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Review

Are fusion peptides a good model to study viral cell fusion?

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Abstract

Fusion peptides are hydrophobic and conserved sequences located within glycoprotein ectodomains that protrude from the virion surface. Direct participation of fusion peptides in the viral membrane fusion phenomenon has been inferred from genetic analyses showing that even a single residue substitution or a deletion within these sequences may completely block the process. However, the specific fusion peptide activities associated to the multi-step fusion mechanism are not well defined. Based on the assumption that fusion peptides are transferred into target membranes, biophysical methodologies have been applied to study integration into model membranes of synthetic fragments representing functional and non-functional sequences. From these studies, it is inferred that, following insertion, functional sequences generate target membrane perturbations and adopt specific structural arrangements within. Further characterization of these artificial systems may help in understanding the molecular processes that bring initial bilayer destabilizations to the eventual opening of a fusion pore.
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1. Introduction

The presence of a fusion peptide (FP) within the ectodomain exposed to the external aqueous medium is a salient feature of viral fusion proteins [1–6]. These hydrophobic sequences, usually located at the N-terminal end of the fusogenic transmembrane subunit, or close to it, are thought to be involved in driving the initial partitioning of the fusion protein into the target membrane. Hence, at a certain stage after fusion activation, the cryptic FP is exposed and likely to insert into the lipid bilayer of the target cell, thus transiently making the viral envelope glycoprotein an integral component of two membranes: that of the virus and that of the target cell [7–10]. Indeed, membrane insertion of FPs within the context of the whole fusion glycoprotein has been experimentally proven through hydrophobic photolabeling techniques [11].

Amino acid homology between fusion proteins of different viruses is usually of less than 20%. In contrast, FP homology for the same proteins can be as high as 90% [1]. FPs are also unusually enriched in Ala and Gly residues [3,4,6]. Comparable high degrees of residue conservation and Ala/Gly content are not found in other membrane-associated hydrophobic protein domains as signal sequences or transmembrane anchors, suggesting that FP sequences have evolved constrained by a selective pressure that cannot be explained by simple requirements of hydrophobicity or amphipathicity. Indeed, mutational analyses have established an absolute requirement for certain residues at defined positions [12,13], although this might not be the general case (see Ref. [14]).

No consensus exists as yet on the roles played by FPs in viral fusion. Some authors suggest that these sequences constitute the ‘‘active center’’ of the fusogenic protein molecule. According to this hypothesis, FPs would directly mediate fusion of the viral envelope with a cellular membrane [7,15]. Information available on the low-energy structures of human immunodeficiency virus (HIV)-1 gp41 and influenza HA2 glycoprotein ectodomains suggests that FPs would be linked through flexible tethers to helical domains [15,16]. Thus FPs need not be linked to those α-helices to

Abbreviations: Chol, cholesterol; FP, fusion peptide; HA, hemagglutinin; HIV, human immunodeficiency virus; IR, infrared spectroscopy; LUV, large unilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; RBC, red blood cell; SFV, Semliki forest virus; SUV, small unilamellar vesicles; TMD, transmembrane domain

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function in membrane fusion. It is then proposed that fusion activity depends solely on the intrinsic capacity of FPs to disrupt the target bilayer architecture after insertion.

An alternative hypothesis states that FPs would function as inert anchors, i.e., "secondary" transmembrane domains (TMDs) translocated into the target membrane by the action of the fusion glycoprotein [17]. Thus, upon fusion activation, FPs and "primary" TMDs would tightly tether target cell and viral membranes, respectively. Fusion would subsequently evolve, directly coupled to the collapse that follows formation of the low-energy helical bundle [17–20]. However, both proposed FP action mechanisms need not be mutually exclusive [21]. Mutant fusion domains have been isolated that are capable of integrating into target bilayers, and yet they do not support fusion activity [12,22], indicating that simple insertion is not enough to unleash FP function. Moreover, a direct role of FPs at facilitating fusion pore formation has been described [22–24], further suggesting the existence of unknown functional membrane-bound structures of FPs.

As discussed by Epand et al. [25] many features of the complex viral systems are not present in isolated peptide–vesicle model systems. In the context of the full-length envelope protein, FPs are linked to other sequences that may condition their conformational behavior. Natural target membranes bear specific characteristics such as composition (presence of particular lipids and proteins) and asymmetry that are difficult to reproduce in vitro. FP cooperativity might as well be promoted through oligomerization of protein complexes and/or by the high local concentrations already present at the viral envelope. Nevertheless, analysis of the molecular mechanisms underlying FP activity in the whole protein would be in most cases not viable. This has justified the development of in vitro FP assays using representative synthetic peptides and model membranes.

2. Evaluation of synthetic FP interaction with membranes

Synthetic FPs have been assayed for their capacity to perturb natural membranes. A good correlation has been observed between the ability of a particular sequence to support fusion in the intact protein and its ability to induce hemolysis [13,26,27]. In addition, HIV-1 FP has been shown to induce cytolysis of viral target $CD4^+$ lymphocytes, and fusion of erythrocytes [28–30]. An advantage of using natural membranes is that their compositions and asymmetry approximate those present in the viral target bilayers. However, specific effects of the lipid environment on FP activity have been more widely analyzed using lipid model systems of defined structure and composition. Large unilamellar vesicles (LUV) represent bilayer systems that closely resemble the lipid packing density and curvature corresponding to biological membranes. In contrast, highly curved small unilamellar vesicles (SUV) produced by sonication mimic the conditions of stressed bilayers as found in certain physiological instances. In addition, phospholipid monolayer systems allow the selection of defined packing density conditions and, therefore, the assessment of lateral pressure effects on the ability of peptides to penetrate the membranes. This option suits the study of FPs particularly well since their interaction appears to be initially restricted to the external monolayer of the target membrane [31–33].

Molecular interactions of FPs with model membranes may be investigated at several stages. First, membrane association is limited by the capacity of the sequence to partition from the aqueous to the membrane phase. Once membrane association is accomplished, peptides attain characteristic locations, conformations and degrees of oligomerization that are thought to condition subsequent induction of bilayer perturbations such as permeabilization or fusion.

2.1. FP transfer to membranes

It is generally assumed that FPs transfer from the aqueous medium to membranes due to spontaneous partitioning. Their tendency to partition from water into membranes is already suggested by their high interfacial hydrophobicity [5,34–36]. When synthetic FPs are diluted in water, aggregation usually occurs due to their hydrophobic character. Under most experimental conditions, aggregation in solution proceeds more slowly than membrane binding, thereby allowing effective incorporation of peptides into target membranes. However, formation of peptide aggregates precludes the experimental characterization of the water–membrane peptide partitioning equilibrium [34,37,38].

Determining the thermodynamic parameters governing this equilibrium might be useful to compute the amount of free energy that could effectively be coupled to membrane merging. This problem has been recently approached by Han and Tamm [37,38] using HA FPs linked to positively charged and unstructured sequences that solubilize the entire constructs. These authors determined an experimental value of -7.6 kcal mol^{-1} for the free energy of partitioning of the 20-aa-long influenza FP into SUVs at pH 5.0. Considering 60–120 kcal mol^{-1} as the free energy for the formation of highly curved lipidic structures that initially mediate membrane merging (see below), it was concluded that binding of 8–16 FPs would provide sufficient energy to stabilize such an intermediate. These calculations would therefore support a necessary and sufficient role of FPs at promoting viral membrane fusion.

2.2. FP insertion into membranes

The relevance of membrane-bound FP structures for viral fusion is supported by the fact that fragmented transmembrane oligomers can reassemble in specific ways (discussed in Ref. [39]). Thus spontaneous FP assembly into model membranes may also reflect the early events that mediate FP

integration into target membranes. A commonly accepted picture has emerged for the structures of monomeric membrane-bound HIV-1 and HA FPs. Based on electron spin resonance data, Gordon et al. [40] assumed the existence of an α-helical monomeric transmembrane state of HIV-1 FP with the N-terminus embedded in the bilayer core. The molecular model developed for this monomeric state by the same authors [40,41] and the NMR structure calculated by Chang et al. [42,43] for the peptide solubilized by SDS, concur in proposing the insertion of residues 1–15 in an α-helical conformation, and the existence of a flexible hinge Ala15–Gly16 that causes redirectioning of the C-terminal sequence. In addition, polarized attenuated total reflection infrared spectroscopy analysis by Martin et al. [44,45] indicated that insertion of the α-helical stretch spanning residues 1–16 occurs at an oblique angle.

Insertion of HA FP restricted to the external membrane monolayer was demonstrated by Brunner [31] using asymmetric hydrophobic photolabeling. This observation was confirmed by spin-labeling electron paramagnetic resonance [32]. According to the latter study, the N-terminus of HA FP also penetrates into the membrane hydrophobic core. The depth profile was consistent with an α-helix tilted from the horizontal plane of the membrane with a maximum depth of 15 Å from the phosphate group. An oblique angle of insertion was also revealed by polarized attenuated total reflection infrared spectroscopy [46,47]. Combining NMR-solved structures in micelles with EPR distance constraints measured in LUV, Han et al. [33] deduced that a 20-mer HA FP inserts into membranes in an "inverted V" conformation (Fig. 1). At low pH, the structure inserts deeper while the C-terminal arm transitions from an extended structure to a shorter 3_{10}-helix. The low-pH-induced structural change is proposed to intensify the FP capacity to distort bilayer architecture. Even though somehow discordant interpretations of structural HA FP NMR data in micelles have been reported [48–50], the "inverted V" model described by Han et al. [33] might constitute a valuable paradigm for future tests of the structural effects exerted by HA FP mutations which interfere with fusion activity (Ref. [21], see also Fig. 1).

2.3. FP oligomerization in membranes

It is generally accepted that viral fusion is promoted by high order complexes or ring-like protein aggregates that would confine fusion-inducing perturbations to localized areas of the interacting bilayers [2,3,51,52]. The rules governing specific self-association of protein stretches immersed into the bilayer milieu are only partially understood [39,53]. FPs have been shown to specifically self-associate in the membrane suggesting that surface oligomerization plays an important role in polypeptide-induced fusion [53–55]. It has been proposed that FPs inserted into target bilayers might assist recruitment of several fusion proteins into a single fusion site [6,38].

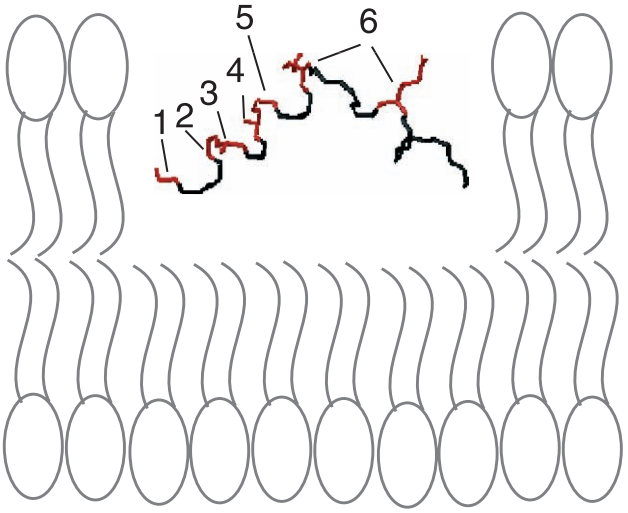

Fig. 1. Membrane-embedded low-pH structure of influenza HA FP: GLFGAIAGFIENGWEGMIDG [33]. Side chains and backbone corresponding to residues identified as affecting peptide–membrane interaction and fusion are indicated in red. (1) Gly1: polar substitutions or deletions abolish HA fusion activity and FP-induced hemolysis. Ala sustains activity while Ser induces "hemifusion" phenotype. Polar G1E substitution impairs membrane penetration and permeabilization, and promotes β-strand formation. Bulkier hydrophobes do not support activity. G1V inserts perpendicular to membrane plane. (2) Gly4: G4E shows reduced activity, synthetic G4E FPs associate to membranes with lower affinity. (3) Ala5: A5V, but not A5G, blocks HA-induced fusion and FP-induced hemolysis, secondary FP structure does not appear to be affected. (4) Ala7: A7G, but not A7V, blocks HA-induced fusion and FP-induced hemolysis, secondary FP structure appears not to be affected. (5) Gly8: in this position Ala does not support HA activity, G8A peptide does not induce hemolysis. (6) Glu11 and Glu14: Val residues in these positions support fusion, enhance RBC lysis and promote more effective membrane penetration at neutral pH.

HIV-1 and HA FP self-association appear mediated by predominantly extended (β-like) conformations [5,6,35,38,56–61]. However, models of helical complexes for membrane-embedded FPs have also been proposed [49]. In particular, the HIV-1 FP has been suggested to alternate membrane-bound α-helical and extended aggregates depending on bilayer physicochemical parameters such as lateral pressure or degree of surface hydration [56,57,62].

2.4. FP-induced bilayer perturbations

FPs inserted into the target membrane may cause the distortion of bilayer organization that is necessary for rupture and merging [25,63]. Viral FPs show lytic effects after binding to either SUV or LUV (see below). In addition, vesicle suspensions treated with these sequences may undergo mixing of lipid components, an activity that may be a correlate of virus-induced membrane fusion. A summary of the lipid vesicle perturbations induced by HIV-1 FP is shown as a function of time in Fig. 2. The earliest perturbation results in leakage of vesicular contents. This is followed by vesicle aggregation and eventual inter-vesicle mixing of lipids. It is important however to discern the leakage of vesicle contents that accompanies the transitional destabilization required for

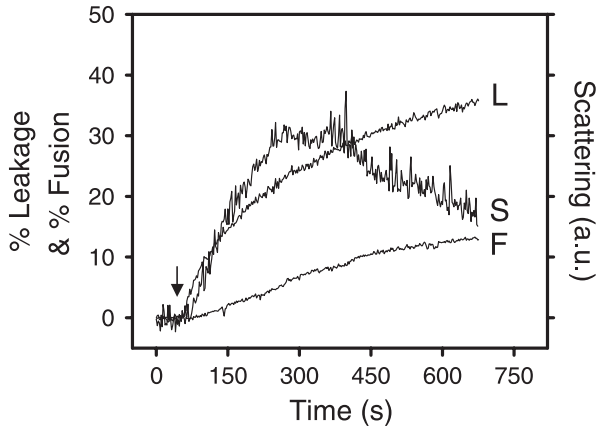

Fig. 2. Typical time-courses of HIV-1 FP-induced vesicle perturbations. At the time indicated by the arrow, the NH_2-AVGIGALFLGFLGAAGSTM-GARS-$CONH_2$ peptide was added to a suspension of PC/PE/Chol (1:1:1) LUV (100 μM) to a final peptide-to-lipid mole ratio of 1:25. L: leakage of aqueous contents (ANTS/DPX assay); S: vesicle aggregation (increase in scattered light with excitation and emission wavelengths set at 500 nm); F: fusion (NBD/Rho assay). For better time-resolution, the perturbing effects were slowed down by increasing the pH to 8.0 and by decreasing temperature to 16 °C.

fusion, as the one illustrated in Fig. 2, from the leakage process occurring through defined pore-like structures [5].

Thus, the HIV-1 FP has been shown to support a leakage process that is not necessarily related to vesicle aggregation or fusion [5,56,62]. The requirements of this process in terms of number of peptides bound per vesicle and structure adopted by the peptide appeared to be different as compared to those exhibited by the fusion phenomenon [56,64]. It was proposed that membrane perturbations leading to fusion were mediated by FPs penetrating shallowly into the external membrane monolayer and containing mainly extended structures [35,59], while pore formation probably required translocation of helices across the bilayer [56,62]. In the light of newly proposed viral fusion mechanisms [65], the functional relevance of both conflicting structures and processes remains to be established.

It is also unclear whether viral fusion involves the formation of defined lipidic intermediates as those mediating thermotropic lamellar-to-non-lamellar phase transitions [63,66–68]. According to this theory of fusion, the process would start with the formation of the "stalk", a transient lipidic intermediate locally connecting the cis monolayers of apposed flat membranes [51,52,66,67]. Stalk formation requires local destabilization of the contacting monolayers and exposure of hydrophobic groups. Stalk enlargement appears facilitated in systems with intrinsic negative curvature. The latter process is required for the formation of local points of hemifusion. While hemifusion represents the energetic barrier of fusion, rupture of the single bilayer diaphragm in these intermediates would constitute the kinetic barrier. The process would terminate with the formation of low-energy fusion pores connecting the initially separated aqueous environments.

In principle, FPs might promote fusion by reducing the stalk and local hemifusion formation energies, and/or by accelerating the decay to fusion pore formation [66,67]. It has been proposed that FPs may modulate the target membrane polymorphism, and therefore promote fusion, by inducing lateral stress in the hydrocarbon region of the external monolayer and/or by selectively inducing an increment of its surface [59,66]. Viral FPs do indeed induce non-lamellar phases at lower temperatures than observed in the pure lipid system [63,66,69]. In addition, the inclusion of FPs is known to drive lamellar-to-non-lamellar (H_{II}) transitions towards the formation of bicontinuous inverted cubic phases (Q_{II}) [70–72]. Siegel and Epand [73] have demonstrated that FPs, in the concentration range of 2–7 mol% (i.e., under conditions that might promote their self-association), do not stabilize non-lamellar structures by reducing the spontaneous radius of curvature of the host lipid. Based on the fact that FPs may lower the membrane rupture tension [74,75], Siegel and Epand [73] propose that a more plausible mechanism of fusion–promotion under these conditions could be the destabilization of bilayer diaphragms at hemifusion intermediates.

3. Physiological relevance of FP studies in model systems

Significant correlations have been established between the effects of FP mutations on the activity of whole fusion proteins, and the effects displayed by the same sequence alterations on the capacity of synthetic analogs to interact with model membranes and perturb them. The significance of the effects displayed in vitro by other physiologically relevant factors affecting fusion activity such as optimal pH, are still a subject of controversy. However, simple systems meant for intracellular delivery, based on the use of FPs have been shown to reproduce several features of the viral entry events upon interaction with cells. These findings support the notion of synthetic peptides being useful models to study viral cell fusion.

3.1. Influenza HA peptides

Structural information at the atomic molecular level available for influenza HA indicates the existence of at least three FP structures: one in uncleaved HA0, one adopted as the N-terminal end of proteolytically processed HA2, and one adopted upon contact with lipids. In combination, these structural requirements impose distinct sequence constraints that are only now starting to be unraveled [13,76]. In what follows, only residue substitutions identified as affecting peptide–membrane interaction and subsequent fusion will be discussed as a possible source of information relevant in the understanding of FP structure–function relationships (Fig. 1).

Gething et al. [22] found that HA2 G1E substitution abolished cell–cell and RBC–cell fusion activity of ex-

pressed HA, while G4E substitution decreased fusion efficiency and elevated the pH threshold for activation of the process. E11G did not affect RBC–cell fusion but impaired syncytium formation. None of these mutations significantly altered the ability of HA to bind SUVs in a pH-dependent fashion, indicating that anchoring the target bilayer might be necessary but not sufficient for fusion to occur. Wharton et al. [77] subsequently demonstrated in the SUV system that variant peptides containing these fusion-impairing mutations had fusion properties similar to those of the corresponding entire HA molecules. Importantly, non-fusogenic peptides still interacted with the membrane but did not cause leakage of liposomal contents. Synthetic 7-aa-long GLFGAIC analogs were subsequently analyzed in their capacity to insert into planar bilayers and LUV [78]. Also in good correlation with functional assays, when compared to the wt-representing peptide, a G1E mutant-representing sequence did not induce conductance increase or release of vesicular contents to the medium. The impairment was not so severe when the substitution was made at position 4.

The effects of G1E and G4E substitutions were also extensively studied by Rafalski et al. [79] using 20-mer GLFGAIAGFIENGWEGMIDG peptides derived from influenza X31 strain, and comparing their ability to interact with POPC monolayers, SUV and LUV. Only fusion-competent wt- and G4E-like peptides inserted at low pH into POPC membrane monolayers at lateral pressures higher than those existing in biological membranes and unstrained large vesicles. Accordingly, only these sequences effectively partitioned into and permeabilized LUV. In sharp contrast, the peptide representing the G1E mutant was unable to penetrate at low pH into monolayers at surface pressures existing in large vesicles and natural membranes, but inserted above pressures existing in the highly curved external monolayer of strained SUV [80]. As predicted, the G1E peptide was experimentally shown to associate efficiently with SUV but not with LUV. In this case, the use of the unstrained LUV was crucial to discriminate between peptides from functional versus non-functional mutant proteins.

Rafalski et al. [79] found no inter-vesicle fusion promoted by the HA peptides under conditions that allowed POPC LUV leakage (i.e., at low-pH and with wt-like sequence). The authors noted that for efficient peptide-induced fusion of LUV, other requirements must be met in addition to bilayer destabilization, specifically the formation of curved lipidic geometries that mediate membrane merger. The dynamics of these curved intermediate lipid structures may be affected by the presence of FPs as discussed above [73]. In fact, the use of lipid mixtures that can undergo thermotropic non-lamellar phase formation made possible the observation of HA FP-induced LUV fusion that was pH-dependent and impaired by the blocking mutations (Nieva and Wilschut, unpublished observations). Also in agreement with these observations, Epand and Epand [81] and Epand et al. [69] showed that wt-like FP impaired lamellar-to-non-lamellar lipid phase transitions at neutral pH, while non-lamellar phase formation was promoted by the same peptide at acidic pH. Most importantly, G1E and G4E peptides were unable to promote such non-lamellar arrangements of the lipids even at the low pH.

Mutagenesis studies were afterwards extended to address FP requirements in terms of charge and length, and to understand the significance of invariable Gly residues present in the sequence [26]. Glu residues at positions 11 and 15 could be substituted by Val without affecting specific HA fusion activity. Ala could substitute for Gly at positions 1 and 4, but not at position 8, without blocking HA expression-induced cell–cell fusion. Bulkier hydrophobes (Ile, Leu, Phe) or polar residues (His, Ser) at position 1 abrogated the fusion activity. Importantly, these results correlated with the hemolytic activity displayed by synthetic analogs of the mutant FPs in that only fusion-supporting sequences induced hemolysis at low pH. It was concluded that both a defined length and a specific N-terminal residue may be necessary for a functional association of the FP with lipid that will influence the efficiency of the fusion process.

The effect of N-terminal Gly substitution or deletion on FP secondary structure and orientation was subsequently studied by means of infrared spectroscopy [47]. Consistent with shallower insertion into unstressed bilayers G1E and ΔG1 variants displayed a larger β-strand content than the wt-like variant. Conformations were not significantly affected by lowering the pH. Interestingly, both α-helical and β-strand segments oriented at oblique angles to the membrane normal in mutant- and wt-like sequences. Also in accordance with different abilities to penetrate into unstressed membranes, measurements in micropipette-aspired giant unilamellar vesicles, showed that deletion of the G1 residue resulted in impairment of low-pH dependent bilayer area expansion and permeation induced by the wt-like sequence [74]. This is in contrast with the Trp fluorescence emission maximum shifts observed for G1E, ΔG1 and wt-like sequences in the presence of SUV, that seem to indicate a similar degree of penetration into these highly curved vesicles for the three variants [47]. A later study by Longo et al. [75] showed that structural changes in synthetic native FP sequences also altered insertion and membrane disruption. Thus, N-terminal modifications lead to diminished insertion and less propensity to pore formation, while carboxyamidation enhanced both abilities. Results by Longo et al. [74,75] are crucial to demonstrate that pore formation is an intrinsic property of HA FP and not a mere consequence of monolayer area expansion due to transfer of mass to membranes. These experiments also put forward an important feature of the FP: its capacity to lower membrane rupture tension.

In a combined mutational and structural study conserved Ala residues at positions 5 and 7 were doubly replaced with Gly (smaller) and Val (bulkier) hydrophobes [27]. Only A5G and A7V substitutions supported cell–cell fusion in cells transfected with mutated HA genes. Similarly, only synthetic peptides representing these mutant sequences induced hemolysis at levels comparable to that displayed

by wt-like sequence. The blocking mutations did not appreciably alter the secondary structure and orientation of membrane-bound sequences.

Analysis by Steinhauer et al. [26] also demonstrated that acidic residues within the FP's first 15 residues were not required for function. A recent report has addressed the question of the greater accessibility to the bilayer at neutral pH of a FP variant containing the Glu residues at positions 11 and 14 replaced by Val [82]. A shift of ca. +0.2 pH units was observed in the ability of the more hydrophobic variant to induce leakage in erythrocyte membranes as compared to the parental wt-representing sequence. Other evidences were provided by these authors indicating that the mutant-like sequence was likely to penetrate deeper into lipid membranes.

A mutational and functional study by Qiao et al. [24] revealed that blocking phenotypes at position 1 in influenza FP were related to the side-chain volume of the particular residues. Only nonpolar small Gly and Ala residues supported lipid and aqueous content mixing, while small polar Ser displayed an only-lipid-mixing or hemifusion phenotype. Bulkier hydrophobic (Val), polar (Gln) and charged residues (Glu, Lys) completely impaired fusion activity. A recent report has clarified several effects of these substitutions on the ability of synthetic FPs to interact with and perturb membranes [69]. A good correlation was found between the ability of peptides to decrease lamellar-to-hexagonal (II) lipid phase transition temperature and their ability to support fusion as part of the intact HA. These measurements correlated better with function than formation of isotropic non-lamellar phases or inter-vesicle mixing of lipids induced by the peptides. The particular case of G1V substitution produced a peptide that inserted perpendicular to the membrane plane as evidenced by polarized infrared spectroscopy.

In conjunction with relevant residue substitutions a potential link with physiology has been established based on the fact that, in a way reminiscent to low-pH-induced fusion activation, synthetic HA FPs interact with membranes more efficiently at acid than at neutral pH. However, conflicting results have been reported, indicating that factors such as peptide length, peptide modifications, selected representative viral strain and model membrane composition may affect the pH dependence of membrane interactions. For instance, while a synthetic GLFGAIAGFIEGGWTGMIDG peptide representing the HA FP from A/PR/8/34 strain was shown by Murata et al. [83] to associate with and induce pH-dependent inter-vesicle lipid-mixing in PC SUV, this dependence on pH was not reproduced by Lear and DeGrado [84] using a closely related but different GFFGAIAGFLEGGWEGMIAG sequence derived from the B/Lee/40 strain. These authors found significant binding and fusion induced by the peptide also at neutral pH. However, interactions with vesicles appeared strengthened at acidic pHs since both, helicity increase and a blue shift of the Trp fluorescence emission maximum, occurred after lowering the pH. Wharton et al. [77] found the synthetic FP-membrane interaction to be pH-dependent only when Chol-containing SUV were used. Synthetic peptides representing the wild-type sequence were found to induce PC SUV fusion and leakage both at neutral and acidic pH. However, this effect was clearly pH-dependent when larger PC/Chol vesicles were used instead. Synthetic 7-aa-long sequences did not respond to acidification of the medium in their capacity to insert into planar bilayers and LUV [78].

Rafalski et al. [79] found that efficient insertion into LUV of synthetic 20-residue peptides representing X31 HA FPs required acid pH to evolve. These authors underscored several factors that might condition the different pH-dependence observed for in vitro detected interactions. For instance, the number of ionizable groups among the four C-terminal residues may affect pH-dependent insertion. The low-pH-dependent peptides derived from A/PR/8/34 and X31 strains contain several ionizable groups within that stretch. In contrast, the electrically neutral carboxy end probably stabilizes more readily perturbing helical conformations in peptides derived from the B/Lee strain, even at neutral pH. This is also consistent with the fact that a shorter 17-aa-long peptide permeabilized PC LUV at neutral and acid pH with almost equal potency [85].

The NMR-solved structures provide structural grounds for a low-pH-induced transition of HA FP bound to lipids, which might be related to the promotion of bilayer destabilization [33]. At both neutral and acid pHs, the structure displays an oblique N-terminal amphipathic helix spanning residues 2–10 and a turn stabilized by H-bonds that redirects the C-terminal portion of the chain (Fig. 1). The C-terminal region structure and orientation relative to the N-terminal helix differ at pH 5 from those at neutral pH. A new network of H-bonds together with an increased hydrophobicity of the membrane-core facing surface result in the two arms of the kinked structure closing further and inserting more deeply into the membrane. This effect is postulated to increase the lateral pressure within the hydrocarbon core and interfacial regions of the membrane monolayer.

An important argument against the relevance of low pH dependence of FP membrane association stems from the fact that the optimum pH of fusion induced by HA always correlates with the optimum pH of its conformational change [76]. No HA has been described so far whose optimal pH of fusion is limited by the optimal pH found for synthetic FP–membrane interaction. Nevertheless, it is possible that certain FP sequences have evolved to become more hydrophobic and fusion-competent, specifically at the pHs they get exposed to the medium.

Finally, it should be mentioned that HA2 FP has been used in gene transfer [86]. Complexes containing plasmid DNA, transferrin–polylysine conjugates and polylysine-conjugated HA2 fusion peptides have been used to transfect different cell lines with expression gene markers. While transferrin is used as a ligand to direct uptake through receptor-mediated endocytosis, the FP is expected to disrupt the endosomal membrane and promote DNA release from endosomes. The

presence of the peptide actually conferred to complexes competence to interact with and permeabilize liposomes in a pH- and sequence-dependent fashion. Its inclusion also resulted in a considerable increase in the efficiency of transferrin–polylysine complexes to mediate gene delivery. Moreover, a close correlation was found between the content of peptide within a complex, percentage of induced liposomal leakage and increase in gene expression. Thus, cell membrane interactions of isolated FP sequences reproduce, at least in part, several aspects of viral entry events.

3.2. HIV-1 gp41 peptides

gp41 FP mutations have been described that specifically affect post-activation membrane merger steps. A mutational analysis by Freed et al. [87] showed that polar substitutions completely abolished syncytium formation induced by the surface expression of HIV-1 env products in transfected HeLa T4 cells. Subsequently, it was shown that one of the described polar substitutions comprising the replacement of hydrophobic Val at position 2 by negatively charged Glu residue, resulted in an inactive gp41 envelope subunit that interfered with both syncytium formation and infection mediated by the wild-type glycoprotein [88].

The ability of the HIV-1 FP to penetrate into membranes was first studied by Rafalski et al. [89] using 23-mer synthetic AVGIGALFLGFLGAAGSTMGARS peptides and PG membranes. These authors showed that these peptides were capable of penetrating lipid monolayers and inducing permeabilization of LUV. In contrast to the wt-like sequence, a peptide bearing the V2E polar substitution was later demonstrated to be unable to destabilize PG vesicles [90]. One interesting finding in that study was that the active peptide, in contrast to the inactive one, was able to adopt a β structure in the membrane, in the presence of cations. Fusion of liposomes with different lipid compositions was also completely abolished by the V2E polar substitution [35,55,91]. However, the wt- and mutant-like sequences seemed to associate to the same extent to vesicles, as inferred from binding assays. Mobley et al. [30] reported that V2E substitution also reduced the ability of the wt-like sequence to induce hemolysis and fusion of erythrocyte ghosts. Interestingly, a different polar substitution (L9R) described by Freed et al. [87] as producing a blocking phenotype, had a more pronounced effect.

Inclusion of a negatively charged residue in position 2 may anchor the N-terminus at the membrane interface and interfere with further penetration into the hydrophobic milieu, thus inactivating the sequence. Indeed, whereas surface-bound 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) emission anisotropy was unaffected by mutant and functional peptides, emission anisotropy of membrane-buried 1,6-diphenyl-1,3,5-hexatriene (DPH) was increased only by the fusogenic sequence, both in neutral [35] and negatively charged vesicles (Pereira and Nieva, unpublished observations). The results in Fig. 3 and Table 1 further illustrate this point.

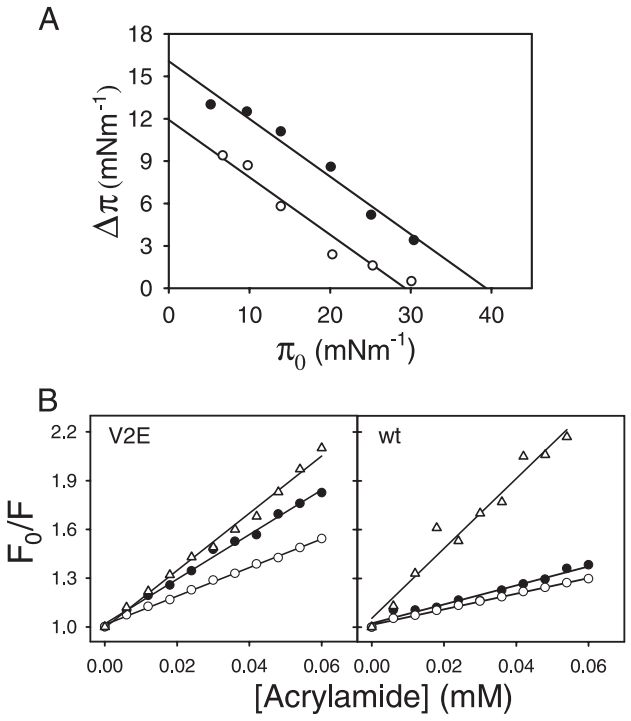

Fig. 3. (A) Penetration of HIV-1 FP sequences into PG monolayers. Maximum increase in surface pressure was measured as a function of the initial pressure of the phospholipid monolayer, upon injection in the subphase of V2E mutant-like NH_2-AEGIGALFLGFLGAAGSTMGAAS-$CONH_2$ sequence (○) or a corresponding wt-like NH_2-AVGIGALFLGFL-GAAGSTMGARS-$CONH_2$ functional peptide (●). At the concentrations used (0.27 μM), peptide alone induced in both cases surface pressure increases below the minimum π_0 tested. (B) Stern–Volmer plot of Trp fluorescence quenching by increasing concentrations of acrylamide in buffer (△) and in the presence of PG LUV (●) or SUV (○). Lipid concentration was 200 μM and peptide-to-lipid molar ratio, 1:200. In these experiments, F8W analogs of previous V2E and wt sequences were used.

The different penetration behavior of wt- and mutant-like peptides depending on the initial lateral pressure of a lipid monolayer (π_0) is shown Fig. 3A. The monolayer exclusion pressures, π_{ex}, or the maximum initial lateral pressures at which membrane-association was accompanied by peptide integration into the monolayer [92] were 29.1 and 39.2 mN m^{-1} for mutant- and wild-type peptides, respectively. Thus, the inactive V2E variant was unable to penetrate monolayers at lateral pressures postulated to arise from the lipid packing density of biological membranes and LUV ($\pi_0 \geq 30$–35 mN m^{-1}, Ref. [80]) but penetrated at lateral pressures existing in the external monolayer of SUV ($\pi_0 \sim 20$–25 mN m^{-1}, Ref. [80]). Accordingly, results in Table 1 indicate a shift in Trp emission of F8W analogs towards shorter wavelengths in the presence of SUV and LUV for the wt-like sequence. A comparable shift for V2E sequence is only observed in presence of SUV. The degree of solvent exposure of the Trp residues in these samples can be inferred from the results obtained using the aqueous quencher acrylamide (panel 3B). The Stern–Volmer quenching constants (K_{SV}) obtained from the plots (Table

Table 1
Maximum emission wavelengths (nm) and Stern–Volmer quenching constants (M^{-1}) for F8W variants of HIV-1 fusion peptides

Peptide	No liposomes		POPG LUV[a]		POPG SUV[a]	
	λ_{max}	K_{SV}	λ_{max}	K_{SV}	λ_{max}	K_{SV}
wt[b]	350	21.5	326	5.8	325	4.8
V2E	350	17.6	347	13.7	326	8.8

[a] The peptide-to-lipid mole ratios were 1:200.
[b] Sequences designated as in caption for Fig. 2.

1) indicate that accessibility of the aqueous quencher to the Trp residue was significantly reduced in both peptides when associated to vesicles. However, the degree of protection from the solvent induced by PG vesicles was lower for the V2E sequence in the presence of LUV. In essence, these data demonstrate that HIV-1 FP V2E and influenza FP G1E N-terminal polar substitutions might provoke a similar blocking effect on peptide penetration into membranes.

An additional aspect of V2E substitution-induced interference was noted by Kliger et al. [55] using 33-aa-long versions of the FP: even if both, wt- and mutant-like sequences, penetrated efficiently into SUV membranes, and self-assembled within, only the wt sequence induced efficient destabilization of these vesicles. This is an important observation because it suggests that the V2E variant is restricted in its interaction with membranes downstream to effective penetration. The different perturbing abilities of the peptides were proposed to result from the different abilities of the sequences to oligomerize. Only the wt-like sequence gave rise to the formation of high order complexes as evidenced by SDS-PAGE.

It is noteworthy here that G1E substitution was also shown to not impair intact HA [22] or synthetic FP [47,79] association with SUV, while the membrane perturbations induced by the wt-like sequence were abrogated. Moreover, Longo et al. [75] identified a pore-formation activity of the functional FP well below the membrane rupture tensions caused by simple transfer of mass to the bilayers. Thus, the ability to perturb the bilayer architecture after insertion seems to be an intrinsic feature of functional viral FPs which probably requires the adoption of structurally defined oligomeric complexes in membranes, most likely formed after insertion.

Evidence for specific HIV-1 FP structures required for fusion activity was provided by the mutagenesis study of Schaal et al. [93]. These authors produced gp41 mutant molecules with N-terminal deletions of increasing lengths. The efficiency of cell–cell fusion activity decreased until it was completely lost with deletion of five amino acids. In addition, the mutants with reduced fusion activity showed dominant interference in co-transfection assays, in a way similar to that displayed by the V2E substitution [88]. This indicates that the first amino acids are dispensable for the oligomerization process, but the preservation of N-terminal identity may be required for optimal fusion. Synthetic peptides representing active and inactive sequences described in this study were later assayed in their capacity to induce lipid mixing and leakage of LUV [45]. Abrogation of fusion in transfected cells correlated with impairment in the ability to perturb vesicles [45] and erythrocyte membranes [30].

To test whether conserved Gly residues scattered along the FP sequence play a role in HIV-1 fusion, Delahunty et al. [94] conducted a mutational analysis using transfected cell–cell fusion functional assays. Gly10 and Gly13 residues were found to be critical for activity. In addition Phe8 and Phe11 within the canonical FLGFLG sequence were found to be important for optimal fusion activity. A combined structure–function study later focused on the effects of replacing Phe11 with Val or Gly [58]. The F11V substitution reduced fusion activity of the whole protein assayed in a cell–cell fusion system but did not abolish it. In contrast, F11G substitution almost completely impaired gp41 fusion activity. Synthetic peptides bearing equivalent substitutions were, respectively, ca. 50% and 100% less fusogenic when assayed in a vesicular system. Fusion activity also correlated with the ability of these sequences to form high order complexes as evidenced by SDS-PAGE, but not with other physicochemical properties such as adopted secondary structure or self-assembly in lipidic environments.

Formation of specific membrane-embedded complexes might also explain the inhibitory effect displayed by HIV-1 FPs in cell systems. A hexapeptide which was identical in amino acid sequence to the gp41 N-terminus was found by Owens et al. [95] to completely inhibit syncytium formation mediated by HIV-1 envelope glycoproteins expressed at the surface of HeLa $CD4^+$ cells. The inhibitory effect was sequence-specific and dose-dependent. These authors suggested that the inhibitors might interact with its homologous FP sequence at gp41 thereby preventing its normal fusogenic function. Longer HIV-1 FPs inhibited HIV-induced syncytium formation and antigen production in infected cells [96]. Solubilization by conjugating the peptide with charged polymers at its C-terminus resulted in augmentation of the inhibitory capacity, while blocking of N-terminus by conjugation with charged polymers hampered the inhibitory capacity of the peptide. Kliger et al. [55] demonstrated inhibition of cell–cell fusion induced by expressed gp120/41 using 33-mer sequences representing gp41 N-terminus. A functional feature of these sequences was shown to be their capacity to assemble high order complexes.

HIV-1 FP has been used as well as a component of complexes designed for intracellular delivery. A chimerical peptide derived from the hydrophobic fusion peptide of HIV-1 gp41 and the hydrophilic nuclear localization sequence of SV40 large T antigen, could also be used for the delivery of oligonucleotides and plasmids into cultured cells [97]. This construct interacts with nucleic acids and protects them from degradation in culture media [98]. The role of the gp41 FP within the conjugate has been ascribed to the promotion of translocation directly through the cell membrane, i.e., independently of the endocytic pathway. In fact,

these conjugates have been shown to localize in the nucleus after internalization. Thus, complexes containing FPs seem to penetrate into cells in a way reminiscent to that of whole virions.

3.3. Sendai F peptides

Many substitutions in the conserved amino acids of SV5 fusion domain are tolerated without loss of syncytium formation activity [14]. In particular, Gly × Ala substitutions exhibited increase in fusion activity in transfected cells. Since other mutations caused increase in hydrophobicity but did not exhibit enhanced fusogenic activity, it was claimed that Gly residues might lessen the potential of the FP to form a fusion-competent α-helical structure.

A peptide representing the Sendai fusion domain as well as one variant containing a G12A substitution were later investigated in their ability to bind to membranes and to induce inter-vesicle fusion [54]. The G12A substitution dramatically increased the fusogenic activity of the peptide, as well as its ability to aggregate membranes. Thus, in vitro lipid mixing induced by these peptides reproduced the syncytium formation results by mutant F proteins. Interestingly, the observed different membrane-aggregating and fusogenic potencies could not be explained in terms of different membrane affinities, adopted conformations or self-aggregation at membrane surfaces. The main differences observed between both sequences were the degree of penetration, shallower for the wt-representing sequence.

3.4. Internal SFV FP

In comparison to N-terminal FPs, little is known on the insertion mechanism of FPs that are internal sequences of the polypeptide chain [2]. A paradigmatic case of internal FP is the fusion domain of the Semliki forest virus (SFV) spike protein [99]. SFV FP has been mapped to residues 75DYQCKVYTGVYPFMWGGAYCFCD97 of E1 subunit and occupies a loop at the tip of its distal domain [100]. Similarly to influenza, SFV penetrates into cells through receptor-mediated endocytosis. Exposure to the low pH present in the lumen of late endosomes activates E1-mediated fusion of virions with the membrane of these organelles. The role of the FP in this process was evaluated through mutagenesis of a spike protein cDNA [99,101,102]. Several mutations were studied in transfected cell–cell fusion assays. In particular, a polar G91D substitution was found to completely block cell–cell fusion activity without affecting spike protein assembly or transport.

In Fig. 4, we analyze the membrane-interacting abilities of 23-aa-long synthetic peptides representing SFV FP as compared to those of a shorter 21-mer derivative lacking the two C- and N-terminal Asp residues. The fluorescence spectra have been obtained utilizing wt- and G91D-like sequences at neutral (7.4) and acid (5.5) pHs. Intrinsic fluorescence of the 21-mer wt-like sequence was found to

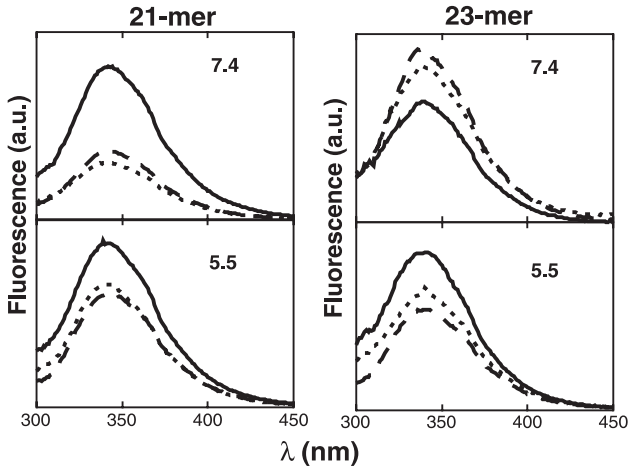

Fig. 4. Fluorescence emission spectra (λ_{ex} = 295 nm) of SFV FPs at different pHs (indicated in panels), in buffer (dotted lines), and incubated with PC/PE/Chol (1:1:1) LUV (continuous lines). 21-mer: NH_2-YQCKVYTGVYPFMWGGAYCFC-$CONH_2$ 23-mer: NH_2-DYQCKVYTGVYPFMWGGAYCFCD-$CONH_2$. Dashed lines correspond to spectra obtained using G91D representing NH_2-YQCKVYTGVYPFMWDGAYCFC-$CONH_2$ (21-mer) and NH_2-DYQCKVYTGVYPFMWDGAYCFCD-$CONH_2$ (23-mer) sequences. For comparison, spectra in buffer for wt- and G91D-representing sequences were normalized to the same emission values. Peptide concentration was 1 μM and, in vesicle samples, the peptide-to-lipid ratio was 1:100.

increase at both pHs in the presence of vesicles, suggesting that the single Trp residue penetrates into the bilayer hydrophobic milieu in both cases. In contrast, the 23-mer reduced its emission intensity at neutral pH upon contact with vesicles, while the opposite effect was observed at low pH. This would be consistent with superficial association and further penetration at neutral and acid pHs, respectively. By comparison, fluorescence of G91D-representing 21- and 23-mer sequences did not appreciably change in presence of vesicles at any pH. Our data suggest that deep insertion into a low polarity environment could only be achieved by wt-representing sequences. In addition, the presence of flanking Asp residues may condition penetration at low pH. These observations imply that the interactions of internal SFV FP with model membranes might resemble in certain aspects those of N-terminal HA FP [47,79].

4. Assisted fusion

Some of the most crucial factors compromising membrane stability reside upon the bilayer lipid–water interface and polar headgroup region. At that level, the forces governing the processes of membrane–membrane interaction and deformability exert much of their action [103]. Given their phospholipid composition, rich in highly hydrated lamellar-type species, bilayers to be fused during viral infection are considered to be refractory to merger. Thus, it is conceivable that FP activity must be assisted in order to modulate the aggregational state of merging bilayers.

HIV-1 FP sequence interacting with negatively charged PG LUV was shown to become fusogenic in the presence of cations [56]. Addition of Ca^{2+} or Mg^{2+} to this system promoted peptide-induced fusion of PG liposomes by supporting aggregation. The occurrence of fusion was supported by the irreversible increase in size of the vesicles observed when the FP was added to a vesicle mixture containing 5 mM Ca^{2+}. The size distribution of the vesicle population (ca. 100 nm) indicated that the mean diameter did not change significantly when the vesicles were treated with 5 mM Ca^{2+} for 10 min and then 10 mM EDTA was added. However, addition of HIV-1 FP after the addition of Ca^{2+}, resulted in an increase of the mean vesicle diameter to ca. 700 nm, as determined after the addition of EDTA. The lipid-mixing kinetics analyzed using a mass-action kinetic model indicated that, at a fixed Ca^{2+} and lipid concentration, increasing the amount of peptide increased the rate constant of fusion. These observations confirmed that the peptide per se constitutes the fusogenic agent in the system.

Following a different strategy, Haque et al. [104] assisted HA FP-induced fusion by aggregating PC vesicles with poly(ethylene glycol). Interestingly, the presence of the FP did promote lipid-mixing and leakage of DOPC LUV, but not the mixing of the vesicular aqueous contents. This emphasizes that in the absence of the HA TMD, the action of the FP might be restricted to the mixing of lipids of interacting bilayers [105].

A relatively new hypothesis postulates that initial molecular events of viral fusion might involve the concerted action of several fusion protein segments interacting with membranes (reviewed in Ref. [53], see also specific chapter in this issue). Thus, the existence in soluble ectodomains of additional regions implied in membrane interactions suggests that more than one protein segment may have a role in the fusion process [106,107]. This also implies that FPs might be assisted in their fusogenic activity by these regions.

5. Concluding remarks

Insertion into target membranes is the common theme among the proposed functions of viral FPs. N-terminal G1E and V2E polar substitutions in influenza and HIV-1 FPs, respectively, impair penetration at the lateral pressures existing in biological membranes (Ref. [79], Fig. 3). The packing state of lipids at the local point where the fusion event develops is not known with certainty, and insertion of these mutant sequences into strained bilayers cannot be excluded. Nevertheless, insertion into membranes is not enough to produce the perturbations that functional FPs induce in model systems. The aforementioned substitutions do not preclude insertion of whole proteins and isolated synthetic FPs into SUV, but hamper the ability of the functional sequences to induce leakage and fusion [22,47,55,77,79]. This suggests that in addition to their function as conditional "secondary" TMDs, fusion-competent FPs must fulfill other functions that are primarily reflected in their capacity to destabilize the bilayer organization.

The membrane perturbations generated by FPs seem to originate from their capacity to form pores and lower bilayer rupture tension [74,75]. The former effect may result in the permeabilization of dispersed vesicles while the latter may lead to fusion of aggregated membranes through the promotion of non-lamellar intermediates with defined geometry. It is possible that both effects arise as a consequence of a common topology and conformation adopted by FPs in the membrane: an amphipathic helix obliquely inserted into one membrane monolayer that is followed by a kink region [33,41]. An oblique angle has been observed for membrane-inserted sequences representing functional helical regions of influenza and HIV-1 FPs (see specific chapter in the same issue). Although necessary, an oblique angle does not seem to be sufficient to attain the functional structure in membranes since several non-functional HA FPs do adopt oblique angles but are unable to induce membrane perturbations [27,47]. On the other hand, the kinked region has not been systematically studied so far and the functional significance of this structure is yet unknown. In this regard, a particularly intriguing observation is that E11V and E15V substitutions in HA FP (Fig. 1) do not significantly affect fusion activity [26,82].

The induction of fusion requires previous close apposition of membranes. It seems that when a particular lipid composition does not bear an intrinsic tendency to aggregate [103], FPs must be assisted in order to induce fusion. Model membrane aggregation has been facilitated using cations [56,90] and more recently poly(ethylene glycol) [104]. It is possible that other regions of the glycoproteins as the helical heptad repeats also assist fusion by acting as linkers that facilitate inter-vesicle contacts [105,108–110]. An alternative, and perhaps complementary, possibility is that these regions actively participate in vesicle perturbation together with the FPs. Thus, these stretches immersed into the surface monolayer might contribute to destabilization and merger of the contacting bilayers [53].

Finally, from the experimental studies using synthetic sequences and model membranes that we have described here, it remains unclear how FPs may contribute to fusion pore opening [22–24]. The approximate four-residue spacing found for Gly residues in FPs might be related to this function [76]. The fact that FPs may assemble oligomeric structures in membranes suggests a certain degree of molecular recognition in which Gly residues might play a direct role. An attractive hypothesis is that eventual opening of the fusion pore is concomitant to the formation of unidentified membrane-embedded protein structures. These structures would be oligomeric in nature and more likely include FPs and transmembrane regions of the fusion glycoproteins. Identification and characterization of those possible organizations might help in unraveling the mechanisms of fusion-inhibition by FP-representing sequences [55,95,96], as well

as the functional implications of many mutations that defy systematic rationalization [21,76].

In conclusion, synthetic FPs are good models to study membrane-associated structures and processes functioning in viral fusion. Assessment of these sequences in model systems has made possible the identification of bilayer perturbations that are specifically promoted by synthetic peptides representing functional FPs. In addition, systematic structure–function analyses based on new structural paradigms of membrane-bound FPs (Fig. 1) will provide a clearer picture on the roles played by these viral sequences during fusion. A landmark finding in this research area is that functional FP-promoted bilayer perturbations are probably required but not sufficient for the occurrence of viral fusion in the physiological context, i.e., that FP action is probably assisted in order to lead these initial processes towards formation of a functional fusion pore. The unraveling of these assistance mechanisms in terms of membrane lipid and fusion protein sequence contributions will definitely benefit from experimental characterization in model systems.

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