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Solid-State NMR Investigation of the Structural Topology and Lipid Interactions a Viral Fusion Protein Chimera Containing the Fusion Peptide and Transmembrane Domain

Hongwei Yao, Myungwoon Lee, Shu-Yu Liao, and Mei Hong*

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139

Revised for Biochemistry

*Corresponding author: Mei Hong
Tel: 617-253-5521, email: meihong@mit.edu

Abstract

The fusion peptide (FP) and transmembrane domain (TMD) of viral fusion proteins play important roles during virus-cell membrane fusion, by inducing membrane curvature and transient dehydration. The structure of the water-soluble ectodomain of viral fusion proteins has been extensively studied crystallographically, but the structures of the FP and TMD bound to phospholipid membranes are not well understood. We recently investigated the conformations and lipid interactions of the separate FP and TMD peptides of the parainfluenza virus 5 (PIV5) fusion protein F using solid-state NMR. These studies provide structural information of the two domains when they are spatially well separated in the fusion process. To investigate how these two domains are structured relative to each other in the post-fusion state, when the ectodomain forms a six-helix-bundle that is thought to force the FP and TMD together in the membrane, we have now expressed and purified a chimera of the FP and TMD, connected by a Gly-Lys linker, and measured the chemical shifts and inter-domain contacts of the protein in several lipid membranes. The FP-TMD chimera exhibits α -helical chemical shifts in all the membranes examined, and does not cause strong curvature to lamellar membranes or membranes with negative spontaneous curvature. These properties differ qualitatively from the separate peptides, indicating that the FP and TMD interact with each other in the lipid membrane. However, no ^{13}C - ^{13}C cross peaks are observed in 2D correlation spectra, suggesting that the two helices are not tightly associated. These results suggest that the ectodomain six-helix bundle does not propagate into the membrane to the two hydrophobic termini. However, the loosely associated FP and TMD helices are found to generate significant negative Gaussian curvature to membranes that possess spontaneous *positive* curvature, consistent with the notion that the FP-TMD assembly may facilitate the transition of the membrane from hemifusion intermediates to the fusion pore.

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Introduction

Enveloped viruses such as HIV and influenza enter host cells by fusing the virus lipid envelope and the cell membrane using viral fusion proteins. For many viruses, the fusion protein is biosynthesized as a homotrimer and is transported to the cell surface in a metastable state after proteolytic cleavage^{1,2}. Virus-cell membrane fusion begins in response to external triggers such as binding to cell-surface receptors and pH changes, which activates the protein to undergo a conformational change that exposes and inserts an N-terminal fusion peptide (FP) into the target membrane. Meanwhile, the C-terminal transmembrane domain (TMD) remains anchored in the virus envelope. This extended intermediate further undergoes a series of conformational rearrangements to bend into a hairpin, in so doing pulling the cell membrane and the viral envelope closer³⁻⁷. During this process, the hydrophobic membrane-anchored FP and TMD destabilize and dehydrate the two lipid bilayers, causing sequential merger of the outer leaflets and inner leaflets. The detailed structures of the membrane intermediates along this protein-mediated fusion pathway are not well understood, and morphologies such as the lipid stalk and hemifusion diaphragm have been proposed based on studies of protein-free membrane fusion. The protein conformational changes, on the other hand, are believed to be important for overcoming the kinetic barrier of virus-cell membrane fusion. After full merger of the lipid bilayers, an aqueous pore opens to connect the cell and the virus. In this post-fusion state, class I viral fusion proteins adopt a characteristic six-helix-bundle (6HB) structure, formed by a trimer of hairpins in the water-soluble ectodomain (*vide infra*).

The structures of the water-soluble domain of many viral fusion proteins have been determined using X-ray crystallography^{2,8,9}. Class I fusion proteins possess two heptad repeats: HR1 follows the N-terminal fusion peptide while HR2 precedes the C-terminal TMD. The pre-fusion state of different viral fusion proteins exhibits non-negligible structural differences. For example, HR2 adopts a compact trimeric coiled coil in paramyxovirus fusion proteins but does not associate into a tight trimer in HIV-1 gp41 and influenza hemagglutinin (HA). In comparison, the post-fusion structures of the ectodomain share significant similarities, and are characterized by a hyper-stable trimer of hairpins, with HR1 forming the interior parallel coiled coil while HR2 draping the exterior in an antiparallel fashion with HR1. This 6HB implies that the FP and TMD may also come into close proximity in the merged membrane. However, the structures of the membrane-associated FP and TMD in the post-fusion state have not been determined. Due to their high hydrophobicity, these two domains are usually excluded in the crystallographic construct, and when included, their signals are not detectable in the crystal structures, indicating high disorder.

Mutagenesis, biochemical, and biophysical studies have shown that the FP and TMD not only serve as structural anchors of the protein in the membrane, but also actively contribute to virus-cell fusion by destabilizing the two lipid bilayers and causing membrane curvature¹⁰⁻¹⁷. Experimental evidence suggests that the fusion peptide facilitates the transition from the prefusion lamellar bilayers to a hemifused structure^{17,18}, while the TMD chiefly facilitates the transition from the hemifusion state to the fusion pore^{13,19}. Theoretical and experimental studies suggest that fusion pore opening and expansion might be the most energy-consuming step in the fusion process, and FP and TMD may facilitate this process by lowering the pore line tension²⁰⁻²³.

Solution NMR and magic-angle-spinning (MAS) solid-state NMR spectroscopy have been used extensively to characterize the structure and topology of the FP of viral fusion proteins in detergent- or membrane-bound states. These studies have shown that membrane composition^{12, 24-28}, pH^{29, 30}, and peptide concentration³¹ have significant influences on the structures of influenza and HIV FPs. Recently, we investigated the conformations and lipid interactions of the FP and TMD of the parainfluenza virus 5 (PIV5) fusion protein F in phospholipid membranes. The data indicate that the backbone conformation of the FP and TMD significantly depend on the membrane composition, with the β -strand conformation increasing with phosphatidylethanolamine (PE) content^{14, 15, 32}. This β -strand conformation causes negative Gaussian curvature and dehydration to the PE membranes. In comparison, predominantly α -helical conformation is found in lamellar membranes, and the helical conformation of the FP and TMD does not incur membrane curvature and dehydration. These results suggest that the β -strand conformation is more fusogenic than the helical conformation. Since the FP and TMD peptides were studied separately, these results most likely reflect the extended intermediate state of the protein, when the two domains are well separated from each other. The fact that the membrane composition shifts the peptide conformational equilibrium implies that the lipids can control the site of membrane fusion, and negative-curvature PE-rich domains are particularly susceptible to curvature induction by the fusion protein.

While studies of the isolated FP and TMD peptides probe early stages of virus-cell fusion, the post-fusion structure of the FP and TMD in the membrane has been much less studied because of the necessity to include or mimic the ectodomain, which range from ~150 residues to ~350 residues long and are thus at or beyond the limit of structure determination using NMR. In this work, we investigate this post-fusion structure for the PIV5 F protein using an engineered chimera of the FP and TMD. By replacing the large (352-residue) F ectodomain with a short flexible linker, we are able to probe the membrane-bound conformation, dynamics, depth of insertion, and inter-domain interactions of the FP and TMD. To compare with the conformations of the separate FP and TMD peptides, we investigate the FPTM chimera structure as a function of the membrane composition. Interestingly, we find that the FP and TMD segments in the chimera adopt a stable and membrane-independent α -helical conformation, in qualitative difference from the separate peptides. Specifically, the chimera is α -helical in negative-curvature PE membranes and in monoolein (MO) membranes, which cause β -strand conformations to the isolated FP and TMD peptides. ³¹P NMR spectra indicate that the α -helical chimera causes limited curvature to most membranes. These differences from the separate peptides suggest FP-TMD interactions. However, no ¹³C-¹³C cross peaks were observed between the two domains, indicating that the FP and TMD helices are not tightly associated with each other in the membrane. These results constrain the possible post-fusion structures of the PIV5 fusion protein, and are compared to current structural information of other class I viral fusion proteins.

Materials and Methods

Cloning, expression and purification of the FPTM chimera

The FPTM chimera connects the FP (residues 103-132) and TMD (residues 485-510) of the PIV5 F protein with an eight-residue linker, GGGKKKKK (G₃K₅). An N-terminal fusion partner, HR52, derived from staphylococcal nuclease³³, was used to increase the chimera solubility and facilitate protein purification (**Fig. 1**). A three-residue segment, DPM, was inserted

N-terminus to the FP to enable formic acid cleavage between Asp and Pro and cyanogen bromide cleavage C-terminal to Met³⁴. Two protease cleavage sites, for thrombin (LVPR↓GS) and human rhinovirus 3C protease (HRV 3C, LEVLFQ↓GP), were also inserted before the FP domain. Two sequences of the chimera, with AIA or SIC at residues 490-492, were expressed. The AIA-FPTM construct has the same TMD sequence as the separate TMD peptide studied before, while the SIC-FPTM construct, without any Ala residues in the TMD, allows us to determine whether observed Ala-Ile cross peaks in 2D correlation spectra result from long-range contacts between the FP and TMD or from sequential contacts. The genes for the HR52-FPTM proteins were synthesized by Genscript and incorporated into a pET28b (Novagen) vector containing a kanamycin resistant gene. The protein was expressed in *E. coli* Rosetta2(DE3)pLysS (Novagen) cells, which contain an additional chloramphenicol resistant gene. The bacteria were grown in LB medium at 37°C until A₆₀₀ reached ~0.8, then 1 mM IPTG was added to induce protein expression. 100 µg/mL kanamycin sulfate (Fisher Scientific) and 37 µg/mL chloramphenicol (Sigma) were used for bacterial screening.

¹³C, ¹⁵N-labeled HR52-FPTM was expressed by growing the bacteria in 1 L LB media to OD₆₀₀ ~0.8, then centrifuging the cells at 5,000 g and 20°C for 7 min. The cell pellet was then resuspended in 1 L M9 minimal medium containing 3 g of uniformly ¹³C-labeled glucose and 1 g of ¹⁵N-labeled ammonium chloride. The cells were grown at 37°C for 30 min, after which 1 mM IPTG was added to induce protein expression. Amino-acid specific labeling of AIA-FPTM was conducted by adding 100 mg each of unlabeled Arg, Asp, Glu, Gln, Gly, His, Lys, Leu, Met, Phe, Pro and Tyr to the 1 L M9 medium. Thus, only non-supplemented amino acids were ¹³C, ¹⁵N-labeled (**Table S1**). For SIC-FPTM, 100 mg each of unlabeled Leu, Lys, Phe, Pro, Arg, Met, His, Trp, Tyr and Cys were added to the 1 L M9 medium. In addition, 50 mg of ¹⁵N-labeled Glu and 50 mg each of ¹³C, ¹⁵N-labeled Gly, Ser and Thr were added to increase the ¹⁵N labeling level and reduce scrambling. After 4 h of protein expression, the cells were centrifuged at 7,000 rpm for 15 min at 4°C and stored at -30°C.

The bacteria were suspended in a pH 8.0 buffer (50 mM Tris-HCl, 250 mM NaCl) and lysed for 30 min in an ice-water bath. The lysed cells were centrifuged at 12,500 rpm and 4°C for 30 min. The protein was mostly expressed in the inclusion body. Previous comparison of the ¹³C chemical shifts of influenza and HIV fusion peptides expressed in the membrane fraction versus inclusion bodies show the same conformation, indicating that viral fusion peptides obtained from inclusion bodies retain their native conformation³⁵⁻³⁷. The pellet was suspended in 5% (v/v) Triton X-100 and incubated with stirring for 10 min at 4°C. The suspension was centrifuged at 10,000 rpm and 4°C for 30 min. The inclusion body was washed two more times in 5% Triton X-100, then washed with water to remove trace amount of detergent. The washed inclusion body was stored at -30°C.

The FPTM chimera was purified with multiple approaches (**Table 2**) and analyzed by gel electrophoresis, mass spectrometry and solution NMR to verify protein purity and homogeneity. All samples were first subjected to inclusion body washes. SIC-FPTM3 was not further purified. AIA-FPTM and SIC-FPTM4 were further purified by HPLC. Since the HPLC yield is low, we alternatively used Ni²⁺ column chromatography to purify the protein (SIC-FPTM2). To assess if residual SDS from the affinity column might affect the protein conformation, we purified one protein sample (SIC-FPTM1) using Ni²⁺ column followed by HPLC.

For preparative reverse-phase HPLC, the inclusion body was dissolved in an acetonitrile/water (67%/33%) solution containing 0.1% TFA and centrifuged. The supernatant was loaded onto a C4 column (Vydac 214TP, 2.2 x 25 cm) and eluted in 1 h with an acetonitrile gradient of 35–99% with a flow rate of 10 mL/min. The target peak was collected and lyophilized, and 15% SDS-PAGE gel was used to verify protein purity. For SDS-PAGE, the protein powder was dissolved in HFIP, the solvent removed with a stream of nitrogen gas, the protein was suspended in 2% SDS (70 mM), sonicated for 6 min, then mixed with the loading buffer to a final concentration of ~10 µg/µL.

For Ni²⁺ column purification, the inclusion body was dissolved in 8 M urea and 1% SDS overnight at room temperature. After 30 min of centrifugation at 12,500 rpm and 20°C, the supernatant was collected and loaded onto the column with a flow rate of 0.5-1.0 mL/min. The column was washed stepwise by 1) 8 M urea and 1% SDS; 2) 6 M urea, 0.5% SDS, and 5 mM imidazole; 3) 4 M urea, 0.1% SDS, and 10 mM imidazole; and 4) 2 M urea and 20 mM imidazole. The protein was finally eluted using 500 mM imidazole. All washing and elution solutions were conducted in pH 8.0 Tris buffer. To remove SDS, the eluent was dialyzed in a dialysis bag with a 3,500 molecular weight cutoff against water for 5 days with water change every 6 or 12 hours. The precipitated protein was collected, lyophilized and analyzed using 15% SDS-PAGE. The protein powder was stored at -30°C.

SIC-FPTM1 was purified by Ni²⁺ column chromatography followed by reverse-phase HPLC using a C4 analytical column (Vydac 218TP, 0.46 x 10 cm) with 50% methanol and 50% isopropanol as the organic phase and water as the aqueous phase. 0.1% TFA was added in both phases. The gradient of the organic phase was 20–99% in 25 min with a flow rate of 1.5 mL/min.

Extensive efforts were made to cleave the HR52 tag using thrombin, formic acid and cyanogen bromide. In most cases, the cleaved product has a high propensity to form high-molecular-weight aggregates, which severely reduce the yield of subsequent purification. Thus, the HR52 tag was retained in most samples, except for SIC-FPTM5, where the HR52 tag was cleaved using cyanogen bromide, which hydrolyzes the peptide bond between Met102 and Phe103. For this sample, 125 mg of purified HR52-FPTM was dissolved in 12 ml of 50% formic acid containing 1.5 g cyanogen bromide. The cleavage reaction was carried out in the dark for 1 hour at room temperature under a stream of nitrogen gas. The solution was then dialyzed against 3 L water for 2 hrs with two water changes to remove cyanogen bromide. After dialysis, the cleavage solution was lyophilized and the dry powder was dissolved in 90% formic acid for reverse-phase HPLC using a C3 column (Agilent Zorbax, 0.94 x 25 cm) with 75% isopropanol and 25% acetonitrile as the organic phase and 95% water and 5% isopropanol as the aqueous phase with 0.1% TFA in both phases. The gradient of the organic phase was 40-100% in 100 min with a flow rate of 2.0 mL/min. The completeness of the cleavage reaction was confirmed by electrospray ionization (ESI) mass spectrometry (**Fig. S1**).

Membrane protein sample preparation

Purified proteins were reconstituted into lipid membranes using organic-phase mixing at protein/lipid mass ratios of 1 : 1 to 1 : 4 (**Table 2**). Three membranes were used to investigate protein conformation: POPC/POPG (4:1), DOPE, and MO/POPC (85% : 15%). These were

chosen because they represent different membrane curvatures and membrane surface changes, which have been shown to have a significant impact on the conformations of the separate FP and TMD peptides^{14, 15, 32}. The negatively charged lamellar POPC/POPG membrane favors the helical conformation of the separate peptides, while the negative-spontaneous-curvature DOPE membrane and the negative-Gaussian-curvature MO/POPC membrane promote the β -sheet conformation. Protein and lipids were co-dissolved in HFIP and chloroform, the organic solvents were removed with a stream of nitrogen gas, and then the mixture was lyophilized overnight. The dry powder was suspended in HEPES buffer (10 mM HEPES, 1 mM EDTA, 3 mM NaN_3 , pH 7.5) and dialyzed against the buffer for a day. The proteoliposome solution was centrifuged at 55,000 rpm or 40,000 rpm at 4°C for 3-4 h. The resulting pellets were equilibrated to ~40% water by mass in a desiccator, then transferred into 3.2 mm or 4 mm MAS rotors. The organic-phase mixing is important for keeping the chimera in a monomeric state before lipid mixing, for ensuring complete reconstitution of the protein into the membrane, and for allowing comparison of the chimera with the separate peptides, which were reconstituted using organic-phase mixing. When organic-phase and aqueous-phase mixing protocols were compared on other membrane peptides such as antimicrobial peptides³⁸⁻⁴¹ and the influenza M2 transmembrane peptide⁴²⁻⁴⁴, they were found to give the same equilibrium structures and dynamics as long as the aqueous-phase mixing is conducted on the monomeric state of the peptide before membrane binding.

To examine the effect of SDS on the protein-lipid interaction, we titrated 10% SDS solution into protein-free and protein-containing DOPE membranes to a DOPE/SDS mole ratio of 2 : 1. The control membrane and the protein-containing membrane were equilibrated to ~55% and ~40 wt% water, respectively. To assess protein conformational homogeneity, we measured solution NMR ^1H - ^{15}N HSQC spectra on SIC-FPTM1 and SIC-FPTM2 (**Fig. S2**). The protein was dissolved in HFIP, vacuum-dried at room temperature to remove the organic solvent, then suspended in 500 μl of 150 mM SDS with 10% D_2O , 20 mM sodium phosphate (pH 7.5), 1 mM EDTA and 1 mM DSS (sodium 2,2-dimethylsilapentane-5-sulfonate), to a final concentration of 0.4 mM for SIC-FPTM1 and 1 mM for SIC-FPTM2.

We also measured the synthetic fusion peptide FPK4 (sequence $^{103}\text{FAGVVIGL AALGVATAAQ VTAVALVK}^{129}\text{-NH(CH}_2\text{CH}_2\text{O)}_2\text{CH}_2\text{CO-KKKK}$) in MO/POPC membranes. The peptide was synthesized as described before^{15, 32}. Two ^{13}C , ^{15}N -labeled samples were measured: GVTAQ-FPK4 with residues labeled at G105, V106, T122, A123 and A124, and AAQV-FPK4 with labeled residues at A118, A119, Q120 and V121. The peptide was reconstituted into MO/POPC (17 : 3 mole ratio) at a peptide/lipid molar ratio of 1 : 40.

NMR experiments

Solid-state NMR experiments were conducted on Bruker spectrometers at ^1H Larmor frequencies of 400, 600, 800 and 900 MHz. ^{13}C chemical shifts were externally referenced to the adamantane CH_2 signal at 38.48 ppm on the TMS scale⁴⁵, ^{15}N chemical shifts were referenced to the Phe ^{15}N signal in formyl-MLF at 110.1 ppm on the liquid ammonia scale⁴⁶, and ^{31}P chemical shifts were referenced to the hydroxyapatite ^{31}P signal at 2.73 ppm on the phosphoric acid scale.

2D ^{13}C - ^{13}C correlation spectra were measured using DARR or PDSD spin diffusion⁴⁷ with short and long mixing times to detect conformation-dependent ^{13}C chemical shifts and inter-residue correlations, respectively. ^{15}N chemical shifts were obtained from 2D ^{15}N - ^{13}C correlation

spectra measured at 298 K under 12 kHz MAS. A REDOR sequence with a 833 μ s mixing time was used for coherence transfer between ^{15}N and ^{13}C ⁴⁸. Partial sequential assignment was obtained from 2D N(CO)CX correlation spectra ⁴⁹ measured at 293 K and 263 K under 11 kHz MAS. To measure protein mobility, we conducted 2D ^{13}C - ^1H DIPSHIFT experiments at 298 K ⁵⁰. To obtain better site solution, we also measured a reduced-3D version of the spectrum containing two ^{13}C chemical shift dimensions ⁵¹. The experiment used 50 ms ^{13}C spin diffusion between the two ^{13}C dimensions and was conducted at 283 K. For these DIPSHIFT experiments, a ^1H FSLG homonuclear decoupling sequence ⁵² with an effective field of 98 kHz was used during the dipolar evolution period, and the sample was spun at 7 kHz. To determine the depth of insertion of the protein, we measured 2D ^1H - ^{13}C spectra correlating the water and lipid ^1H chemical shifts with the protein ^{13}C chemical shifts ^{53, 54}. The ^1H spin diffusion mixing times were 25 ms to 100 ms. The effect of the chimera on the membrane order was probed from 1D ^{31}P static spectra and 2D ^{31}P - ^1H correlation spectra under 5 kHz MAS.

2D ^1H - ^{15}N HSQC spectra of SIC-FPTM1 and SIC-FPTM2 in 150 mM SDS solution were measured at room temperature on the 900 MHz spectrometer. ^1H chemical shifts were referenced to internal DSS and ^{15}N chemical shifts were indirectly referenced to DSS ⁵⁵.

Results

Expression and purification of HR52-FPTM

The combination of FP and TMD renders the chimera extremely hydrophobic, thus an effective solubility tag is essential for purification. With HR52, we were able to obtain ~100 mg of Ni^{2+} -column purified protein from 1 L of minimal media. SDS-PAGE of the lysed whole cell before purification showed both monomer and dimer bands (monomer molecular weight 16.2 kDa) (**Fig. 1c**). After purification, the protein purity was 80-95% based on the sum of the oligomer bands up to the tetramer. When the inclusion body was washed with 5% Triton X-100, the purity was ~80% (SIC-FPTM3). With additional Ni^{2+} column chromatography, the purity increased to ~85% (SIC-FPTM2). HPLC purification increased the purity to ~95% (SIC-FPTM1). HFIP solubilization before SDS-PAGE significantly reduced the amount of high-order oligomers, while without HFIP, the gel is dominated by high-molecular-weight aggregates (**Fig. 1d**). Some of the gels resolve two dimer bands, suggesting two slightly different conformations of the protein under the SDS-PAGE condition. To assess if the presence of the dimer gives rise to conformational heterogeneity in NMR spectra, we compared the HSQC spectra of fully purified SIC-FPTM1 and affinity-column purified SIC-FPTM2 in SDS micelles. The spectra are indistinguishable (**Fig. S2**), and both exhibit a single sidechain N-H cross peak for the single Trp in the protein, indicating that the protein conformation is homogeneous, and the purity is the same with or without HPLC.

FP and TMD in the chimera are α -helical in POPC/POPG membranes and exhibit no inter-domain cross peaks

Our general approach for investigating the FPTM chimera structure is to measure 2D correlation spectra, to determine whether the protein conformation is sensitive to the lipid composition of the membrane, whether there is significant protein motion in the liquid-crystalline phase of the membrane, and whether there is close spatial contact between the FP and TMD segments based on long-range correlation spectra. In the chimera sequence, the FP contains most Ala residues, the only Asn of the protein, and lacks Ser, while the TMD contains

most Ile residues the only Trp of the protein, and lacks Thr (**Table 1**). Thus, if Ala, Asn and Thr exhibit cross peaks with Ile, Trp or Ser, then the FP and TMD are in close contact ($< 10 \text{ \AA}$). Based on the number of each amino-acid type, the most diagnostic long-range cross peaks that would indicate FP-TMD association are Ala-Ile and Ala-Ser.

Fig. 2 and Fig. S3 show 2D ^{13}C - ^{13}C correlation spectra of POPC/POPG-bound AIA-FPTM. Characteristic chemical shifts of Ala, Ile, Val, Thr, Ser and Glu are observed and can be readily assigned^{56, 57}. Among these residues, Ile gives exclusively α -helical chemical shifts while Ala and Val show predominantly helical chemical shifts, with additional weak intensities at β -strand chemical shifts. Thr exhibits α -helical chemical shifts in the carbonyl region and random coil chemical shifts in the aliphatic region. The Ile intensities are significantly higher at low temperature than at high temperature, suggesting that the Ile-rich TMD segment may undergo intermediate timescale motion in the membrane. Since Ile, Ala and Val are mainly found in the FP and TMD (**Table 1**), while nine out of eleven Thr residues are located in HR52, these results suggest that the FP and TMD are α -helical while the solubility tag is a random coil. The unstructured nature of the tag is also reflected by FT-IR and CD data⁵⁸. Based on C α -C β cross peak intensities in the short mixing-time 2D spectra at low temperature (**Fig. S3**), we estimate the non-helical fractions of the Ala and Val signals to be $\sim 13\%$ and $\sim 42\%$, respectively, which match well the percentages of Ala and Val in the non-FP and non-TMD regions of the protein (20% and 38%). Thus, the non-helical chemical shifts can be mostly attributed to HR52 and the protease cleavage sites.

SIC-FPTM showed similar α -helical chemical shifts as AIA-FPTM in the POPC/POPG membrane (**Fig. 2c and Fig. S3**). More cross peaks were observed due to the more extensive isotopic labeling. Ile shows completely α -helical chemical shifts, while Ala, Val, Ser, Thr, Gly and Gln show mixed-conformation chemical shifts. Due to the larger linewidths at low temperature, we used the high-temperature spectra to estimate the helical fractions of the labeled residues. Based on one- and two-bond cross peak intensities in the short-mixing-time spectra, the helical fractions of Ala, Val and Gly are $\sim 95\%$, $\sim 68\%$ and $\sim 32\%$, respectively, again in excellent agreement with the percentages of these residues in the FP and TMD, indicating that these two domains are predominantly helical.

The amino-acid specific chemical shifts of FP- and TMD-enriched residues allow us to probe FP-TMD spatial contacts in the membrane. **Fig. 2b, d** show 2D ^{13}C - ^{13}C correlation spectra with a long mixing time of 500 ms. For AIA-FPTM, clear Ala-Val and Ala-Ile cross peaks were observed. The former can be assigned to intra-FP contacts, since the FP contains two sequential pairs of Ala and Val, while the Ala-Ile cross peaks can result either from two sequential Ala-Ile pairs in the TMD (**Fig. 1b**) or from inter-domain FP-TMD contacts. To remove this ambiguity, we measured the spectra of SIC-FPTM1, which eliminated the two Ala residues in the TMD. **Fig. 2d** shows that the Ala-Ile cross peaks disappeared in the 500 ms spectrum, indicating that the Ala-Ile cross peaks in the AIA-FPTM spectrum are due to intra-TMD sequential correlations rather than inter-domain contacts.

The SIC-FPTM1 sample shows numerous other inter-residue cross peaks such as A-T, A-Q, A-G, G-V, S-I, S-G and S-V in the 500 ms spectrum (**Fig. 2d**). However, sequential pairs of these residues can be found in either the FP domain or the TMD, thus these peaks most likely

result from intra-domain correlations. The A-T, A-V, A-Q, A-G and G-V cross peaks can be assigned to the FP domain, while the S-I, S-G and S-V cross peaks can be assigned to the TMD. These residue pairs span most of the FP and TMD sequences, confirming that the two domains are α -helical throughout their entire lengths in the POPC/POPG membrane.

FP and TMD interactions are unaffected by the solubility tag

To verify whether the above structural results for the FP and TMD hold in the absence of the solubility tag, we cleaved the solubility tag using cyanogen bromide and measured the 2D ^{13}C - ^{13}C correlation spectra of the cleaved protein in the POPC/POPG membrane. ^{13}C 1D and 2D correlation spectra (**Fig. 3a, b**) show that all α -helical chemical shifts of the FP and TMD residues remain, but the non-helical chemical shifts of the tag disappeared. 2D correlation spectra measured with long mixing times of 0.5 s and 1.0 s show only intra-domain cross peaks such as between Ala and Val in the FP and between Ser and Ile in the TMD, but no cross peaks between the FP and TMD residues (**Fig. 3c**), identical to the findings of the uncleaved protein. Therefore, these spectra confirm that the FP and TMD segments remain loosely associated with each other in the absence of the solubility tag, and the spectra of the uncleaved protein reflect the intrinsic conformation and three-dimensional fold of the FP-TMD segment. In the cleaved protein, a few β -sheet chemical shifts of Ile and Val residues are observed, which overlap with the β -sheet signals of the solubility tag in the uncleaved protein. These minor β -sheet signals can be assigned to the TMD, whose C-terminus was shown to have a higher propensity to form β -sheet structure in the isolated peptide¹⁴.

FP and TMD in the chimera remain helical in DOPE and MO/DOPC membranes

Since the separate FP and TMD peptides shift their conformational equilibria to β -strand in negative-curvature membranes^{14, 15, 32}, it is important to determine if the FPTM chimera's conformation is also membrane dependent. The β -strand conformation of the isolated TMD converts the negative-curvature DOPE to a bicontinuous cubic phases (Q_{II}) with negative Gaussian curvature (NGC), which is the characteristic membrane topology in hemifusion intermediates⁵⁹. Similarly, the isolated FP exhibits predominantly β -strand chemical shifts and is well inserted into cubic-phase MO/POPC membranes (**Fig. S4**)⁶⁰. To investigate if these negative-curvature and negative-Gaussian-curvature membranes change the chimera's conformation, we measured 2D ^{13}C - ^{13}C correlation spectra of the protein bound to DOPE and MO/POPC membranes. Importantly, the protein shows nearly identical chemical shifts in the DOPE membrane (**Fig. 4, Fig. S5**) as in the POPC/POPG membrane, with Ala, Val and Ile showing predominantly α -helical chemical shifts while Gly, Ser, Thr and Gln exhibiting mixed helix and coil chemical shifts. Low-temperature $\text{C}\alpha$ - $\text{C}\beta$ cross peak intensities indicate helical fractions of $\sim 91\%$, $\sim 64\%$ and $\sim 82\%$ for Ala, Val and Ile, respectively, similar to the values for the POPC/POPG-bound protein. Also similar to the POPC/POPG-bound proteins, Ile intensities are weaker at high temperature while other residues do not have this temperature dependence, indicating that the Ile-rich TMD undergoes intermediate-timescale motion in the DOPE membrane. At a long mixing time of 1.0 s, inter-residue A-T, A-V, A-Q, A-G, G-V, S-I and S-V cross peaks are observed and can be assigned to either the FP or the TMD, but no inter-domain A-I and A-S cross peaks are present. Changing the membrane to MO/POPC only moderately affected the helical content of the protein, with Val and Ile exhibiting lower helical fractions of $\sim 61\%$ and $\sim 57\%$, but Ala remains 90% helical. Again, no inter-domain cross peaks are present. Finally, to verify whether residual SDS in the affinity-column-purified samples might be

responsible for the persistent α -helical conformation, we reconstituted the protein from inclusion body washes directly into the DOPE membrane. **Fig. S5** shows the spectra of SIC-FPTM3, where Ala, Ile, and Val exhibit the same α -helical chemical shifts as the other samples, confirming that the helical conformation is inherent to the FP-TMD sequence. These data, taken together, indicate that tethering the FP and TMD stabilizes the α -helical conformation and prevents the helix-to-strand conformational change seen in the separate peptides, but the FP and TMD do not come into sub-nanometer contact of each other.

The α -helical FP and TMD are immobilized and inserted into the membrane

In these 2D ^{13}C - ^{13}C correlation spectra, residues that are significantly populated in the HR52 and the G₃K₅ linker such as Thr and Ser show mainly random coil chemical shifts. C-H dipolar coupling measurements (**Fig. 5**) indicate low order parameters of 0.55–0.76 for C α -H α and $\sim$ 0.48 for C β -H β for residues in the solubility tag and the linker, while α -helical Ala, Val and Gly residues yield higher C α -H α and C β -H β order parameters of 0.86–0.95 and 0.66, respectively, indicating that the FP and TMD are more immobilized.

2D ^{13}C - ^1H correlation spectra (**Fig. 6**) of the POPC/POPG-bound protein show clear cross peaks between the lipid CH₂ and the helical FP and TMD residues at short spin diffusion mixing times of 25 ms and 49 ms, indicating that the FP and TMD are well inserted into the membrane. The intensity distribution between the lipid CH₂ and water cross sections differ: the random coil Thr, Asp/Glu/Gln, and Gly signals show much higher water cross peaks than lipid cross peaks, indicating their proximity to water. These results indicate that HR52 and the linker are dynamically disordered and well exposed to water, while the FP and TMD domains are immobilized and inserted into the membrane.

2D ^{15}N - ^{13}C correlation spectra of DOPE-bound SIC-FPTM2 (**Fig. 7**) allow sequential assignment of several residue pairs and reveal different mobilities of the FP and TMD. The REDOR-based 2D spectrum shows all one-bond ^{15}N - $^{13}\text{C}\alpha$ cross peaks while the N(CO)CX spectra exhibit sequential cross peaks between the amide ^{15}N of the i th residue and ^{13}C of the i -1th residue were observed. By comparing with the ^{15}N chemical shifts obtained from the REDOR-based 2D ^{15}N - ^{13}C correlation spectra and the ^{13}C chemical shifts from 2D ^{13}C - ^{13}C correlation spectra, we can assign a number of sequential cross peaks. Since the TMD does not contain Thr and Ala residues, and the FP does not contain Ser, all helical chemical shifts of Thr and Ala can be assigned to the FP and all helical chemical shifts of Ser can be assigned to the TMD. Only one pair of Val-Thr and Ala-Thr exist in the protein, thus the T_N-V_{Ca} cross peak can be assigned to T122-V121 and the T_N-A_{Ca} peak can be assigned to T117-A116 in the FP. Similarly, only one pair of Ser-Val and Gly-Ser exist in the protein, thus we can assign a V506-S505 and S495-G494 cross peak. Importantly, the TMD cross peaks are only observed at low temperature (263 K) and are absent in the room-temperature spectra, while the FP cross peaks are observed at both temperatures. Thus, the TMD is more dynamic than the FP in the chimera, consistent with the weaker intensities of the TMD-enriched Ile signals in the 2D ^{13}C - ^{13}C correlation spectra.

FPTM chimera generates negative Gaussian curvature to membranes with positive spontaneous curvature

To determine if the FPTM chimera causes membrane curvature and dehydration, we measured static 1D ^{31}P spectra and 2D ^1H - ^{31}P correlation spectra (**Fig. 8**). POPC/POPG and POPE membranes are unperturbed by the chimera, as shown by their uniaxial powder lineshapes. For the DOPC/DOPG membrane, which has a small negative spontaneous curvature due to the disordered acyl chains, the protein partially narrowed the ^{31}P lineshape. For the hexagonal-phase DOPE membrane, the protein caused a small isotropic peak that amounts to $< 15\%$ of the total spectral intensity. This perturbation is much weaker than the membrane perturbation caused by the isolated TMD, which converted $\sim 85\%$ of the DOPE membrane to a cubic phase¹⁴. Interestingly, when a small amount of SDS was added to DOPE, which created a lamellar membrane, the ^{31}P spectrum of the protein-containing membrane shows a strong isotropic peak. Since SDS causes positive curvature, this result suggests that the chimera induces significant curvature to membranes with spontaneous *positive* curvature. This behavior differs qualitatively from the isolated TMD, which incurs curvature preferentially to membranes with spontaneous *negative* curvature^{14, 15}. ^{31}P T_2 relaxation measurement of the DOPE/SDS sample showed a short T_2 of 1.8 ± 0.4 ms (data not shown), indicating cubic-phase formation, since isotropic micelles exhibit much longer T_2 values⁶¹. Therefore, the FPTM chimera generates NGC to membranes with spontaneous positive curvature. The ^1H cross sections of the 2D ^1H - ^{31}P correlation spectra of FPTM-containing POPC/POPG and DOPE membranes show broadened water cross peaks, indicating that both membranes are hydrated, but the water dynamics is slower in the presence of the protein than in protein-free membranes.

Discussion

In the post-fusion state of class I viral fusion proteins, the ectodomain forms a trimer of hairpins, which implies that the hydrophobic fusion peptide at the N-terminus and the transmembrane domain at the C-terminus lie in close proximity in the membrane. However, these crystal structures were solved in the absence of lipids or detergents, thus the trimer of hairpin structure was never subjected to the environment of a membrane, and few direct experimental data exist about the structure and packing of the FP and TMD in lipid bilayers. The current FPTM chimera is designed to directly interrogate the FP-TMD structure and assembly in phospholipid membranes. Comparisons of the chimera's chemical shifts in different lipid membranes further allow us to probe the influence of the membrane environment on the protein structure. The results are interesting in three ways. First, the conformation of the FP and TMD in the chimera is stably α -helical, mostly independent of the membrane composition, which contrasts with the conformational plasticity of the separate FP and TMD peptides. The isolated peptides are helical in lamellar POPC/POPG membranes but shift significantly or predominantly to the β -strand conformation in negative-curvature PE membranes^{14, 15, 32}. The persistence of the helical structure of the FP and TMD in the chimera implies that inter-domain FP-TMD interactions, and possibly intermolecular protein-protein interactions, are stronger than the interaction of each domain with the lipids. Second, although the stability of the α -helical conformation of the chimera suggests mutual interactions between the FP and the TMD, no inter-domain ^{13}C - ^{13}C cross peaks are observed. Thus, tethering the FP and TMD by an eight-residue linker is insufficient for causing a tight hetero-oligomeric helix bundle with inter-helical separations of less than ~ 7 Å, which is the approximate distance upper limit of ^{13}C spin diffusion⁶². The weak interaction between the FP and TMD persists in the absence of the solubility tag, thus is an inherent property of the two hydrophobic segments. Finally, ^{31}P spectra indicate that the FPTM chimera induces little curvature to lamellar and negative-curvature membranes, but

causes significant negative Gaussian curvature to mixed DOPE/SDS membranes, which have *positive* spontaneous curvature. This differs from the behavior of the separate TMD and FP peptides, which create NGC only to membranes with spontaneous *negative* curvature.

2D ^1H - ^{13}C correlation spectra confirm that the FP and TMD residues are well inserted into the membrane whereas the polar residues in the N-terminal tag lie in the aqueous phase and are dynamically disordered. The weak FP-TMD interaction in the PIV5 fusion protein chimera is consistent with recent data of the influenza and HIV fusion proteins. For influenza HA, mixed FP and TMD peptides in lipid membranes caused different EPR lineshapes and different membrane ordering from those of the separate peptides. On the other hand, isothermal titration calorimetry data of the mixed peptides in small unilamellar vesicles showed only a small enthalpy of mixing of -5.7 kCal/mol, indicating that the intermolecular interaction is weak⁶³. Fluorescence resonance energy transfer (FRET) data indicate that the FP and TMD of influenza HA associate in an antiparallel fashion in DMPC/DMPG membranes⁶⁴, but the complex is not strong enough to survive 0.1% SDS and the electric field.

For the HIV fusion protein gp41, FP-TMD interactions were evidenced by FRET between the FP and TMD, the slowing down of FP degradation upon addition of the TMD, and the inhibition of the self-assembly of the TMD by exogenously added FP⁶⁵. Mutagenesis data indicate that the interaction regions lie in the N-terminal half of the FP and the C-terminal half of the TMD, suggesting an antiparallel arrangement. However, solution NMR studies of several gp41 constructs in DPC micelles found no evidence of stable FP-TMD association. In full-length gp41¹⁻¹⁹⁴, the NMR signals of the N-terminal half of the protein containing the FP are readily detectable, whereas the signals of the C-terminal half of the protein containing the TMD are broadened²⁶. In a shorter gp41 construct²⁷⁻¹⁹⁴ without the FP, relaxation NMR data indicate that the TMD is significantly immobilized, which differs from the observed large-amplitude motion of the FP in the full-length protein. Thus, these two domains of gp41 do not form tight complexes in DPC micelles²⁷. Paramagnetic relaxation enhancement data of spin-labeled gp41¹⁻¹⁹⁴ showed that the C-terminus of the TMD only moderately increased the transverse relaxation rates of FP and FPPR, also suggesting only a weak interaction²⁶.

The weak interaction between FP and TMD in the PIV5 chimera differs from analytical ultracentrifugation data that indicate hexamer formation of the FP and TMD peptides in DPC micelles⁶⁶. We hypothesize that this difference may result from the positive curvature of DPC micelles, which is absent in most of the membranes used in this study. It is well established that transition from the separate membranes to a lipid stalk state requires negative curvature of the outer leaflet, while transition from the stalk to the post-fusion pore requires positive curvature of the inner leaflet^{20, 22, 67}. The positive-curvature DPC micelles may mimic the post-fusion membrane environment more than earlier states of the fusion pathway. Thus, the detected hexamer formation of the PIV5 FP and TMD in DPC micelles suggests that FP-TMD association may be facilitated by positive membrane curvature.

The weak yet helix-preserving FP-TMD interaction in the PIV5 fusion protein chimera gives insight into the mechanism of fusion-pore formation and constrains the possible post-fusion structures of the intact protein. A recent study of the F protein of the measles virus, another member of the paramyxovirus family, found that an ectodomain 6HB was not absolutely

required for complete fusion⁶⁸. When disulfide bonds were engineered at the C-terminus of HR2 to prevent 6HB formation, virus-cell fusion still occurred⁶⁸. This result suggests that either the FP and TMD assemble into an intramembrane 6HB to provide the energy for fusion, or partial assembly of the trimeric ectodomain, with only five or four helices in the bundle or with only partial zippering of the HR1 and HR2, may be sufficient to enlarge and open the fusion pore. The current solid-state NMR data indicate that the FP and TMD do not form tight complexes in the membrane, thus the second interpretation is more likely.

The exact relative orientation and displacement of the FP and TMD in the chimera are not known from the current data, and several scenarios can be envisioned. One possibility is that the two helices may be tilted from each other and associate loosely in a wedge shape. Such a wedge may serve the function of promoting the appropriate curvature to one leaflet of the membrane to facilitate fusion-pore opening (**Fig. 9**). Alternatively, the two helices may be translationally displaced from each other without a significant tilt. Our data so far do not probe homo-oligomeric association, thus we include both monomers and oligomers in the model. Future experiments will be required to determine homo-oligomeric association of this chimera.

Since in the intact F protein, the FP and TMD are tethered to the adjacent HR1 and HR2, which are capable of forming a helix bundle under certain conditions, it is reasonable to ask whether the replacement of these segments by a flexible linker might weaken the FP-TMD interactions. This possibility cannot be ruled out without studying the full-length protein, which is challenging for the large PIV5 F protein. However, solution NMR data of HIV gp41 found that ectodomain HR1 and HR2 each interacts strongly with the membrane surface but much less with each other^{69,70}, implying a lack of FP-TMD association. Together with the disulfide-bond study of the measles virus F protein, these results consistently suggest that the ectodomain may not be a strong enforcer of FP-TMD association in the membrane, and loose FP-TMD contact may be operative for a significant fraction of the lifetime of these viral fusion proteins in the fusion pathway. It is also possible that close contact between the FP and TMD may require an even shorter linker than used here. Future experiments will be required to test this hypothesis. We note that an eight-residue random coil is not sufficiently long to traverse the water layer between two lipid bilayers, thus the FP and TMD should reside in the same bilayer.

In summary, the hydrophobic FP and TMD of the PIV5 F protein, when placed in spatial proximity by a covalent linker, interact with each other to adopt an α -helical conformation but do not interact so strongly to form a tight helix bundle. The membrane-spanning helices are found in low-curvature PC/PG membranes as well as negative-curvature PE membranes, which differ qualitatively from the β -strand-rich conformation of the isolated peptides in PE membranes. The helix-rich FP-TMD chimera induces little curvature to lamellar and negative-curvature membranes, but generates significant NGC to membranes that initially possess spontaneous positive curvature. This behavior differs from the separate peptides, which incur NGC to membrane with spontaneous negative curvature. These results support a model where the separate β -sheet FP and TMD transform negative-curvature membrane domains to NGC, which are characteristic of hemifusion intermediates, while the tethered and loosely associated α -helical FP-TMD transforms positive-curvature regions of lipid membranes to NGC. Therefore, both the membrane and the protein have extremely fine tuned structures during the multi-step transformations that accomplish virus-cell fusion.

Supporting Information

Additional NMR spectra and tables include:

MALDI and ESI mass spectrometry data (Figure S1);

^1H - ^{15}N HSQC spectra (Figure S2);

Additional 2D spectra of FPTM in POPC/POPG and DOPE membranes (Figures S3, S5);

Additional spectra of FPK4 in MO/POPC membranes (Figure S4).

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Table 1. Amino acid composition and distribution in the FPTM chimera.

Residues	Amino acid distribution			
	FP	TMD ^a	Other ^b	Total ^a
Ala (80%)	10	2 (0)	3	15 (13)
Asn	1	0	0	1
Arg	0	0	2	2
Asp	0	0	5	5
Cys	0	0 (1)	0	0 (1)
Gln	1	0	2	3
Glu	1	0	4	5
Gly (31%)	3	2	11	16
His	0	0	9	9
Ile (78%)	1	6	2	9
Leu	3	7	9	19
Lys	1	1	14	16
Met	0	0	5	5
Phe	1	0	2	3
Pro	0	0	8	8
Ser	0	3 (4)	4	7 (8)
Thr	2	0	9	11
Tyr	0	0	2	2
Trp	0	1	0	1
Val (62%)	6	4	6	16
Total	30	26	97	153

^a Numbers in brackets are the values for SIC-FPTM.

^b Other includes the HR52, protease cleavage sites, and the G₃K₅ linker.

Table 2. Isotopic labeling and purification of FPTM chimera samples.

Protein ^b	Labeled residues ^a	Purification	Purity	Membranes	P/L mass ratio
AIA-FPTM	AINSTVW	inclusion body washes, HPLC	≥ 95%	POPC/POPG (4 : 1)	1 : 3
SIC-FPTM1	ADGINQSTV	inclusion body washes, Ni ²⁺ column, dialysis, HPLC	≥ 95%	POPC/POPG (4 : 1)	1 : 3
SIC-FPTM2	ADGINQSTV	inclusion body washes, Ni ²⁺ column, dialysis (minor amount of SDS)	~85%	DOPE; MO/POPC (85:15)	1 : 1
SIC-FPTM3	All residues	inclusion body washes	~80%	DOPE	1 : 1.5
SIC-FPTM4	Unlabeled	inclusion body washes, HPLC	≥ 95%	POPC/POPG (4 : 1), DOPC/DOPG (4 : 1), POPE, DOPE	1 : 3
SIC-FPTM5	ADGINQSTV	inclusion body washes, cyanogen bromide cleavage, HPLC	≥ 95%	POPC/POPG (4 : 1)	1 : 4

^a The labeled residues are based on the unlabeled amino acids added to the M9 medium.

^b SIC-FPTM5 does not contain the HR52 tag while all other constructs contain the tag.

Figure 1. Expression and purification of the PIV5 FPTM chimera. (a) Schematic of the chimera sequence, including the fusion partner, the FP domain, the G₃K₅ linker, and the TMD. Residue numbering at the top (1-153) refers to the construct, while numbering at the bottom (103-132 and 485-510) corresponds to the numbering in full-length PIV5 F. (b) Amino acid sequence of HR52-FPTM. Two protease cleavage sites were placed between HR52 and FPTM. The AIA and SIC variants differ at residues 490-492, where the wild-type protein has AIC. (c) SDS-PAGE gel showing protein expression in *E. coli*. After IPTG induction, two bands corresponding to the monomer and dimer molecular weights (16.2 and 32.4 kDa) were observed. (d) SDS-PAGE gel comparing protein purification results. Without solubilization in HFIP (left), the protein forms high-order aggregates. With HFIP solubilization (right), the monomer band dominates and accounts for 50-90% of the total intensity. Two weak dimer bands are resolved under some of the conditions, suggesting two slightly different conformations. The most complete purification protocol (SIC-FPTM1) gave the purest protein, but even the simple procedure of inclusion body washes (SIC-FPTM3) gave a similar gel result, with the monomer being the major product.

Figure 2. 2D ^{13}C - ^{13}C correlation spectra of AIA-FPTM (a, b) and SIC-FPTM1 (c, d) in POPC/POPG membranes. (a) AIA-FPTM spectrum at 293 K and 20 ms mixing. (b) AIA-FPTM spectrum at 248 K and 500 ms mixing. (c) SIC-FPTM1 at 298 K and 100 ms mixing. (d) SIC-FPTM1 at 263 K and 500 ms mixing. All spectra show predominantly α -helical chemical shifts, and all inter-residue cross peaks can be assigned to within the FP or within the TMD. The Ala-Ile cross peaks in the AIA-FPTM spectrum (b) are not observed in the SIC-FPTM1 spectrum (d), indicating that they result from intra-domain correlations. Intra-residue assignments are shown in green for FP, red for TMD, and black for mixed FP and TMD signals. Non-helical chemical shifts (assigned in brown) can be attributed to the solubility tag and the linker. Only inter-residue cross peaks are assigned in the long mixing-time spectra.

Figure 3. 1D and 2D ^{13}C spectra of HR52-cleaved SIC-FPTM5 in POPC/POPG membranes. (a) 1D ^{13}C direct polarization (DP) spectra of unclesaved SIC-FPTM1 and cleaved SIC-FPTM5 at 298 K. The random-coil signals of the solubility tag are absent in the spectrum of cleaved sample, confirming cleavage. (b) 2D ^{13}C - ^{13}C correlation spectrum of SIC-FPTM5 at 263 K with a 100 ms mixing time. (c) 2D ^{13}C - ^{13}C correlation spectrum with 0.5 s and 1 s mixing. Non-helical signals (assigned in brown) of the solubility tag disappeared in the cleaved SIC-FPTM5 spectra, while the helical chemical shifts of the FP and TMD residues are unchanged. Cross peaks are assigned in green for FP residues, red for TMD residues, and black for mixed FP and TMD signals. Non-helical chemical shifts of HR52 and the linker are assigned in brown. Only inter-residue cross peaks are assigned in the long mixing-time spectra.

Figure 4. 2D ^{13}C - ^{13}C correlation spectra of SIC-FPTM2 in (a, b) DOPE and (c, d) MO/POPC membranes. (a, c) 263 K spectra with 100 ms mixing. (b, d) 298 K spectra with 1.0 s mixing. Both the FP and TMD exhibit predominantly α -helical chemical shifts. The β -strand signals of Ile and Val are most likely from the TMD. Superscripts "h", "s", and "c" denote helix, strand, and coil, respectively.

Figure 5. ^{13}C - ^1H dipolar order parameters of SIC-FPTM1 in the POPC/POPG membrane. (a) The first slice of the ^{13}C dimension of the 2D ^1H - ^{13}C DIPSHIFT spectrum. Compared to the 1D ^{13}C DP spectrum, the α -helical signals of the FP and TMD are preferentially enhanced, indicating that the FP and TMD are immobilized while the solubility tag and the linker are dynamic. (b) Representative dipolar dephasing curves of C α and C β sites. FP and TMD residues show larger S_{CH} values than water-exposed HR52 residues (brown).

Figure 6. 2D ^1H - ^{13}C correlation spectrum with spin diffusion of SIC-FPTM1 in POPC/POPG membrane to determine the depth of insertion. (a) 2D spectrum with ^1H spin diffusion mixing times of 25 ms and 49 ms and a ^1H T_2 filter of 5.7 ms. Lipid-protein cross peaks are observed, indicating that the chimera is well inserted into the membrane. (b) ^{13}C cross sections from the water (blue) and lipid CH_2 (black) ^1H chemical shifts. High water cross peaks (assigned in brown) are observed at non-helical chemical shifts and can be attributed to the disordered solubility tag and the linker.

Figure 7. 2D ^{15}N - ^{13}C correlation spectra of SIC-FPTM2 in the DOPE membrane. (a) REDOR transferred spectrum showing intra-residue N-C α cross peaks. (b, c) N(CO)CX correlation spectra showing sequential cross peaks between the ^{15}N of residue *i* and the ^{13}C chemical shifts of residue *i*-1. The spectra were measured at 293 K (b) and 263 K (c).

Figure 8. Effects of the FPTM chimera on membrane curvature and hydration. (a) ^{31}P static spectra of POPC/POPG, DOPC/DOPG, POPE, DOPE and DOPE/SDS membranes in the absence (black) and presence (red) of the protein. The temperatures were chosen to be above the phase-transition temperature of each membrane by a similar extent. The protein slightly narrowed the spectra of POPC/POPG and DOPC/DOPG membranes, did not perturb the POPE membrane, and caused a minor isotropic peak to the DOPE membrane. In comparison, the protein caused a strong isotropic peak to the DOPE/SDS membrane. (b) 2D MAS ^{31}P - ^1H correlation spectra of POPC/POPG and DOPE membranes. ^1H spin diffusion mixing times were 225 ms for the POPC/POPG sample and 100 ms for the DOPE sample. (c) ^1H cross sections of the 2D ^{31}P - ^1H correlation spectra showing broad water cross peaks, indicating that the membranes are hydrated but the water dynamics is slower than in protein-free membranes.

Figure 9. Schematic model of the conformation and membrane-bound topology of the PIV5 FPTM chimera in the membrane. The FP and TMD are α -helical under a variety of membrane compositions, but the two domains do not form a tight complex. A wedge shape may be adopted by the FP and TMD helices to incur membrane curvature to mediate fusion-pore opening.

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