resonance Structure Calculations and Determination—Synthetic p15 peptides were dissolved in buffered 95:5 or 0:100% H2O/D2O with and without 300 mM perdeuterated dodecylphosphocholine (DPC-d45; 98 atom %D). The aqueous buffer used for sample preparation contained 50 mM K2HPO4/KH2PO4 at pH 6.0 or 8.0. For all samples, the concentration of peptide was 5 mM. One- and two-dimensional 1H NMR data sets were collected on a Bruker AVANCE 500 spectrometer and processed as previously described (33). For structure calculations, distance restraints determined from the integration of NOE cross-peaks (80 ms for myristoylated p15 peptide and 250 ms for non-myristoylated p15 peptide) were classified into four groups: strong, medium, weak, and very weak, corresponding to inter-proton distance ranges of <2.3, 2.0–3.5, 3.3–5.0, and 4.8–6.0 Å, respectively. All structural calculations were based on previous studies (34) and were performed using the XPLOR 3.1 software package. The overall quality of these refined structures was examined with the program PROCHECK. Except for random-coil sections, all backbone dihedral angles resided in the well defined, acceptable regions of the Ramachandran plot. For structural determination, spin systems were identified through chemical shifts and characteristic TOCSY cross-peak patterns (60 ms for myristoylated p15 and 120 ms for non-myristoylated p15). Sequence-specific assignments were determined as described previously (30, 34). The structure of the non-myristoylated p15 ectodomain at pH 6.0 was deposited with the Protein Data Bank (PDB code 2LKW).

Peptide-mediated Lipid Mixing—Liposomes were prepared as previously described (30) using a 1:1:1 molar ratio of 1,2-dioleoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and cholesterol (Avanti Polar Lipids) and extruded through a polycarbonate filter in a hand-held extruder (Avestin, Ottawa) to generate 100 nm liposomes. Fluorescent liposomes were similarly generated, except 4 mol % of DOPE in each liposome was replaced with 2 mol % each of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N’-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N’-(lissamine rhodamine B sulfonyl) (Rho-DOPE). The lipid concentration of each preparation was confirmed using a lipid-phosphorus assay (35). Non-fluorescent and fluorescent liposomes were mixed in a 9:1 ratio in HEPES-buffered saline (HBS: 150 mM NaCl, 10 mM HEPES, pH 7.4) to obtain a final concentration of 100 μM lipid. The peptides were dissolved in DMSO and assayed for lipid mixing in the indicated final concentrations, using the same volume of DMSO as a solvent control. For temperature shift experiments, peptide and liposomes were mixed together and incubated at 20°C for 0 or 10 min, then rapidly heated to 37°C by addition of an equal volume of buffer preheated to 56°C. Measurements were made at 37°C using an excitation wavelength of 460 nm and an emission wavelength of 535 nm. Maximum lipid mixing was determined as previously described (36), using liposomes containing NBD-DOPE and Rho-DOPE at the theoretical maximum dilution if 100% lipid mixing occurred (i.e. 0.2 mol % of each). Percent lipid mixing was determined using the formula (Fexp − Fmin)/(Fmax − Fmin), where Fexp represents experimental fluorescence, Fmin represents minimum fluorescence (zero fluorescence), and Fmax represents maximum fluorescence.

Liposome Binding and Turbidity Measurements—Optical densities of 100 μM liposome suspensions mixed with a 40 μM final concentration of the indicated peptides were monitored at 600 nm over time using a Varian Cary 50 UV-visible spectrophotometer. To quantify peptide binding to liposomes, peptide/liposome mixtures were adjusted to 30% (w/v) sucrose in a final volume of 100 μl, then overlaid with 200 μl of sucrose-free HBS. Samples were centrifuged at 240,000 × g for 30 min in a Beckman TLS-55 swing bucket rotor. The bottom (200 μl), middle (50 μl), and top (200 μl) fractions were harvested and analyzed by Tricine SDS-PAGE and Coomassie staining, or by analytical reverse phase HPLC on a Waters 2680 Separations Module using a water:acetonitrile (A:B) mobile phase gradient with solvent B progressing from 5 to 95% at a flow rate of 1 ml/min for 25 min (5-μm particle, 100 Å, 3.9 × 150-mm Waters Symmetry C18
The p15 Ectodomain Proline-rich Motif Is Essential for Cell-Membrane Fusion

Aside from an essential N-terminal myristate moiety (5), the only other notable feature of the 19-residue p15 ectodomain is a PRM of undetermined function (Fig. 1A). To investigate a possible role for this PRM in p15-induced membrane fusion, a p15 construct (p15pro1) was created in which the five Pro residues in the PRM (PPAPPP) were replaced by Ala. Microscopic examination of Giemsa-stained QM5 cells transfected with p15 or p15pro1 revealed that Ala substitutions of the PRM abrogated the ability of p15 to induce cell-cell membrane fusion and multinucleated syncytium formation (Fig. 1B). As previously reported (32), a p15G2A construct lacking the myristoylation consensus sequence was also defective for syncytogenesis. Immunoblotting indicated both mutant versions of p15 were expressed at similar levels as authentic p15 (Fig. 1C). To determine whether any particular residue(s) contained within the p15 PRM was essential, a series of constructs were created to substitute individual Pro residues with Ala. The preceding Gln residue at position 9 (Gln-9) was also changed to Ala, or the intervening Ala residue at position 12 (Ala-12) was converted to Pro. Each construct was transfected into cells and the extent of syncytium formation was quantified using a syncytial index based on the average number of syncytial nuclei present in random microscopic fields (32). Although the extent of syncytogenesis induced by the various mutants was variable at a given time point, all of the Pro substitution constructs retained substantial levels of cell-cell fusion activity and induced extensive syncytium formation, including a mutant that contained two Ala substitutions (PPAPP converted to APAPAP) (Fig. 1D). The slight differences in the rate of syncytogenesis could reflect either modest changes in expression levels or fusion activities of the different Pro mutants. The preceding Gln-9 and the intervening Ala-12 residues were also not required for cell-cell fusion. Thus, whereas the p15 PRM is essential for membrane fusion activity, no single Pro residue is required implying the functional importance of the p15 PRM reflects something other than its primary sequence.

The p15 PRM Exists in a Polyproline Type II Helix Conformation—PRMs frequently assume a PPII helix conformation. The PPII helix is an extended, left-handed helix composed of backbone dihedrals of $(\phi, \psi) = (−75^\circ, +145^\circ)$ and an axial translation of $3.2 \AA$. With precisely three residues per turn, amino acids that are three positions apart align along one side of the helix (37, 38). Circular dichroism (CD) spectroscopy is widely considered the most reliable method for the identification of PPII helix structures in peptides (39). The far-UV spectrum of a poly-L-proline peptide (Fig. 2A) demonstrates the two spectral features of a PPII helix; a weak positive maximum at $-228 \text{ nm}$ along with a negative maximum in the vicinity of 205 nm (40). The former is considered to be a hallmark feature of the PPII structure. To investigate whether the PPII structure is likely to occur in the PRM of p15, a synthetic peptide (VQPPAPPVPNAPFAVE) corresponding to residues 8 to 17 of the 19-residue p15 ectodomain was analyzed by CD spectroscopy, acquiring spectra between 250 and 200 nm. The spectrum of the peptide contained a maximum absorbance at 228 to 230 nm and a minimum at $-205 \text{ nm}$ (Fig. 2B). Similar spectral analysis was conducted on the p15 peptide in the presence of 3 M urea or 4 M NaCl. Due to the absorbance and light scattering in these solutions at short wavelengths, measurements were taken to 210 nm. The CD spectrum in the presence of 3 M urea led to an increase in intensity of the maximum at 228 nm, whereas 4 M NaCl reduced the maximum (Fig. 2B). Such enhancing and
The inhibitory effects of denaturant and high salt concentration, respectively, are diagnostic of peptides having PPII helix structure (41–43). The p15 PRM therefore assumes a PPII helix conformation in solution.

The p15 Ectodomain Comprises a Polyproline Type II Helix Flanked by Disordered N and C Termini—To examine structural features of the p15 PRM when present within the entire p15 ectodomain, synthetic peptides representing the 19-residue p15 ectodomain, either with or without a myristoylated N terminus, were analyzed by NMR spectroscopy in both aqueous and membrane mimetic (i.e. DPC) environments. In the aqueous environment, the myristoylated p15 peptide formed large aggregates, most likely due to the hydrophobic nature of the N-terminal myristate. The NMR signals from these samples were severely broadened and thus, structural analysis of the myristoylated peptide in an aqueous environment could not be performed. The myristoylated peptide in a DPC environment was soluble, as was the non-myristoylated peptide in both aqueous and DPC environments, allowing for NMR structural analysis under these three conditions. Based on structural constraints derived from the NMR NOESY data, the p15 ectodomain peptide contained a well defined PPII helix from Gln-9 to Pro-15 under all three experimental conditions (Fig. 3). The helix was determined by resolved, strong cross-peaks within the NOESY spectra between the \( \text{H}^2 \) protons of Proi-1 to the \( \text{H}^2 \) and \( \text{H}^2 \) protons of Proi. The strong cross-peaks for the \( \text{H}^2 \) to \( \text{H}^2 \) and \( \text{H}^2 \) to \( \text{H}^2 \) protons were equal in intensity (Fig. 4 A), indicative of a PPII helix. Based on the lack of NOESY cross-peaks between residues outside of the PPII helix, no well defined secondary structure could be determined for these regions. Except for expected shifts in peak positions due to pH changes (e.g. the \( \alpha \)-\( \beta \) proton correlations for His-5), the chemical shifts, NOESY cross-peak patterns, and NOESY cross-peak volumes of the myristoylated and non-myristoylated p15 peptides were virtually identical at pH 6.0 and 8.0, and in the aqueous and membrane mimetic environments (Fig. 4, B and C). Thus, for samples that were amenable to NMR structural analysis, regardless of the environment or the myristoylation state of the peptide, the 19-residue ectodomain of the p15 FAST protein comprises a central PPII helix flanked by unstructured N and C termini.

FIGURE 2. The CD spectra of the p15 PRM indicates a polyproline type II helix. A, CD spectrum of a polyproline peptide indicating the characteristic features of a PPII helix (maximum at ~230 nm and minimum at ~205 nm). The inset presents unnormalized data measured with two different peptide samples at peptide concentrations of 1.0 or 0.35 mg/ml. Increasing peptide concentration accentuated the positive signature of the PPII helix at 230 nm. B, CD spectra of a synthetic peptide (VQPPAPPPNA) that encompasses the p15 ectodomain PRM. Reading downwards at 230 nm, the spectra were obtained in the presence of 3 M urea, buffer, or 4 M NaCl. The inset shows a magnification of the spectra in the vicinity of 230 nm to better indicate that urea and NaCl, respectively, increased or decreased the spectral peak at 230 nm, indicative of a PPII helix.

FIGURE 3. NMR-derived structures of the 19-residue p15 ectodomain reveal a polyproline type II helix flanked by disordered N and C termini. Based on the NOESY structural constraints, the p15 ectodomain peptide consists of a well defined PPII type helix with flexible disordered flanking regions. The top panel is an overlay of the NMR-derived structures under different experimental conditions. Structures of the p15 non-myristoylated peptide at pH 8.0 (green) or 6.0 (yellow) in aqueous conditions and at pH 6.0 with DPC-d_{18} (purple) are depicted, along with the p15 myristoylated peptide in DPC-d_{18} at pH 6.0 (black) or 8.0 (pink). The bottom panel is the structure of the non-myristoylated p15 ectodomain peptide in DPC-d_{18} at pH 6.0 (PDB code 2LKW). Residues are color coded to indicate the amphiphilic nature of the peptide; white (hydrophobic), green (polar/hydrophilic), blue (basic), and red (acidic) with the PPII helix in orange.
Several other noteworthy observations were obtained from the NMR data. First, from diffusion ordered spectra, the myristoylated p15 peptide and DPC diffused at the same rate, indicative of a strong association between the peptide and membrane mimetic. No such peptide-DPC interactions were observed for the non-myristoylated peptide (supplemental Fig. S1), indicating the myristate is required for p15 ectodomain interactions with membrane mimetics. Second, in addition to the DPC-myristate interactions, some amino acid-DPC interactions were observed within the NOESY spectra of the myristoylated peptide. Gly-2, His-5, Ile-7, and Phe-18 all showed NOESY cross-peaks between the NH and the choline methyl headgroup of the DPC molecules (Fig. 4D). In addition, the chemical shift changes of the Val-8 and possibly Val-19 residues (Fig. 4E) are suggestive of DPC association. Because the NOESY cross-peak patterns and volumes were virtually identical for all spectra (Fig. 4F), these amino acid interactions with the membrane mimic did not result in significant structural changes within the peptide. Finally, the \(\alpha-\beta\) proton correlations among the Pro residues were unaffected by the presence of DPC (Fig. 4C), suggesting residues within the PPII helix are unlikely to associate strongly with membranes even following peptide-membrane interactions mediated by the N-terminal myristate.

**Cell-Cell Fusion Activity of p15 Correlates with Presence of Ectodomain Polyproline Type II Helix**—Because the PPII helix structure can be propagated through two adjacent alanines (44), the PPII helix conformation was likely maintained in all of the functional alanine substitution mutants (Fig. 1D). To explore the relevance of the PPII helix conformation to p15-induced membrane fusion, an additional p15 construct was created (p15pro2) that contained three adjacent alanine residues in the PRM. CD spectroscopy of a synthetic peptide representing the modified PRM present in this construct (VQPAAA-PANA) indicated the three adjacent alanine residues disrupted the PPII helix structure, evident by the loss of the positive maxima at 228 nm (Fig. 5A). Immunostaining transfected cells expressing p15pro2 revealed only single antigen-positive cells that failed to form the multinucleated syncytia characteristic of
cells transfected with authentic p15 (Fig. 5B). The loss of PPII helix content within the p15 PRM therefore correlates with a loss of fusion activity.

To investigate whether the nonfusogenic proline mutants were compromised in some aspect of p15 function prior to the membrane fusion step, the expression, myristoylation, membrane association, topology, and plasma membrane localization of the p15pro1 and p15pro2 constructs were analyzed. The nonfusogenic proline constructs incorporated [3H]myristic acid at a level equivalent to authentic p15, using the myristoylation-minus p15G2A construct as a negative control for the specificity of myristoylation (supplemental Fig. S2A). These constructs also existed exclusively as integral membrane proteins following high-salt buffer extraction of in vitro translations performed in the presence of microsomal membranes (supplemental Fig. S2B). Furthermore, using a protocol previously used to determine the topology of p15 in the membrane (32), the p15pro1 and p15pro2 constructs assumed correct Nexo/Ccyt membrane topology, evidenced by the accessibility of the polybasic and C-terminal domains (but not the translocated N-terminal ectodomain) to cytoplasmic phosphorylation by protein kinase A (supplemental Fig. S2, C and D). The PRM therefore does not affect the ability of the p15 transmembrane domain to function as a reverse signal anchor. Last, confocal microscopy of GFP-tagged p15pro1 and p15pro2 constructs revealed no qualitative difference in plasma membrane localization of these constructs and p15 containing an authentic PRM (supplemental Fig. S3), similar to what was previously reported for a fusion-dead myristoylation-minus mutant of p15 (5). The membrane fusion activity of p15 is therefore directly dependent on a myristoylated ectodomain containing a PPII helix.

FIGURE 5. Mutations that disrupt the p15 PPII helix inhibit p15-induced syncytogenesis. A, CD spectra of synthetic peptides representing the authentic p15 PRM (VQPPAPPNA) and the p15pro2 mutant (VQPAAAPANA), reading downwards at 230 nm, respectively. Loss of the PPII helix structure in the pro2 mutant peptide is evident by the loss of the weak positive peak at 230 nm. B, transfected cells expressing authentic p15 or p15pro2 were immunostained using anti-p15 antiserum. Arrows in the p15 panel indicate syncytia, whereas those in the pro2 panel indicate single antigen-positive cells.

FIGURE 6. The p15 ectodomain demonstrates time-delayed fusion peptide activity. A, a synthetic peptide representing the 19-residue p15 ectodomain was assessed for peptide-induced lipid mixing using liposomes (100 μM lipid) and the indicated final concentrations of peptide. Results are reported as the percent of maximum fluorescence relative to control liposomes composed of pre-diluted fluorescent lipids. Lipid mixing induced by the solvent control (DMSO) is also shown. B, lipid mixing was assessed as described in panel A using three different p15 ectodomain peptides at 40 μM: the myristoylated p15 ectodomain peptide (+myr), the same peptide minus the N-terminal myristate (−myr), or a myristoylated scrambled version of the p15 ectodomain (−myrScr). All readings were corrected for the low level of lipid mixing induced by the DMSO peptide solvent.
The kinetics of p15-induced lipid mixing displayed a long lag phase of 100–400 s before the onset of lipid mixing, a feature not typically observed with other viral FPs. Peptide titrations revealed dose-dependent lipid mixing at peptide concentrations of 20–40 μM, with the peptide dose affecting the rate of lipid mixing and the length of the lag phase (Fig. 6A). As with p15-induced cell-cell fusion (5), peptide-induced lipid mixing was dependent on an N-terminal myristoyl moiety (Fig. 6B). A myristoylated peptide comprising a scrambled p15 ectodomain sequence induced some lipid mixing, although to substantially lower levels than the authentic peptide (Fig. 6B). The p15 ectodomain therefore possesses the lipid mixing potential typical of viral FPs, and this FP activity is dependent on both the sequence and the N-terminal myristate.

Liposome Aggregation Induced by p15 Ectodomain Is the Rate-limiting Event in Lipid Mixing Reaction—The kinetics of lipid mixing induced by the p15 ectodomain FP were exceptional and suggested a biphasic reaction, where rate-limiting peptide-peptide or peptide-liposome interactions are required prior to the onset of lipid mixing. To distinguish between these possibilities, the authentic p15 ectodomain peptide was incubated for 10 min, either by itself or in the presence of liposomes, at a temperature that restricts the rate of lipid mixing (20 °C). These mixtures were then rapidly heated to 37 °C and monitored for lipid mixing. Preincubating the peptide with liposomes eliminated the lag (Fig. 7A), whereas preincubating the peptide alone had no effect on the lag phase (data not shown). Time-dependent peptide-liposome interactions are therefore responsible for the lag phase in p15-induced lipid mixing.

Further analyses revealed a direct correlation between liposome aggregation and the lag phase. Addition of the myristoylated p15 ectodomain peptide to liposomes induced rapid aggregation of the liposomes, as evidenced by a dose-dependent increase in the turbidity of the suspension (Fig. 7B). The end of the exponential phase of liposome aggregation at each peptide dose corresponded closely to the end of the lag phase and the onset of lipid mixing (Fig. 6A). The non-myristoylated peptide failed to induce liposome aggregation (Fig. 7C), whereas the myristoylated, scrambled version of the p15 ectodomain peptide led to a decrease in turbidity (Fig. 7C), due to partial liposome lysis, a decrease in liposome size and/or conversion of the liposomes to lipid micelles. The decrease in turbidity suggested the low level of lipid mixing induced by the myristoylated, scrambled p15 ectodomain peptide (Fig. 6B) was likely the result of partial liposome lysis rather than liposome fusion. Liposome aggregation alone was not sufficient to induce lipid mixing, as indicated by the ability of divalent cations to promote aggregation of anionic liposomes but not lipid mixing (supplemental Fig. S4). Peptide-membrane interactions required for liposome aggregation are therefore the rate-limiting step in the lipid mixing reaction induced by the p15 ectodomain.

Membrane Interactions of p15 Ectodomain Are Initiated by Myristate Moiety and Stabilized by Amino Acid-Membrane Interactions—The NMR prediction that the N-terminal myristate promotes initial membrane interactions by the p15 ectodomain was confirmed using liposome-binding assays. Liposome flotation on sucrose gradients was used to separate the liposome-associated p15 ectodomain peptide from free peptide. SDS-PAGE analysis allowed a qualitative assessment of peptide association with liposomes, although the small size of the peptide resulted in its migration to the same region of the gel as the overwhelming mass of lipids present in the liposome, confounding a quantitative analysis (Fig. 8A). HPLC analysis cleanly separated the peptide and lipid components, which
allowed a quantitative assessment of the relative distribution of peptide in the liposome-containing and liposome-free fractions of the sucrose gradient (Fig. 8B). Over 80% of the myristoylated peptide but <30% of the non-myristoylated peptide associated with liposomes, indicating the myristate moiety is required for efficient peptide-membrane interactions. However, less than half of the myristoylated scrambled p15 peptide stably associated with liposomes (Fig. 8). Because myristate reversibly associates with membranes (45), interactions of specific amino acids with liposomes following the initial myristate-membrane interaction are likely needed to stabilize association of the p15 ectodomain with membranes. The scrambled peptide results imply such amino acid-membrane interactions are dependent on the linear sequence of the p15 ectodomain, suggesting the PPII helix may be needed to promote these interactions. The NMR results implicate residues that flank the PPII helix (i.e. Gly-2, His-5, Ile-7, Val-8, Phe-18, and possibly Val-19), rather than the prolines in the helix, as the residues most likely to promote stable peptide-membrane interactions.

DISCUSSION

Using a combination of CD and NMR spectroscopy, protein-mediated cell-cell fusion, peptide-liposome binding, and peptide-induced lipid mixing approaches, we investigated the structural and functional attributes of the 19-residue ectodomain of the baboon reovirus p15 FAST protein. Results indicate that the p15 ectodomain comprises a PPII helix flanked by unstructured N and C termini, both in the absence and presence of the N-terminal myristate moiety and in aqueous or membrane mimetic environments. The N-terminal myristate and the PPII helix are both required for efficient membrane binding, liposome aggregation, and FP activity of the p15 ectodomain. These peptide-membrane interactions, which induce the liposome aggregation that precedes lipid mixing, are the rate-limiting event in p15-induced lipid mixing. The p15 FAST protein is the first example of a viral fusogen whose mechanism of membrane fusion is directly dependent on a myristoylated PPII helix. These results provide important insights into how this rudimentary fusion module may function to induce cell-cell membrane fusion.

The essential role of the p15 PPII helix reflects its function as a novel FP motif. Although the p15 ectodomain induces lipid mixing, a hallmark feature of viral FPs, there are notable differences between the p15 ectodomain FP and typical FPs. In contrast to most viral FPs (46), the p15 ectodomain is more hydrophilic than hydrophobic, it is not enriched in glycine and alanine residues, and it is highly tolerant of substitutions. Functional attributes also distinguish the p15 FP from all other characterized FPs. The most notable difference was the long lag phase before the onset of p15-induced lipid mixing. This lag was eliminated by pre-mixing the p15 ectodomain peptide with liposomes under conditions that restrict the rate of lipid mixing (Fig. 7A), and the length of the lag phase correlated with the time needed for maximal liposome aggregation (Fig. 7B). Although liposome aggregation is a priori for peptide-induced lipid mixing, the p10 and p14 FAST protein FPs induce both events concurrently with no lag time in lipid mixing (30, 31).

The same situation applies to enveloped virus FPs, where peptide addition leads to lipid mixing with little (<20 s) to no lag (47–49). Lipid mixing induced by the p15 ectodomain is therefore a biphasic reaction, where the rate-limiting step is peptide-membrane interactions that trigger liposome aggregation followed by additional interactions that drive lipid mixing.

The other major functional difference between the p15 FP and enveloped virus fusion peptides was the extent and rate of lipid mixing under similar experimental conditions (e.g. temperature, lipid formulation, and peptide and liposome concentrations). The p15 peptide induced ~70% lipid mixing within ~1500 s at a peptide:lipid ratio of 1:5 (Fig. 6). In contrast, the 80–100% lipid mixing induced by the HIV FP occurs within 20–30 s at a peptide:lipid ratio of 1:10, and ~40% lipid mixing occurs in ~150 s at a peptide:lipid ratio of 1:60 (36, 49, 50). Thus, the p15 ectodomain FP has a lower specific activity in lipid mixing assays compared with the FP of HIV, and to the FPs of influenza virus and Sendai virus (48, 51). This may reflect the fact that the p15 FP is considerably less hydrophobic than enveloped virus fusion peptides. However, hydrophobicity alone does not explain p15-induced lipid mixing because the myristoylated, scrambled version of the p15 ectodomain induced only a low level of lipid mixing, due most likely to detergent-like effects on liposome solubilization (Figs. 6B and 8).
p15 Polyproline Helix Fusion Peptide

7C). The linear sequence arrangement of the p15 ectodomain, and by inference the presence of the PPII helix, is therefore required for the p15 ectodomain to function as a FP. We note that the results obtained using synthetic peptides in standard lipid mixing assays may not directly reflect the efficiency or mechanism of action of a FP functioning as a component of a fusion protein. For example, the lipid mixing event is frequently accomplished by extensive content leakage and may actually arise in part due to liposome lysis, as we have shown for the myristoylated scrambled p15 ectodomain peptide. However, the authentic p15 peptide induced lipid mixing in the absence of liposome lysis (Fig. 7B). Moreover, alterations of the p15 peptide that led to a loss of lipid mixing (i.e. removal of the N-terminal myristate or altering the primary sequence of the p15 polyproline helix) (Fig. 6B) also lead to a loss of cell-cell fusion activity when similar changes were made in the context of the p15 protein (Figs. 1B and 5B). The myristoylated, PPII helix-containing ectodomain is therefore required for both lipid mixing and cell-cell membrane fusion, consistent with its inferred role as a novel FP motif.

The diversity of FAST protein ectodomain FPs is quite remarkable, with a myristoylated, proline-hinged loop in p14 (30), a non-myristoylated cystine loop in p10 (29), and now a myristoylated PPII helix in p15. Unexpectedly, the 19-residue p15 ectodomain can be replaced by the 37-residue ectodomain of p14 with no adverse effects on cell-cell fusion activity (28), suggesting the two very different FPs present in these ectodomains may provide an equivalent function in the fusion reaction. The loop structures of the p10 and p14 FPs presumably result in solvent exposure of hydrophobic residues, particularly a Val and Phe residue at the apex of these loops (29, 30). These loops share features with the fusion loops contained within several enveloped virus fusion proteins, which also expose hydrophobic anchors at their apices (8). Shallow insertion of exposed hydrophobic amino acid side chains into the outer leaflet of membranes displaces lipid headgroups and can promote membrane curvature and fusion (52–54), suggesting the p10 and p14 FPs may function through a similar “wedging” mechanism. However, whereas the FAST protein transmembrane domains are functionally interchangeable in a p14 backbone (26), this is not the case in the p15 backbone (28). The unique properties of the p15 PPII helix FP motif may therefore require additional essential features of the p15 transmembrane domain (i.e. glycine and β-branched residues and a triserine motif) to generate a functional fusogen.

The p15 FP forms an extended PPII helix, not a loop, and the present results provide several insights into the mechanism of action of this novel FP motif. Liposome binding studies and CD and NMR spectroscopy revealed complex interactions between the p15 ectodomain and membranes or membrane mimetics. In the absence of myristate, the p15 ectodomain had limited capacity to bind to liposomes (Fig. 8), displayed no close association with DPC micelles (Fig. 4), and was inactive for both lipid mixing (Fig. 6B) and liposome aggregation (Fig. 7C). Although the N-terminal myristate apparently mediates initial interactions with membranes, specific amino acid-membrane interactions are needed to stabilize membrane association of the p15 ectodomain. These stabilizing interactions depend on the linear sequence of the ectodomain and, by inference, the presence of the PPII helix. These conclusions are supported by the liposome binding properties of the authentic versus the scrambled p15 ectodomain peptides (Fig. 8), and by the NMR results that indicated close association of several residues that flank the PPII helix with DPC (Fig. 4). Two-step models for peptide interactions with membranes are not uncommon, as occurs with amphipathic cationic antimicrobial peptides that use electrostatic interactions to promote initial peptide-membrane interaction followed by membrane lysis or pore formation (55). Lipid anchors have also been used in contrived peptide systems to promote liposome aggregation and membrane fusion (47, 56, 57), although the fatty acid did not exert these enhancing effects by promoting peptide-liposome binding as occurs with the p15 ectodomain.

The p15 PPII helix does not directly interact with membranes, suggesting its essential role is to facilitate membrane interactions of other residues that drive the fusion process. NMR spectroscopy implicated residues that flank the PPII helix as the residues most likely to promote stable peptide-membrane interactions and lipid mixing (Fig. 4, C and D). These flanking residues include a Phe-Val dipeptide, the same residues present at the apex of the p10 and p14 fusion loops. As with the fusion loops of the enveloped virus fusogens and the p10 and p14 FAST proteins, formation of the p15 PPII helix may force solvent exposure of these flanking hydrophobic residues for membrane interaction. A similar role for a PPII to force solvent exposure of an aromatic residue was recently proposed for binding of the substance P neuropeptide to the neurokinin 1 receptor (58). In addition, structural plasticity is an inherent, and possibly required, feature of FPs (59, 60), including the cystine loop FP of the p10 FAST protein (29). Dynamic structural changes in the PPII helix may also be needed for fusion activity and would be facilitated by the absence of stabilizing backbone hydrogen bonds that allow the PPII helix to fluctuate around an idealized PPII conformation (61). The aliphatic pyrrolidine rings in the PPII helix are interspersed by the polar carbonyl oxygens of the peptide backbone, which are solvent exposed to form hydrogen bonds with water molecules or adjacent polar lipid headgroups (37). Localized conformational changes of this dynamic amphiphile in the vicinity of lipid headgroups would alter the interfacial boundary that serves as a barrier to lipid mixing. Such structural fluctuations may also promote membrane insertion of the hydrophobic residues to the correct depth needed to induce membrane curvature.

Either or both of these effects would be expected to contribute to the ability of this novel FP motif to mediate lipid mixing and the onset of the membrane fusion reaction.

An unresolved question is precisely how p15 ectodomain interactions with membranes result in liposome aggregation and why there is a kinetic delay between extensive liposome aggregation and the onset of lipid mixing? It is improbable that the p15 ectodomain “bridges” the two membranes being fused, using myristate insertion in one membrane and insertion of hydrophobic amino acid side chains into the other membrane to anchor the peptide in both membranes. This scenario is somewhat akin to the FP-transmembrane domain anchor model of the enveloped virus fusogens (8). The weak, reversible
interaction of myristate with membranes and the limited ability of the non-myristoylated p15 peptide to associate with liposomes (Fig. 8) argue against this model. It is more plausible that initial peptide interactions with a membrane mediated by the myristate result in subsequent insertion of the hydrophobic amino acid side chains into the same membrane. Altered stability of the interfacial region and/or membrane curvature changes due to these peptide-membrane interactions could expose lipid acyl chains, resulting in liposome aggregation to mask these hydrophobic defects. Partially exposed acyl chains at the tip of a highly curved membrane protrusion is an inherent feature of the wedging mechanism for membrane fusion (52). In the case of p15, the hydrophobic defects must be such that they promote liposome aggregation but not lipid mixing. A straightforward explanation is that fewer peptides are needed to alter the membrane sufficiently to promote aggregation versus lipid mixing. Assuming the rate of stable p15 peptide association with membranes is the actual limiting step in the fusion reaction, then additional time would be needed for the membrane-associated peptide concentration to reach the threshold level needed to initiate membrane merger. It is also conceivable that the fusion reaction is a cooperative event, where initial peptide-membrane interactions promote the subsequent association of additional peptides and/or alter the nature, or increase the extent of, the hydrophobic defects required for membrane merger. Further studies are needed to resolve these issues.

The myristoylated, PPII helix of p15 adds a third example to the list of unusual FPs utilized by members of the FAST protein family to induce cell-cell fusion. Most importantly, the present results provide the first significant insights into the mechanism of action of the p15 ectodomain. At only 19 residues, this ectodomain defies many of the pre-conceived notions of how viral and cellular fusogens function to promote membrane fusion. The unusual features of the p15 ectodomain provide additional perspectives on the role of polyproline helices as membrane interaction motifs and on how this motif can function as a FP to mediate membrane merger. Last, the FAST proteins are modular fusogens with the ecto-, endo-, and transmembrane domains all playing an essential role in the fusion reaction. Further insights into how these three fusion modules function in a coordinated manner, and from both sides of the membrane, to induce membrane fusion should continue to reveal what may be a diversity of approaches by which proteins can mediate cell-cell membrane fusion.

Acknowledgments—We thank Jingyun Shou for excellent technical assistance. We thank Martin St-Maurice for expert technical assistance with the CD spectroscopy, and Christian Hoy for initial characterization of several proline mutants. Magnetic Resonance data were collected at the Biomolecular Magnetic Resonance Facility (BMRF) housed at the National Research Council of Canada’s Institute for Marine Biosciences.

REFERENCES

1. Chappell, J. D., Duncan, R., Mertens, P. P., and Dermody, T. S. (2005) Orthoreovirus, Reoviridae. Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses (Fauquet, C., Mayo, M. A., Maniloff, J., Desselberger, U., and Ball, L. A., eds) pp. 455–465, Elsevier/Academic Press, London
2. Thalmann, C. M., Cummins, D. M., Yu, M., Lunt, R., Pritchard, L. L., Hansson, E., Cramer, S., Hyatt, A., and Wang, L. F. (2010) Broome virus, a new fusogenic Orthoreovirus species isolated from an Australian fruit bat. Virology 402, 26–40
3. Attou, H., Fang, Q., Mohd Jaafar, F., Cantaloube, J. F., Bigani, P., de Micco, P., and de Lamballerie, X. (2002) Common evolutionary origin of aquareoviruses and orthoreoviruses revealed by genome characterization of golden shiner reovirus, grass carp reovirus, striped bass reovirus, and golden ide reovirus (genus Aquareovirus, family Reoviridae). J. Gen. Virol. 83, 1941–1951
4. Corcoran, J. A., and Duncan, R. (2004) Reptilian reovirus utilizes a small type III protein with an external myristylated amino terminus to mediate cell-cell fusion. J. Virol. 78, 4342–4351
5. Dawe, S., and Duncan, R. (2002) The Sigma40 gene product of baculovirus Vero virus is bicistronic and encodes a novel fusion-associated small transmembrane protein. J. Virol. 76, 2131–2140
6. Racine, T., Hurst, T., Barry, C., Shou, J., Kibenge, F., and Duncan, R. (2009) Aquareovirus effects syncytogeneisis by using a novel member of the FAST protein family translated from a noncanonical translation start site. J. Virol. 83, 5951–5955
7. Shimulevitz, M., and Duncan, R. (2000) A new class of fusion-associated small transmembrane (FAST) proteins encoded by the non-envelope aquareovirus genome. EMBO J. 19, 902–912
8. White, J. M., Delos, S. E., Brecher, M., and Schornberg, K. (2008) Structures and mechanisms of viral membrane fusion proteins. Multiple variations on a common theme. Crit. Rev. Biochem. Mol. Biol. 43, 189–219
9. Kielland, M., and Rey, F. A. (2006) Virus membrane-fusion proteins. More than one way to make a hairpin. Nat. Rev. Microbiol. 4, 67–76
10. Melikyan, G. B. (2008) Common principles and intermediates of viral protein-mediated fusion. The HIV-1 paradigm. Retrovirology 5, 111
11. Roche, S., Albertini, A. A., Lepault, J., Bressanelli, S., and Gaudin, Y. (2008) Structures of vesicular stomatitis virus glycoprotein. Membrane fusion revisited. Cell. Mol. Life Sci. 65, 1716–1728
12. Harrison, S. C. (2008) Viral membrane fusion. Nat. Struct. Mol. Biol. 15, 690–698
13. Lai, A. L., Park, H., White, J. M., and Tamm, L. K. (2006) Fusion peptide of influenza hemagglutinin requires a fixed angle boomerang structure for activity. J. Biol. Chem. 281, 5760–5770
14. Li, Y., and Tamm, L. K. (2007) Structure and plasticity of the human immunodeficiency virus gp41 fusion domain in lipid micelles and bilayers. Biophys. J. 93, 876–885
15. Lorieu, J. L., Louis, J. M., and Bax, A. (2010) The complete influenza hemagglutinin fusion domain adopts a tight helical hairpin arrangement at the lipid-water interface. Proc. Natl. Acad. Sci. U.S.A. 107, 11341–11346
16. Gibbons, D. L., Vaney, M. C., Roussel, A., Vigoureux, A., Reilly, B., Lepault, J., Kielland, M., and Rey, F. A. (2004) Conformational change and protein-protein interactions of the fusion protein of Semliki Forest virus. Nature 427, 320–325
17. Gregory, S. M., Harada, E., Liang, B., Delos, S. E., White, J. M., and Tamm, L. K. (2011) Structure and function of the complete fusion loop from Ebola virus glycoprotein 2. Proc. Natl. Acad. Sci. U.S.A. 108, 11211–11216
18. Kadlec, J., Loureiro, S., Abrescia, N. G., Stuart, D. I., and Jones, I. M. (2008) The postfusion structure of baculovirus gp64 supports a unified view of viral fusion machinery. Nat. Struct. Mol. Biol. 15, 1024–1030
19. Modis, Y., Ogata, S., Clements, D., and Harrison, S. C. (2004) Structure of the dengue virus envelope protein after membrane fusion. Nature 427, 313–319
20. Rey, F. A. (2006) Molecular gymnastics at the herpesvirus surface. EMBO Rep. 7, 1000–1005
21. Roche, S., Bressanelli, S., Rey, F. A., and Gaudin, Y. (2006) Crystal structure of the low pH form of the vesicular stomatitis virus glycoprotein G. Science 313, 187–191
22. Epand, R. M. (2003) Fusion peptides and the mechanism of viral fusion. Biochim. Biophys. Acta 1614, 116–121
23. Chernomordik, L. V., and Kozlov, M. M. (2008) Mechanics of membrane fusion. Nat. Struct. Mol. Biol. 15, 675–683
p15 Polyproline Helix Fusion Peptide

24. Boutilier, J., and Duncan, R. (2011) in Membrane Fusion (Chernomordik, L. V., and Koslov, M. M., eds) Vol. 68, pp. 107–140, Elsevier, San Diego, CA

25. Barry, C., and Duncan, R. (2009) Multifaceted sequence-dependent and -independent roles for reovirus FAST protein cytoplasmic tails in fusion pore formation and syncytigenesis. J. Virol. 83, 12185–12195

26. Clancy, E. K., and Duncan, R. (2009) Reovirus FAST protein transmembrane domains function in a modular, primary sequence-independent manner to mediate cell-cell membrane fusion. J. Virol. 83, 2941–2950

27. Shmulevitz, M., Salsman, J., and Duncan, R. (2003) Palmitoylation, membrane-proximal basic residues, and transmembrane glycine residues in the reovirus p10 protein are essential for syncytium formation. J. Virol. 77, 9769–9779

28. Clancy, E. K., and Duncan, R. (2011) Helix-stabilizing, β-branched, and polar residues in the baboon reovirus p15 transmembrane domain influence the modularity of FAST proteins. J. Virol. 85, 4707–4719

29. Barry, C., Key, T., Haddad, R., and Duncan, R. (2010) Features of a spatially constrained cystine loop in the p10 FAST protein ectodomain define a new class of viral fusion peptides. J. Biol. Chem. 285, 16424–16433

30. Corcoran, J. A., Svytški, R., Top, D., Epand, R. M., Epand, R. F., Jakeman, D., and Duncan, R. (2004) Myristylation, a protruding loop, and structural plasticity are essential features of a non-enveloped virus fusion peptide motif. J. Biol. Chem. 279, 51386–51394

31. Shmulevitz, M., Epand, R. F., Epand, R. M., and Duncan, R. (2004) Structural and functional properties of an unusual internal fusion peptide in a non-enveloped virus membrane fusion protein. J. Virol. 78, 2808–2818

32. Dawe, S., Corcoran, J. A., Clancy, E. K., Salsman, J., and Duncan, R. (2005) Unusual topological arrangement of structural motifs in the baboon reovirus fusion-associated small transmembrane protein. J. Virol. 79, 6216–6226

33. Syvitski, R. T., Burton, I., Mattatall, N. R., Douglas, S. E., and Jakeman, D. L. (2005) Structural characterization of the antimicrobial peptide pleurocin-din from winter flounder. Biochemistry 44, 7282–7293

34. Nilges, M., Kuszelewski, J., and Brunger, A. T. (1991) Computational Aspects of the Study of Biological Macromolecules by NMR, Plenum Press, New York

35. Stewart, J. C. (1980) Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal. Biochem. 104, 10–14

36. Pereira, F. B., Gori, F. M., Muga, A., and Nieva, J. L. (1999) Interbilayer lipid mixing induced by the human immunodeficiency virus type 1 fusion peptide on large unilamellar vesicles. The nature of the nonlamellar intermediates. Chem. Phys. Lipids 103, 11–20

37. Koizumi, M. M., McMahon, H. T., and Chernomordik, L. V. (2010) Protein-driven membrane stresses in fusion and fission. Trends Biochem. Sci. 35, 699–706

38. Groffen, A. J., Martens, S., Diez Arazola, R., Cornelisse, L. N., Lozovaya, N., de Jong, A. P., Goriouanova, N. A., Habets, R. L., Takai, Y., Borst, J. G., Brose, N., McMahon, H. T., and Verhage, M. (2010) Doc2b is a high-affinity Ca2+ sensor for spontaneous neurotransmitter release. Science 327, 1614–1618

39. Hui, E., Johnson, C. P., Yao, J., Dunning, F. M., and Chapman, E. R. (2009) Synaptotagmin-mediated binding of the target membrane is a critical step in Ca2+-regulated fusion. Cell 138, 709–721

40. Findlay, B., Zanel, G. G., and Schweizer, F. (2010) Cationic amphiphiles, a new generation of antimicrobials inspired by the natural antimicrobial peptide scaffold. Antimicrob. Agents Chemother. 54, 4049–4058

41. Joseph, M., and Nagaraj, R. (1995) Interaction of peptides corresponding to fatty acylation sites in proteins with model membranes. J. Biol. Chem. 270, 16749–16755

42. Pécœur, E. L., Hockstra, D., Sainte-Marie, J., Maurin, L., Bienvenûe, A., and Philippot, J. R. (1997) Membrane anchorage brings about fusogenic properties in a short synthetic peptide. Biochemistry 36, 3773–3781

43. Foroutan, A., Lazarova, T., and Padros, E. (2011) Study of membrane-induced conformations of substance P. Detection of extended polyproline II helix conformation. J. Phys. Chem. B 115, 3622–3631

44. Tamm, L. K., Crane, J., and Kiessling, V. (2003) Membrane fusion. A structural perspective on the interplay of lipids and proteins. Curr. Opin. Struct. Biol. 13, 453–466

45. Reichert, J., Grassnick, D., Afonin, S., Buercrk, J., Wadwhani, P., and Ulrich, A. S. (2007) A critical evaluation of the conformational requirements of fusogenic peptides in membranes. Eur. Biophys. J. 36, 405–413

46. Zagrovic, B., Lipfert, J., Sorin, E. J., Millett, I. S., van Gunsteren, W. F., Doniach, S., and Pande, V. S. (2005) Unusual compactness of a polyproline type II structure. Proc. Natl. Acad. Sci. U.S.A. 102, 11698–11703