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Review

# Viral fusion proteins: multiple regions contribute to membrane fusion

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**Abstract**

In recent years, the simple picture of a viral fusion protein interacting with the cell and/or viral membranes by means of only two localized segments (i.e. the fusion peptide and the transmembrane domain) has given way to a more complex picture in which multiple regions from the viral proteins interact with membranes. Indeed, possible roles in membrane binding and/or destabilization have been postulated for the N-terminal heptad repeats, pre-transmembrane segments, and other internal regions of fusion proteins from distant viruses (such as orthomyxo-, retro-, paramyxo-, or flaviviruses). This review focuses on the experimental evidence and functional models postulated so far about the role of these regions in the process of virus-induced membrane fusion.


*Keywords:* Viral entry; Fusion peptide; Protein dissection

## 1. Introduction

Infection of eukaryotic cells by enveloped viruses requires the fusion of the viral and plasma or endosomal membranes. Highly specific viral envelope glycoproteins, named ‘‘fusion proteins’’, catalyze this reaction, thus overcoming its inherent energy barriers. The current accepted hypothesis postulates that, at one stage during the fusion process, viral fusion proteins interact simultaneously with both the viral and cell membranes, therefore bridging the gap between them [1]. The interaction between most known fusion proteins and the viral envelope is mediated by a transmembrane domain that connects the intraviral (or cytoplasmic) region with the ectodomain. On the other hand, the interaction with the target membrane has been thought to involve a hydrophobic stretch of about 15 residues called ‘‘the fusion peptide’’ [2]. These segments were initially identified either at the N-terminus, as in most orthomyxoviruses, paramyxoviruses, and several retroviruses [2–4], or in the interior of the fusion proteins, as in Rous Sarcoma virus [5], Vesicular Stomatitis virus [6], or Ebola virus [7]. It was generally accepted that each viral fusion protein contains a single fusion peptide and that this segment was the solely responsible for the destabilization of the target cell membrane. However, in recent years, new evidence has surfaced, indicating that, in addition to classical fusion peptides, other regions from viral fusion proteins directly interact with membranes, contributing to their merging. This review focuses on the role of these regions in membrane fusion.

## 2. The role of the N-terminal heptad repeat

Structural studies of fragments from some fusion proteins from ortho-, paramyxo-, as well as retroviruses have unraveled a common structural theme shared by the fusion machines of these unrelated viruses: a heptad repeat region that follows the N-terminal fusion peptide folds, at an unknown stage during the fusion process, into a trimeric helical coiled coil [8] (see Fig. 1). Indeed, formation of the coiled coil has been postulated to relocate Influenza hemagglutinin N-terminal fusion peptide close to the target cell endosomal membrane [9]. Furthermore, the subsequent packing of C-terminal helices against the grooves of the coiled coil would bring the viral and the target membranes close to one another [10]. Thus, for already a number of years, N-terminal heptad repeats have been known to play a crucial role in the structural organization of fusion proteins. However, recently, a growing number of studies have pointed to an additional role for the N-terminal heptad repeat of

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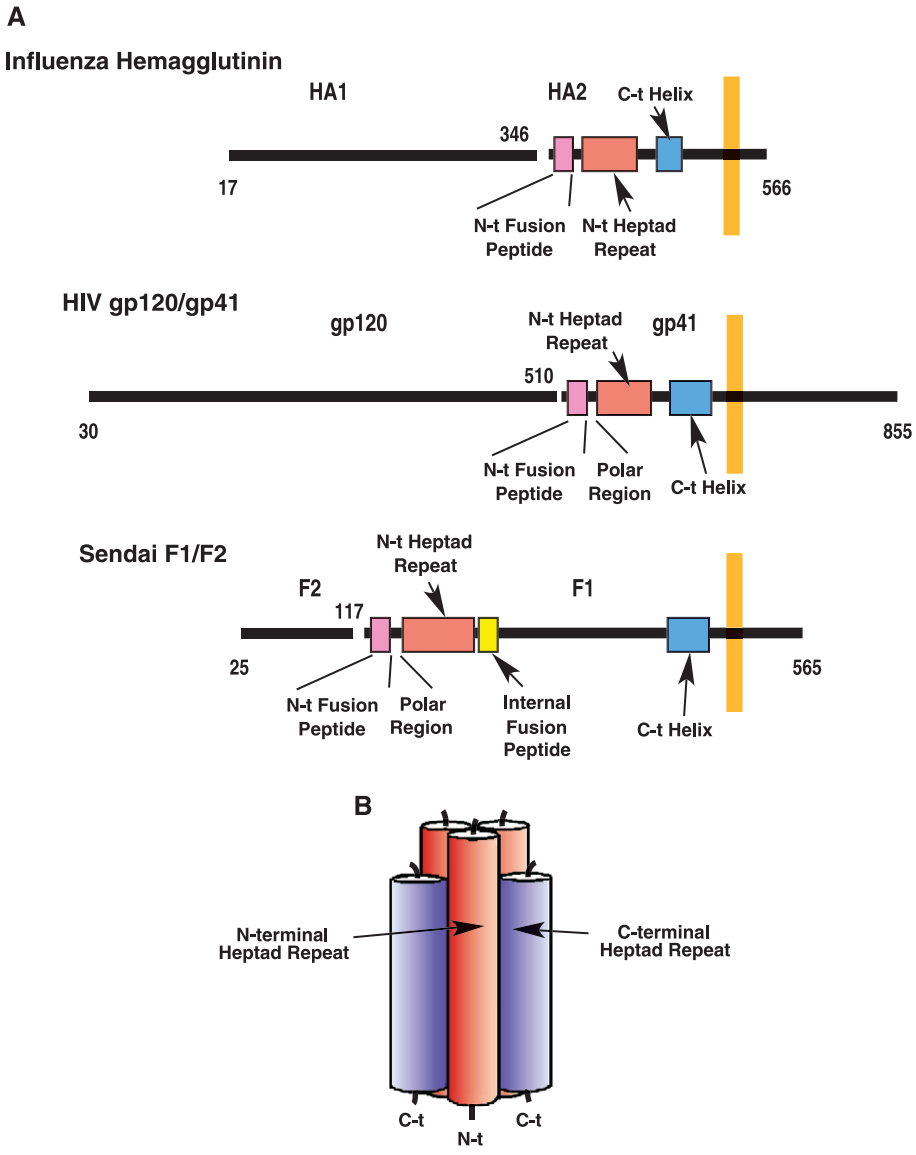


Fig. 1. Panel A, schematic organization of the fusion proteins from Influenza, HIV, and Sendai virus. The colored boxes represent the N-terminal fusion peptide, in pink; the N-terminal heptad repeat, in red; and the C-terminal helix, in blue. The orange vertical strip represents the viral envelope. In Sendai F1, the additional yellow box represents the putative internal fusion peptide. Note that the residue numbers in the three proteins correspond to the mature polypeptides, after processing of the signal sequences. Panel B, structural scheme based on the crystal structure of fragments from SV5 F1 and HIV-1 gp41. In both cases, three N-terminal heptad repeats (in red) form an internal coiled coil against which three C-terminal helices are packed (in blue). The crystal structure of a fragment of Influenza hemagglutinin in the low pH conformation shows a very similar 3D structure: an internal trimeric coiled coil formed by three N-terminal heptad repeats and three short helices that surround its C-terminal end.

Influenza hemagglutinin, Sendai F protein, and HIV gp41 in the fusion process. These studies are discussed in the following paragraphs.

### *2.1. Influenza hemagglutinin*

Epand et al. [11] studied the ability of different constructs corresponding to the ectodomain of Influenza virus hemagglutinin to induce membrane fusion of large unilamellar vesicles. Specifically, they compared the activity of the 20-residue N-terminal fusion peptide, a 127-residue segment (FHA2) comprising both the N-terminal fusion peptide, the following coiled coil, the loop that reverses the chain and a consecutive short helix, and a 95-residue truncated form of FHA2 lacking the N-terminal fusion peptide (named SHA2). They found that FHA2 promotes pH-dependent fusion of liposomes to an extent significantly higher than the N-terminal fusion peptide alone. Interestingly, this activity was abolished when the fusion peptide was removed, but also when residues D109, D112, or E114, all part of the loop region, were replaced by Cys. Furthermore, although SHA2 did not induce membrane fusion, it promoted the aggregation of lipid vesicles, proving that regions apart from the fusion peptide bind to lipid membranes. These results suggest that,

although the N-terminal fusion peptide is necessary for inducing rapid membrane fusion, the following coiled coil and loop have an important role in the fusion process [11]. Furthermore, the fact that the lipid mixing activity of a segment of HA2 showed a pH dependence similar to the observed for the intact virions suggests that changes in pH not only affect the interaction between the HA1 and HA2 subunits and the conformation of HA2. The authors suggested that protonation of specific groups in FHA2, presumably in the N-terminal fusion peptide and within an acidic cluster in the loop region, increased the fusogenic activity of FHA2. As a result of the protonation, the protein may change its conformation and/or penetrate deeper into the membrane. In a subsequent study, Leikina et al. [12] demonstrated that FHA2 is also capable of inducing cell–cell hemifusion (fusion of the outer leaflets of the membrane). That FHA2 does not promote full fusion is not surprising: GPI-anchored constructs of HA lacking the transmembrane and cytoplasmic regions also induce hemifusion only [13]. Interestingly, the FHA2 hemifusion activity required concentrations of FHA2 similar to those required for HA-mediated cell–cell fusion. Moreover, FHA2-induced hemifusion was reversibly blocked by lysophosphatidylcholine and low temperature, factors that have equivalent effects on HA fusion activity. Thus, the authors hypothesized that both HA-induced full fusion and FHA2-induced hemifusion might share similar mechanisms and that, considering that FHA2 is in the low-pH stable conformation, hemifusion requires only part of the energy released in the low pH-induced conformational change of HA [12].

### 2.2. Sendai F protein

Infection by paramyxoviruses, a family that includes severe human respiratory tract pathogens, results in the formation of large multinucleated cells [14]. In paramyxoviruses, both virus–cell as well as cell–cell fusion are mediated by the F protein. F is synthesized as an inactive precursor that, after protease activation, is formed by two disulfide-linked subunits, F1 and F2 [15] (see Fig. 1). Crystallographic studies of fragments corresponding to the SV5 F protein N-terminal and C-terminal heptad repeats have shown that, as with other fusion proteins, three N-terminal heptad repeats form an internal coiled coil against which three C-terminal helices are packed [16]. This conformation is thought to form late during the fusion process. In addition to their structural role, heptad repeats from the F protein of Sendai virus, another member of the paramyxoviridae family, were shown to have high affinity toward phospholipid membranes [17], suggesting a possible involvement in the actual fusion event. In order to further understand this possible role, Ghosh and Shai [18] compared the ability to induce fusion of large unilamellar vesicles of constructs of different lengths corresponding to segments from the F protein. Specifically, they compared the fusogenic activity of a 33-residue segment that includes the N-terminal fusion peptide, a 70-residue segment elongated to include also the N-terminal heptad repeat, and two mutants, one with a mutation in the region of the N-terminal fusion peptide (G119K) and the other within the heptad repeat (I154K). These mutations had been shown to drastically reduce the fusogenic ability of the homologous fusion protein of Newcastle disease virus. Ghosh and Shai [18] found that only the 70-residue wild-type peptide was able to induce membrane fusion of PC/PG large unilamellar vesicles. The G119K mutation could directly affect the proper interaction between the N-terminal fusion peptide and the membrane. On the other hand, the I154K mutation is far away from the fusion peptide and could only affect it in an indirect way, presumably by altering the required structure and/or oligomerization of the heptad repeat. Thus, they postulated that the heptad repeat assists the N-terminal fusion peptide during membrane merging.

### 2.3. HIV gp41

The N-terminal domain of HIV gp41 contains a ~15-residue hydrophobic fusion peptide, followed by a segment of about 15 mostly polar residues and a consecutive heptad repeat (see Fig. 1). Both NMR and crystallographic studies have shown that three N-terminal heptad repeats fold into a trimeric coiled coil surrounded by three C-terminal helices [8,19,20]. This structure, strikingly similar to the one described for paramyxovirus F protein, has been suggested to form at a late stage during the fusion process.

Protein dissection studies have focused on the role of gp41 N-terminal domain on membrane fusion. As found with several fusion peptides, a 16-residue peptide corresponding to the gp41 N-terminal fusion peptide induces lipid mixing of PC/PE/Cho (1:1:1) large unilamellar vesicles [21]. Furthermore, it has been shown that extension of this peptide to include the following 17 mostly polar residues significantly enhanced its fusogenic activity. As many other fusion peptides, the N-terminal 16-residue peptide decreased the bilayers to hexagonal phase transition temperature of dipalitoleoylphosphatidylethanolamine ($T_H$), suggesting its ability to promote negative curvature in membranes; surprisingly, the longer 33-residue peptide that includes both the N-terminal fusion peptide as well as the following polar region, raised the $T_H$. Analysis of the different segments by means of FTIR and fluorescence spectroscopy lead to the hypothesis that the first 16 residues of the full-length peptide are inserted into the membrane, promoting negative curvature, thus facilitating the formation of a stalk intermediate in membrane fusion [21]. The 17 consecutive residues lie near the surface of the membrane, contributing to the correct oligomerization of the peptide, further enhancing its fusogenic activity [21]. Initial evidence supporting a more direct role for the N-terminal heptad repeat in membrane fusion came from protein dissection studies that showed that peptides cor-

responding to gp41 N-terminal heptad repeat bind to phospholipid bilayers with high affinity [22,23]. Indeed, elongation of the mentioned 33-residue peptide (that comprised the fusion peptide and the consecutive polar region) to include the full N-terminal heptad repeat resulted in a 70-residue segment with dramatically increased fusogenic activity [24]. Interestingly, the I62D mutation in the C-terminal region of the heptad repeat, far away from the N-terminal fusion peptide and known to render the virus noninfectious [25], resulted in a significant reduction of the fusogenicity of the 70-residue peptide. This mutation is believed to block the ability of the heptad repeat to form a stable trimeric coiled coil, suggesting that the correct structure and/or oligomeric state of the full-length peptide is necessary for its proper activity.

## 3. The role of the "pre-transmembrane" region

The importance of viral fusion proteins transmembrane and pre-transmembrane segments on the mechanism of viral entry has been postulated long time ago. In a pioneering work, Salzwedel et al. [26] showed that replacement of the transmembrane segment of gp41 by a by a glycosyl-phosphatidylinositol anchor (GPI) resulted in a loss of syncytium forming capacity, although the protein was still attached to the membrane. Along this line, it was demonstrated that GPI-anchored Influenza hemagglutinin promotes cell–cell hemifusion [13]. This suggested a role for hemagglutinin transmembrane domain in the formation of a stable fusion pore and in its expansion. Furthermore, mutational studies with the full-length HIV-1 gp41 showed that the pre-

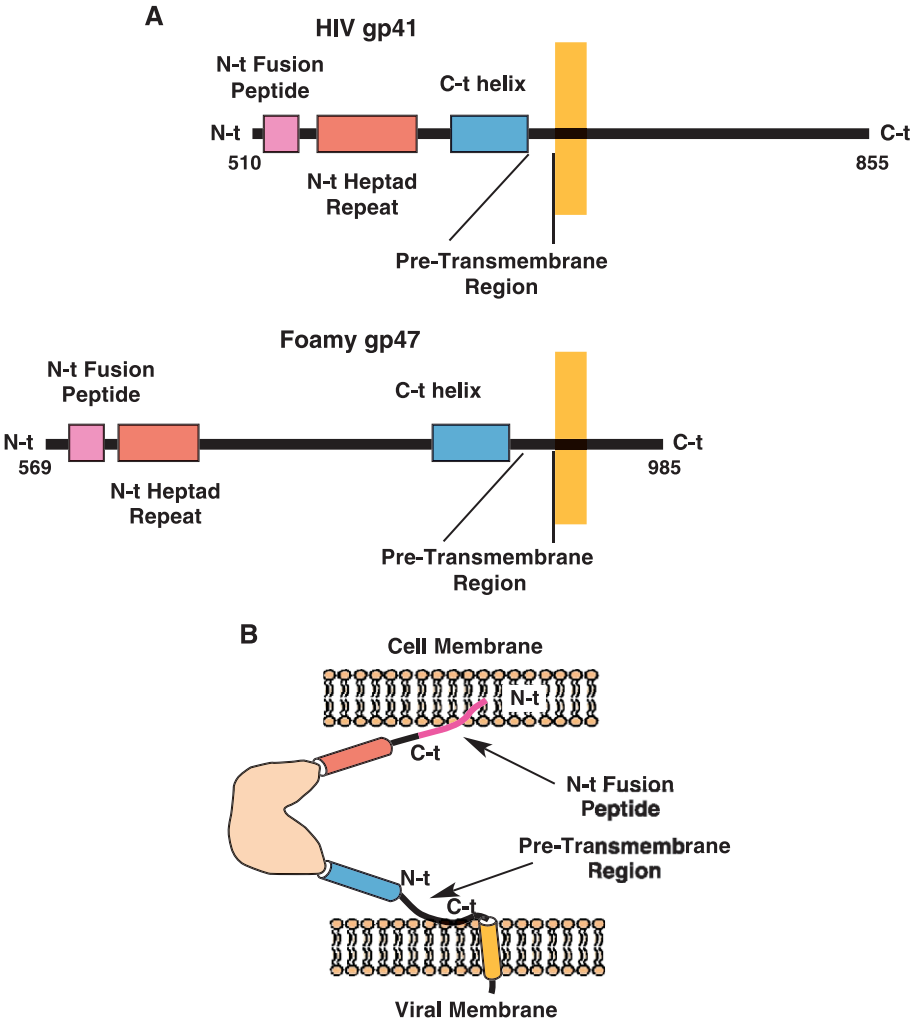


Fig. 2. Panel A, schematic representation of HIV gp41 and Foamy gp47, the fusion proteins from two different retroviruses. The colored boxes represent the N-terminal fusion peptide, in pink; the N-terminal heptad repeat, in red; and the C-terminal helix, in blue. The orange vertical strip represents the viral envelope. The pre-transmembrane regions are located between the C-terminal helices and the viral envelopes. Panel B, cartoon highlighting the opposite topology in the membrane interactions of N-terminal fusion peptides and C-terminal pre-transmembrane regions. The N-terminal fusion peptides have their N-termini closer to the bilayer, while their C-termini are further away from the membrane. On the contrary, the pre-transmembrane regions have their C-termini closer to the membrane (in fact, they may be already part of the transmembrane domain). In the scheme, the N-terminal heptad repeat is symbolized by a red cylinder, the C-terminal helix by a blue cylinder, and the transmembrane segment by a light brown cylinder.

transmembrane region of HIV-1 gp41 ectodomain is crucial for complete fusion [27,28]. These studies highlighted the importance of the C-terminal regions of fusion proteins ectodomains in the process of membrane merging. More recently, new reports pointed to a direct role in membrane destabilization.

### 3.1. HIV

Suarez et al. [29] first suggested a direct involvement of the pre-transmembrane region of a fusion protein in the actual membrane destabilization process. They observed that a Trp-rich segment located at the C-terminus of HIV-1 gp41 ectodomain, displayed a high tendency to partition into the membrane interface as revealed by the interfacial hydrophobicity scale developed by Wimley and White [30]. They showed that synthetic peptides corresponding to a 20-residue segment that immediately precedes the gp41 transmembrane domain, and includes the Trp-rich region (see Fig. 2A), induces destabilization of PC/PE/Cho (1:1:1) liposomes (i.e. permeabilization and lipid mixing). Furthermore, mutations within this region that render gp41 nonfunctional resulted in inactive peptides. The authors concluded that the membrane-perturbing activity of the gp41 pre-transmembrane region mirrors that of the polar segment that follows the N-terminal fusion peptide [29]. Indeed, the structural organization of the N-terminus and C-terminus of gp41 ectodomain share two elements: a hydrophobic segment that inserts into the membrane (the fusion peptide at the N-terminus and the transmembrane domain at the C-terminus), followed by (in the case of the N-terminus) or preceded by (in the case of the C-terminus) more polar helical regions that assist in the fusion process.

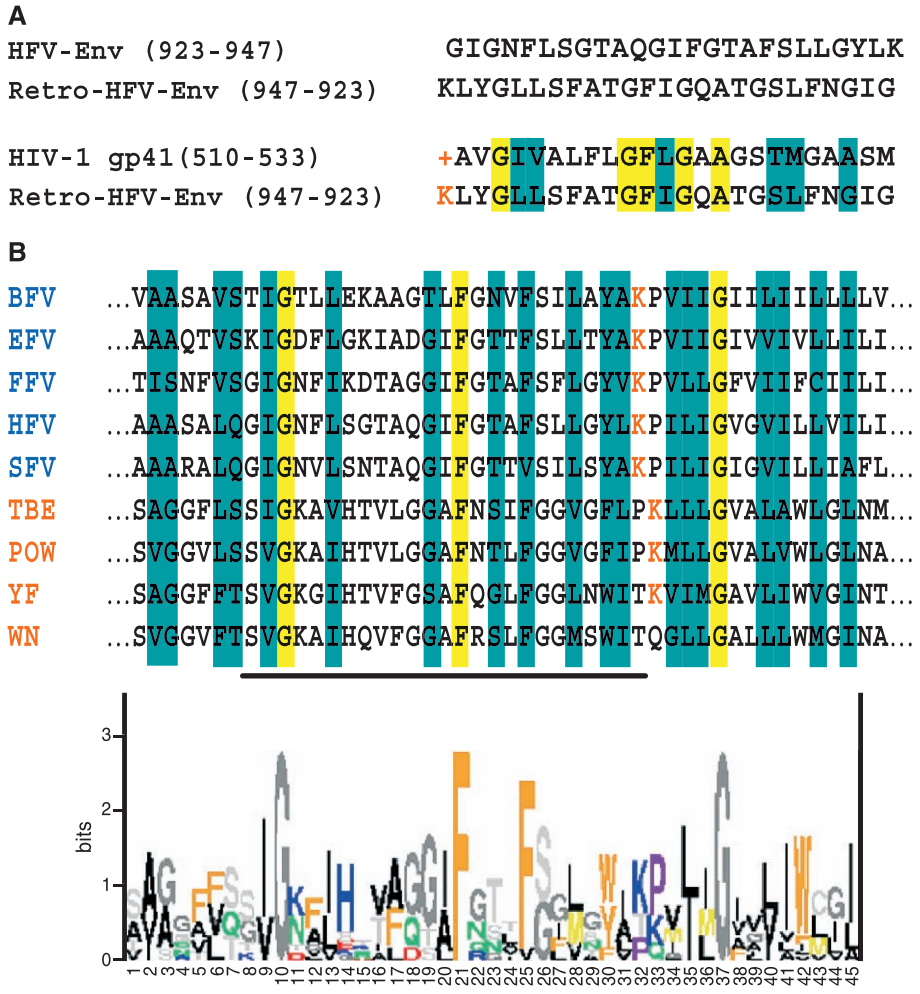


Fig. 3. Panel A, on top, the pre-transmembrane sequence of Human Foamy Virus Env (HFV-Env, as a representative of all Foamy viruses) and its retro-sequence (Retro-HFV-Env) are shown; at the bottom, the alignment between Retro-HFV-Env and the N-terminal fusion peptide of HIV-1 gp41 is shown. Identical positions are marked in yellow, similar positions in blue. A Lys in the Foamy protein and the N-terminal positive charged from the HIV protein are marked in red. Panel B, alignment between the pre-transmembrane regions of five Foamy virus fusion proteins (bovine, BFV; equine, EFV; feline, FFV; human, HFV; and simian, SFV) and four Flavivirus Envelope Protein E (Tick-borne Encephalitis virus, TBE; Powasan virus, POW; Yellow Fever virus, YF; and West Nile virus, WN). As before, identical positions are marked in yellow and similar positions in blue. The region in the Foamy proteins analogous to the HIV-1 gp41 N-terminal fusion peptide is underlined. At the bottom, the multiple alignment is shown in a Logo representation.

### 3.2. Foamy and flaviviruses

Gp47, the protein responsible for Foamy virus-induced membrane fusion, contains a putative N-terminal fusion peptide and a putative C-terminal transmembrane domain [31], the latter unusually long for retroviral fusion proteins (see Fig. 2A). This prompted Epand and Epand [32] to investigate a possible role in membrane destabilization for the most external portion of the putative transmembrane domain. They found that, as with other viral fusogenic segments, a synthetic peptide corresponding to the pre-transmembrane domain region induces fusion of model PC/PE/Cho (1:1:1) membranes [32]. The membrane-destabilizing activity of the pre-transmembrane segment from Foamy virus is similar to that reported for the equivalent region from HIV-1 gp41 and suggests that other retroviral fusion proteins might destabilize both the target membrane (with the N-terminal fusion peptide) and the viral membrane (with the pre-transmembrane region) during the fusion process.

Retroviral N-terminal fusion peptides have common features at the level of their amino acid sequences [4]. They have a high content of Ala, Gly, and Phe and, sometimes, tandem repeats of the tripeptide FLG are present [4]. The similarities observed between the membrane destabilizing activities of N-terminal and pre-transmembrane segments from different retroviruses suggest that these regions might also have commonalities in their primary sequences. However, sequence comparison between these two regions must consider that the interactions between target cell bilayers and the N-termini of viral fusion proteins and between the viral bilayer and the pre-transmembrane regions have "opposite" topologies [33] (see Fig. 2B). The N-terminal fusion peptides have their N-termini closer to the bilayer, while their C-termini are further away from the membrane. On the contrary, the pre-transmembrane regions have their C-termini closer to the membrane (in fact they may be already part of the transmembrane domain). Indeed, classical sequence alignments between N-terminal fusion peptides and the Foamy virus pre-transmembrane region did not yield significant similarities [33]. However, alignments between the "reversed" Foamy virus pre-transmembrane sequence (writing it from C-t to N-t, instead of from N-t to C-t, thus correcting for the opposite topology of N-terminal fusion peptides and C-terminal pre-transmembrane regions) and N-terminal fusion peptides from retroviruses lead to the identification of highly similar regions belonging to the Foamy pre-transmembrane region and the N-terminal fusion peptide of HIV gp41 [33] (see Fig. 3A). Strikingly, the tripeptide FIG in the retro-Foamy sequence aligned with the HIV-1 FLG tripeptide and about 50% of the matches corresponded to Ala or Gly, residues known to be important for the activity of fusion peptides. Furthermore, sequence analysis studies detected a region in the C-terminal end of the ectodomain of the Envelope Protein E from Flaviviruses, particularly among members of the Tick-Borne Encephalitis virus group, highly similar to that of Foamy viruses [33] (see Fig. 3B). As in Foamy viruses, this analogous region comprises the pre-transmembrane as well as part of a putative transmembrane segment [34]. This strongly suggests that the pre-transmembrane region of the Flavivirus Envelope Protein E might play a role in membrane destabilization during the fusion process. The sequence similarity between pre-transmembrane regions of FV-Env and Flavivirus Protein E is unexpected, since the structure of the latter is very different from all known retroviral fusion proteins [34].

## 4. Paramyxovirus internal fusion peptide

The ectodomain of the paramyxovirus F protein is composed of about 500 residues. As mentioned before,

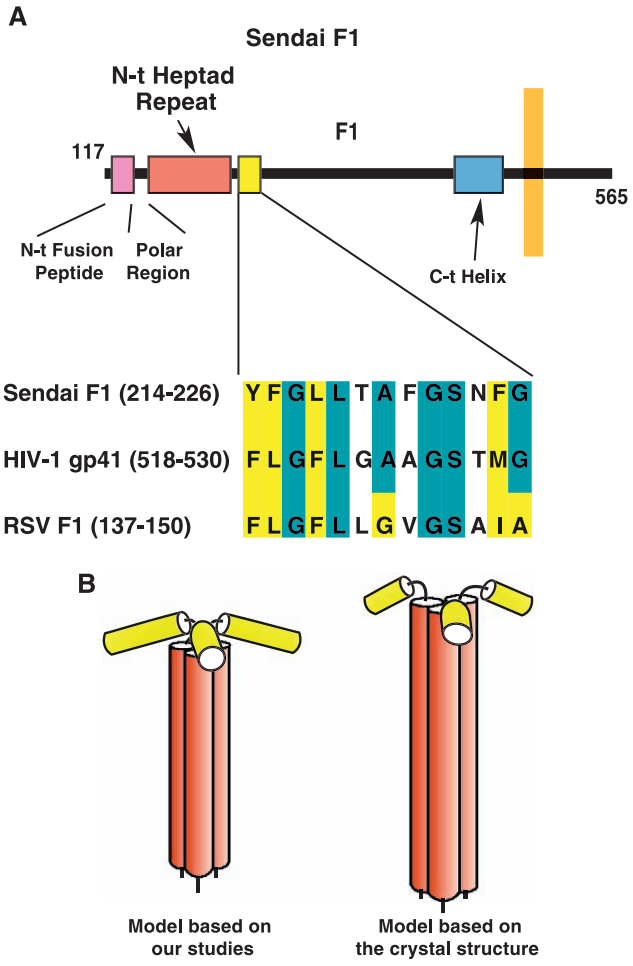


Fig. 4. Panel A, schematic representation of Sendai F1 protein indicating the region that corresponds to the putative internal fusion peptide. As observed, this segment is highly similar to the N-terminal fusion peptides of HIV-1 gp41 (a retroviral protein) and Respiratory Syncytial virus F1 (a paramyxoviral protein). Panel B, left, model based on studies done with peptides from Sendai F1; right, model based on the crystal structure of Newcastle disease virus. The coiled coils formed by the N-terminal heptad repeats are shown in red and the putative internal fusion peptides in yellow.

the highly conserved first 20 residues of the F1 subunit form an N-terminal fusion peptide that is followed by a heptad repeat (see Fig. 1). It has been postulated that, consecutive to the heptad repeat, the F1 protein from Sendai virus contains a second fusogenic domain, highly homologous to known N-terminal fusion peptides [35] (see Fig. 4A). Experimental evidence suggesting a role for this region in paramyxovirus-induced membrane fusion came from protein dissection studies that showed that peptides encompassing this region could induce fusion of large unilamellar vesicles [35,36]. Moreover, some of these peptides inhibited Sendai virus infection of red blood cells, presumably blocking conformational changes in the F protein [37]. When the existence of a second internal fusion peptide in the F1 protein was postulated, the structure of the pre-fusion conformation of the F protein was not known. Thus, a structural model based on functional studies done with different peptides was postulated [35,37]. The model was based on the following information: (i) The peptides corresponding to residues 208–229 or 201–229 of Sendai virus F protein (see Fig. 4A) bind to the surface of the membrane, adopting α-helical structure. (ii) The peptide corresponding to residues 201–229 oligomerizes in aqueous solution, whereas removal or mutation of its N-terminal residues results in the loss of its oligomerization ability [37]. Based on these results, and considering that the coiled coil formed by the N-terminal heptad repeats is believed to be perpendicular to the membrane during the onset of viral entry, it was hypothesized that the internal fusion peptide could form a helix perpendicular to the coiled coil [37]. The different abilities of the peptides to homo-oligomerize indicated that the N-terminal region of each internal fusion peptide could be close to the trimeric coiled coil, while the C-terminal ends could extend radially, away from the central axis (see Fig. 4B). This hypothetical model required that upon binding of the virion to cell receptors, the consequently induced conformational change in F2/F1 results in the exposure of the internal fusion peptide region and its subsequent interaction with the target membrane [38].

The recent determination of the crystal structure of the pre-fusion conformation of the Newcastle Disease virus (NDV, a paramyxovirus) F protein [39] validated this model: the internal fusogenic region is part of an α-helix that extends radially from the internal coiled coil and is almost perpendicular to it (see Fig. 4B). In the crystal structure, a "head" formed by segments from the F1 and the F2 subunits hides the internal fusogenic region. Therefore, for this region to be exposed during an intermediate stage in the fusion process, a conformational change that moves the head away is required. Indeed, there is ample evidence supporting the opening of the head region [38–41].

It is interesting to note that the location of the paramyxovirus putative internal fusogenic region, on top of the N-terminal trimeric central coiled coil, is equivalent to the location of the Influenza hemagglutinin loop that connects the N-terminal coiled coil with the short helix that follows and also to the HIV/SIV gp41 loop that connects the N-terminal coiled coil with the C-terminal helices. Indeed, as described here for the paramyxovirus putative internal fusion peptide, both the Influenza hemagglutinin and HIV/SIV gp41 loops have been postulated to bind to phospholipid membranes [11,42,43].

## 5. Concluding remarks

In recent years, the simple picture of a viral fusion protein interacting with the cell and/or viral membranes by means of only two localized segments (i.e. the fusion peptide and the transmembrane domain) has given way to a more complex picture in which multiple regions from the viral proteins interact with membranes. Although still largely hypothetical, the roles played by these regions could vary, from simply assisting N-terminal fusion peptides to adopt their correct structure and/or oligomeric state, to promote membrane apposition, to directly destabilize the viral and/or target membranes leading to fusion. Future experiments shall shed light into the precise function each region has in the context of the intact virions.

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