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Minireview

Virus membrane fusion

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Abstract **Membrane fusion of enveloped viruses with cellular membranes is mediated by viral glycoproteins (GP). Interaction of GP with cellular receptors alone or coupled to exposure to the acidic environment of endosomes induces extensive conformational changes in the fusion protein which pull two membranes into close enough proximity to trigger bilayer fusion. The refolding process provides the energy for fusion and repositions both membrane anchors, the transmembrane and the fusion peptide regions, at the same end of an elongated hairpin structure in all fusion protein structures known to date. The fusion process follows several lipidic intermediate states, which are generated by the refolding process. Although the major principles of viral fusion are understood, the structures of fusion protein intermediates and their mode of lipid bilayer interaction, the structures and functions of the membrane anchors and the number of fusion proteins required for fusion, necessitate further investigations.**

Keywords: Membrane fusion; Hemifusion; Influenza virus hemagglutinin; HIV-1 gp41; Paramyxovirus F; SFV E1; TBE E; VSV G; Herpes simplex virus gB; Class I, II, III membrane fusion protein

1. Introduction

Enveloped viruses contain a lipid bilayer that serves as an anchor for viral glycoproteins and protects the nucleocapsid containing the genetic information from the environment. The lipid bilayer is acquired from host cell membranes during the process of virus assembly and budding. Consequently, infection of host cells requires that enveloped viruses fuse their membrane with cellular membranes to release the nucleocapsid and accessory proteins into the host cell in order to establish a new infectious cycle. Glycoproteins from enveloped viruses evolved to combine two main features. Firstly, they contain a receptor binding function, which attaches the virus to the host cell. Secondly, they include a fusion protein function that can be activated to mediate fusion of viral and cellular membranes. Both tasks can be achieved by a single or by separate glycoproteins acting in concert.

Depending on the viral family, fusion occurs either at the plasma membrane, where receptor binding triggers conformational changes in the glycoprotein, or in endosomes upon virus uptake by endocytosis. In the latter case the low pH environment of the endosome leads to protonation of key residues, most probably histidines, which induces conformational changes. These conformational changes result in the exposure of hydrophobic peptides, loops or patches (the so-called "fusion peptides"), which then interact with and destabilize one or both of the participating membranes. Triggering the conformational change in the absence of a target membrane leads to inactivation of the fusion properties of the viral glycoprotein.

2. Fusion protein structures

The determination of the atomic structure of complete ectodomains or core regions of many viral fusion proteins in their pre- and/or post-fusion states has revealed a large diversity of conformations (see below). Nevertheless, in all the cases studied so far, the structural transition from a pre- to a post-fusion conformation leads to a stable hairpin conformation resulting in the positioning of the two membrane anchors, the transmembrane and the fusion peptide domains, at the same end of a trimeric elongated rod-like structure. Three different classes of viral fusion proteins have been identified to date based on their common post-fusion structural motifs (Table 1 and Fig. 1). These include class I fusion proteins, characterized by trimers of hairpins containing a central α-helical coiled-coil structure and class II fusion proteins, characterized by trimers of hairpins composed of beta structures [1–5]. A third class of fusion proteins has been described, that also forms trimers of hairpins by combining two structural elements. Similar to class I fusion proteins, the post-fusion trimer displays a central α-helical trimeric core; however, each fusion domain exposes two fusion loops located at the tip of an elongated β-sheet revealing a striking convergence with class II fusion proteins [6,7] (Table 1 and Fig. 1). Although the importance of the hairpin arrangement for membrane fusion was first recognized based on the crystal structure of the post-fusion conformation of HIV-1 gp41 [8], the conclusions drawn were largely based on the known conformational transitions of influenza virus hemagglutinin upon exposure to low pH [1,9].

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Table 1
Classification of viral fusion proteins based on the structural motifs of their post-fusion conformations

Virus family	Virus species	PDB code
Class I		
Orthomyxoviridae	Influenza A virus HA	1HA0, 3HMG, 1HTM, 1QU1
	Influenza C virus HEF	1FLC
Paramyxoviridae	Simian parainfluenza virus 5 F	2B9B, 1SVF
	Human Parainfluenza virus F	1ZTM
	Newcastle disease virus F	1G5G
	Respiratory syncytial F	1G2C
Filoviridae	Ebola virus gp2	1EBO, 2EBO
Retroviridae	Moloney Murine leukemia virus TM	1AOL
	Human immunodeficiency virus 1 gp41	1ENV, 1AIK
	Simian immunodeficiency virus gp41	2SIV, 2EZO
	Human T cell leukemia virus 1 gp21	1MG1
	Human syncytin-2 TM	1Y4M
	Visna virus TM	1JEK
Coronaviridae	Mouse hepatitis virus S2	1WDG
	Sars corona virus E2	2BEQ, 1WYY
Class II		
Flaviviridae	Tick-borne encephalitis virus E	1URZ, 1SVB
	Dengue 2 and 3 virus E	1OK8 IUZG, 10AN, 1TG8
Togaviridae	Semliki forest virus E1	1E9W, 1RER
Class III		
Rhabdoviridae	Vesicular stomatitis virus G	2GUM
Herpesviridae	Herpes simplex virus gB	2CMZ

3. Structural transitions leading to fusion

Even though the mode of activation, the structural motifs used and the differences in initial oligomerization states of viral fusion proteins, namely native trimeric conformations in case of class I and class III fusion proteins versus homo- or hetero-dimeric conformations in case of class II fusion proteins, are substantial, the common mechanism for refolding allows to suggest a few generic steps, which are supposed to be common to viral glycoprotein mediated fusion. Firstly, activation upon receptor binding or acidification of the endosomal compartment exposes the fusion peptide that is projected toward the top of the glycoprotein, allowing the initial interaction with the target membrane (Fig. 2B and C). Secondly, the folding back of the C-terminal region onto a trimeric N-terminal region (Fig. 2D) leads to the formation of a post-fusion protein structure with the outer regions zipped up against the inner trimeric core (Fig. 2E and F). The final step also requires further refolding of the membrane proximal and transmembrane regions in order to obtain a full-length post-fusion structure where both membrane anchors are present in the same membrane [10,11]. Notably, paramyxoviruses and retroviruses fusion occurs concomitantly with the formation of the post-fusion core structure [12,13].

Refolding of class II fusion proteins generates trimers assembled from intermediate monomer conformations (Fig. 2B). Whether monomerization occurs during class I and class III protein refolding remains a matter of debate. Although the initial steps leading to fusion peptide exposure and its interaction with the target membrane may maintain strict trimeric symmetry, the folding back of the C-terminal part of the molecule requires breaking the three-fold symmetry of the molecule at least for the C-terminal half (Fig. 2C and D). It is also worth to note that the trimer contacts observed in the pre- and post-fusion conformations of both VSV G and paramyxovirus F protein differ [6,14–16]. In contrast, the trimer interface is very similar in case of influenza virus HA2 pre- and post-fusion conformations [1].

4. Lipidic intermediates and lipids in viral membrane fusion

The biophysics of membrane fusion is dominated by the stalk hypothesis. According to this view, fusion of two lipid bilayers in an aqueous environment requires that they come into close contact. This process involves local membrane bending creating a first site of contact. Dehydration of the initial contact site induces monolayer rupture that allows mixing of lipids from the two outer leaflets, resulting in a hemifusion stalk, i.e., a local lipidic connection with negative curvature. In a next step, the model predicts that radial expansion of the stalk leads to either direct fusion pore opening or to the formation of another intermediate, the hemifusion diaphragm, a local bilayer resulting from the contact between the two internal leaflets of the fusing membranes. Depending on the experimental system, hemifusion might be restricted (i.e., without lipid exchange between both membranes) or unrestricted (i.e., without any restriction of lipid diffusion). The break of the hemifusion diaphragm also results in pore formation. The initial fusion pore is small and is characterized by an opening and closing (“flickering”). Finally, pore enlargement, which requires most of the energy, leads to complete fusion [17].

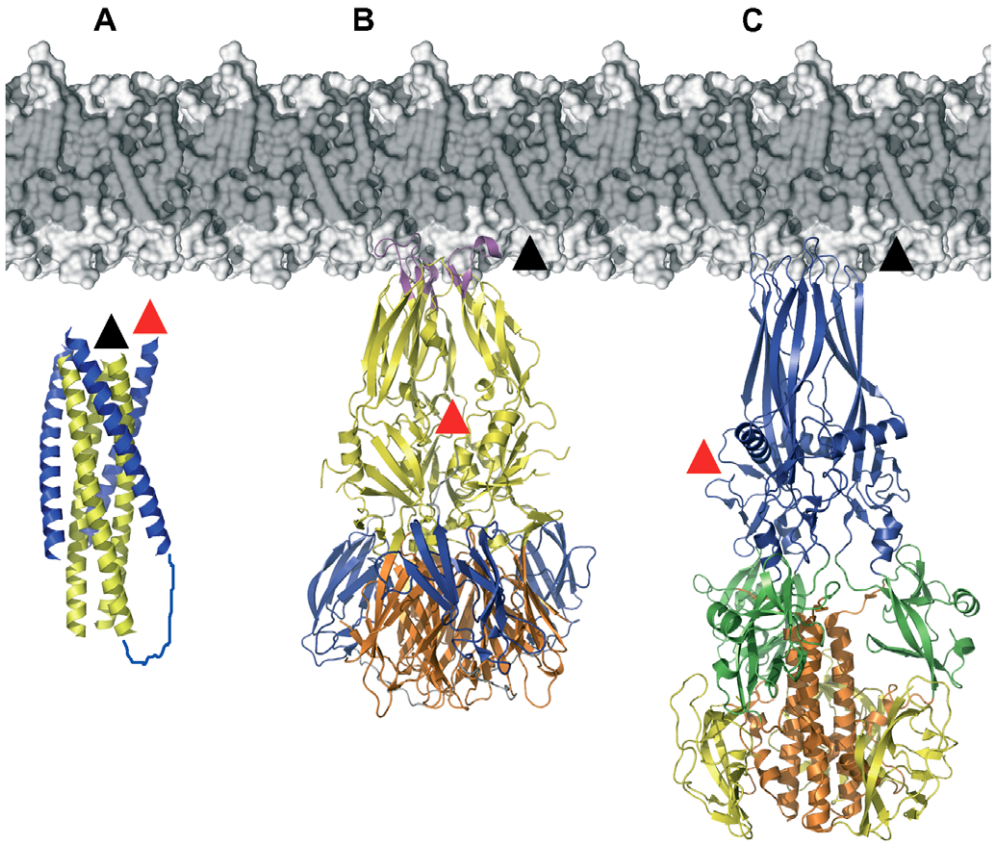

Fig. 1. Structural motifs of viral fusion proteins. Ribbon diagrams of representative structures of class I, II and III fusion proteins in their proposed post-fusion conformations positioned with respect to the lipid bilayer. The positions of both membrane anchors at the tip of the elongated structures are indicated by black (fusion peptide, fp) and red (transmembrane, TM) arrows. (A) HIV-1 gp41 core structure; (B) Flavivirus fusion protein E and (C) VSV glycoprotein G. Structural elements, which undergo change from pre-fusion to post-fusion are show in different colors (B and C). The structural changes of gp41 from a pre-fusion to a post-fusion conformation are still unknown.

The applicability of the stalk/pore model to viral membrane fusion processes is largely supported by experimental results. First evidence for a hemifusion intermediate in viral fusion resulted from the replacement of the hemagglutinin membrane anchor by a glycosylphosphatidylinositol (GPI) anchor, which revealed the importance of the transmembrane region in the transition from the stalk and/or hemifusion structure to fusion pore opening and expansion (Fig. 2E and D) [18]. Furthermore, hemifusion intermediates, i.e., lipid mixing without content mixing, have been detected in case of HIV-1 env mediated fusion [19], class II protein driven fusion, such as alphavirus E1 [20] and paramyxovirus F fusion [12]. Finally, such hemifusion intermediates were also observed with peptide inhibitors such as HIV-1 gp41 T-20, which target a pre-fusion or pre-hairpin structure [21,19]. Interestingly, peptide inhibitors derived either from the N-terminal region or from the C-terminal region inhibit either lipid mixing or content mixing, indicating that C-terminal inhibitors allow sufficient membrane bending of the fusion protein to support lipid mixing [12,19,22]. Similarly to the N-terminal gp41 peptide, recombinant domain III of alphavirus E1 and flavivirus E inhibit fusion also at the early lipid mixing step [23].

The stalk/pore model has also been challenged using lipids of different shapes. When present in the outer leaflets of the fusing membranes, lipids such as inverted cone-shaped lysophospholipids induce a micellar positive spontaneous curvature and inhibit stalk formation, while cone-shaped phosphatidylethanolamines or oleic acid induce negative curvature and promote hemifusion in membrane fusion assays. In contrast, when present in the inner leaflets of the membranes, the lipid effect on the opening of the fusion pore is the opposite. Thus lipid shapes affect the membrane fusion process as predicted by the stalk/pore hypothesis [5,17,24].

Although cholesterol and sphingolipid requirements for class II glycoprotein mediated fusion have been described, no coherent process has yet evolved [25–28]. A potential lipid dependence of virus entry processes has been deduced from experiments suggesting the implication of lipid rafts [29], which otherwise serve as efficient platforms for virus assembly and budding [30]. This is indirectly supported by the fact that HIV-1, whose envelope lipid content resembles lipid raft microdomains, becomes less infectious when the virus is grown in cells with a defect in sphingolipid and cholesterol synthesis [31]. However, it remains to be determined whether the lipid composition of the viral membrane has an influence on viral fusion efficiency or on a certain stage of virus entry.

In any case, cholesterol and sphingolipids are not known to induce specific membrane curvature and the local lipid content at the site of fusion does not change dramatically during the fusion process. Therefore, the energy necessary for membrane deformation and bending has to be solely provided by the glycoproteins. This idea is consistent with the observation that

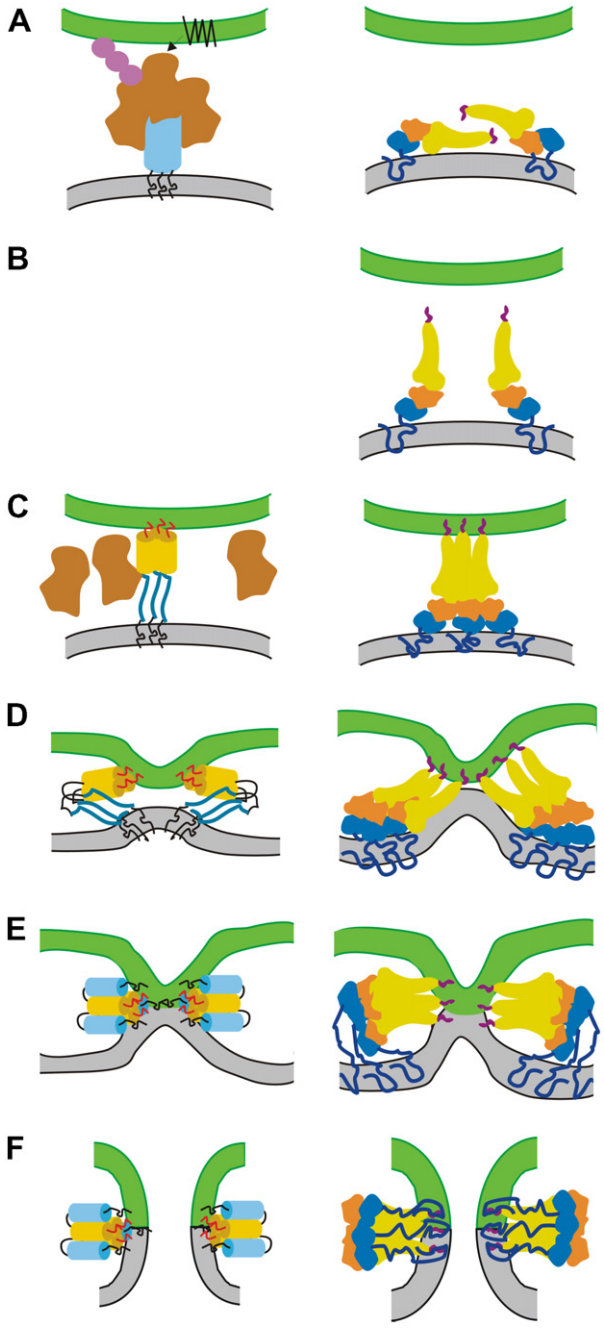

Fig. 2. Similar fusion models evolved for class I (left panel) and class II (right panel) fusion proteins. (A) Receptor binding alone (e.g., HIV-1, CD4 and CXCR4 or CCR5) or coupled to endozytosis (e.g., influenza virus HA, TBE E) leads to conformational changes outlined in panels B to F. (B) A transition in oligomeric state is accompanied by fusion peptide target membrane interaction in case of class II. Intermediate monomeric structures have to be also postulated for Rhabdovirus G and paramyxovirus F. Whether they play a role in other class I mediated fusion reactions (influenza virus HA, HIV-gp41) remains to be determined and if so it might be very short lived. (C) Transient intermediate states where the fusion peptide is anchored in the membrane might induce initial curvature. This might involve several fusion proteins, which might cluster via fusion peptide interactions. (D) Initial refolding of the C-terminal region leads to further apposition of the bilayers. Although this step may keep strict trimeric symmetry at the N-terminus, its C-terminal region must be flexible. (E) Final zipping up of the outer layers might induce hemifusion controlled by both membrane anchors. (F) The membrane anchors also play a critical role in fusion pore opening and possibly expansion.

many viruses such as influenza virus and rhabdoviruses can fuse with liposomes of various compositions indicating that no natural lipid is absolutely required for efficient fusion [17,32].

In contrast to virus fusion, intracellular vesicle fusion processes might be regulated by the lipid composition at the site of fusion, which includes a role for phosphatidic acid, phosphatidylinositol-4,5-bisphosphate (PIP2) and phospholipase D1 [33–35].

5. Membrane anchors and membrane curvature

The low pH structures TBE virus E, dengue fever virus E, SFV E1 (class II), vesicular stomatitis virus G and possibly also herpes simplex virus gB (class III) indicate that only hydrophobic side chains of the loops insert into the hydrocarbon chains of the outer leaflet of a target membrane [6,7,25]. Remarkably, all these fusion proteins have at least one polar aromatic residue in their fusion loops. Tyrosines and tryptophans are residues typically found at the interface between the fatty acid chains and head group layers of lipids. Such an interfacial interaction is thus sufficient to anchor the fusion protein to the host cell membrane. Intercalation of hydrophobic side chains into one bilayer leaflet might be also a general way to induce membrane curvature, similar to that induced by amphipathic helices of cellular membrane bending proteins such as BAR domain containing proteins [36].

Due to the large number of spikes at the viral surface and the oligomeric status of fusion proteins, multiple fusion loops might interact with the external leaflet, potentially initiating membrane deformation. Multimerization of fusion loops has also been suggested to induce a nipple-like structure, which might initiate apposition of two bilayers as suggested by the stalk model [17,25].

In this context it is probably reasonable to speculate that class I fusion peptides also do not penetrate deep into the bilayer structure but rather induce local membrane curvature similar to class II and class III fusion loops. Indeed, some class I proteins utilize also internal fusion loops such as Ebola virus Gp2 whose fusion loop could expose a conserved WIPYF sequence at its tip [37].

Besides the initial anchoring and potential membrane bending function of fusion peptides, mutagenesis data implicate fusion peptides in the transition from hemifusion to fusion [38]. Together with the experiments performed with GPI anchored fusion proteins such as HA, it is evident that both the transmembrane regions as well as the fusion peptides/loops are directly involved in several steps of the fusion process.

6. A network of fusion proteins versus single trimeric complexes

For class I and class II viral fusion proteins, the pre-fusion state is metastable and it has been proposed that the free energy released during the structural transition is used to overcome the high energetic barrier encountered during the fusion process. Indeed, energy recovery via refolding of a single env trimer is theoretically sufficient to overcome the free energy barrier of fusion [39]. In case of rhabdoviruses, however, there is a pH dependent equilibrium between pre- and post-fusion conformations of G. Thus, the energy released by the struc-

tural transition of one trimer is not sufficient to catalyze fusion which indicates that a larger number of spikes act cooperatively during fusion [40].

Numerous experiments suggest a similar scenario for other fusion proteins. Work on influenza virus hemagglutinin indicates that HA surface density is important for fusion [41] and that a decrease in HA surface density arrests fusion at the hemifusion stage [4,17]. The restriction to lipid diffusion observed downstream of stalk formation has been attributed to the formation of a ring of HA surrounding the initial membrane contacts [42]. Furthermore, HA outside the direct contact zone contributes to the fusion activity thus exerting a synergistic effect [43]. Finally, quick freezing electron microscopy images of influenza virus particles fusing with liposomes have revealed local micro contacts between viral and liposomal membranes, resembling stalk intermediates, which are organized in regular polygonal arrangements [44]. In contrast to HA, data on HIV-1 env suggest that a single env glycoprotein trimer is sufficient for fusion [45]. On the other hand cellular receptor density as well as env density affect the kinetics of HIV-1 env mediated fusion [46].

The role of a potential cooperative function of fusion proteins is clearer in case of class II mediated fusion. Homo- or hetero-dimeric class II fusion proteins already form a protein shell covering the complete viral membrane in the native pre-fusion state [47,48]. Upon activation *in vitro*, both, soluble SFV E1 protein and flavivirus E proteins, insert their fusion loops into liposomes and form arrays of trimers organized in a lattice composed of rings of five or six, which either determine the curvature of coated liposomes or form flat hexagonal arrays *in vitro* [11].

7. Conclusions

Functional and structural studies have shown that membrane fusion processes catalyzed by virus GPs are in effect largely similar and follow the same fundamental principles. These are indeed also highly similar to SNARE mediated fusion processes, where folding of a four helical bundle leads to the apposition of two membranes and provides the energy for the fusion reaction [49]. However, to understand the fine tuning of fusion further structural studies of complete post-fusion conformations of viral glycoproteins containing both membrane anchors should aid in understanding their precise roles. This might shed light on the open question whether both regions cooperate to regulate the transition from hemifusion to fusion pore opening and/or pore enlargement. In addition, functional studies are needed to test the hypothesis that the membrane anchors are responsible and sufficient to induce curvature. These data could then be used for simulating fusion reactions *in silico*, which might help to determine the energy requirements and reveal the potential folding path of intermediate states. Finally, understanding the structures of fusion intermediates might be also crucial for the development of fusion inhibitors interfering with virus infection for therapeutic applications.

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