Cell Entry of Enveloped Viruses

Richard K Plemper[*]

Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322, USA and Children's Healthcare of Atlanta, Atlanta, GA 30322, USA

Abstract

Infection of cells by enveloped viruses requires merger of the viral envelope membrane with target cell membranes, resulting in the formation of fusion pores through which the viral genome is released. Since lipid membranes do not mix spontaneously, the fusion process is energy-dependent and mediated by viral envelope glycoprotein complexes. Based on their structural and mechanistic properties, three distinct classes of viral fusion proteins have been identified to date. Despite their diversity, basic principles of viral membrane fusion, simultaneous engagement of both donor and target membrane and refolding into hairpin-like structures, have emerged as universally conserved. This article provides an overview of the basic principles of viral membrane fusion common to all enveloped viruses and discusses the specific structural and functional features of the different fusion protein classes by example of the paramyxovirus, flavivirus and rhabdovirus families.

Introduction

As obligatory intracellular parasites, viruses must transfer their genetic information across cellular membranes to initiate an infectious cycle that ultimately results in the production of progeny virions. For membrane-wrapped enveloped viruses, this requires merger of the viral envelope with target cell membranes and formation of a fusion pore through which the genome of the particle is released. Maintaining membrane integrity is imperative for cellular life, however, and high repulsive energy between approaching membranes prevent spontaneous fusion of distinct lipid vesicles.

Basic principles of viral membrane fusion

Similar to the well-documented vesicular fusion machinery in eukaryotic cells [1], membrane fusion for viral entry is mediated by membrane protein complexes. These envelope glycoproteins are typically observed as characteristic protein "spikes" on the surface of purified virions in electron microscopic images. Significant progress in the structural analysis of spike proteins derived from representatives of a variety of enveloped virus families has resulted in the identification of, thus far, three distinct classes of viral fusogenic membrane glycoprotein (FMG) based on their structural organization and mechanism of fusion (table 1). Independent of this differentiation and the nature of the particular cellular membrane (intracellular versus plasma membrane) that is targeted for viral entry, however, two basic mechanistic principles of membrane fusion have emerged as conserved among enveloped viruses, target membrane engagement and refolding into hairpin-like structures ([2] illustrated in figure 1).

[*]address for correspondence: Division of Infectious Diseases, Department of Pediatrics, 520 Emory Children's Center, 2015 Uppergate Drive, Emory University School of Medicine, Atlanta, GA 30322, Phone: +1-404-727-1605, Fax: +1-404-727-9223, rplempe@emory.edu.

Firstly, upon particle attachment to target cells, conformational rearrangements of the fusogenic spike protein complex take place that result in insertion of largely hydrophobic membrane attack domains, termed the fusion peptides or fusion loops, into the target membrane. The resulting pre-hairpin intermediate conformation thus engages both the viral envelope and the host cell membrane.

Secondly, refolding of the trimeric fusogenic complex onto itself into, ultimately, a trimeric hairpin structure is considered to then bring the attack and transmembrane domains, and thus the donor and target membranes, into close proximity. Involving the concomitant engagement of several complexes [3,4,5,6,7,8,9], a current consensus model (however, see [10,11] for alternative hypotheses) proposes progressing of the membranes towards each other through formation of a lipid funnel [12], which, ultimately, sets the stage for merger of the outer leaflets of the approaching monolayers in regions of negative spontaneous curvature [13,14]. This hemifusion step is followed by fusion of the inner leaflets, forming a pore. As alternative pathway to this stalk-diaphragm model, direct transition from stalk to fusion pore was proposed, based on Monte Carlo and coarse-grain ensemble molecular dynamics algorithms [15,16,17].

Once a fusion pore has formed, subsequent pore enlargement appears associated with the most significant energy penalty of the process, resulting in the possibility that newly opened pores regress to the hemifusion stage rather than proceed to expansion for fusion completion and viral infection [7,8,18].

Energy dependence of the process

As depicted schematically in figure 1, membrane fusion requires the introduction of a high degree of local curvature into the lipid bilayer, rendering it an energy-dependent process overall [19]. In the absence of external energy sources, refolding of the FMGs into their thermodynamically stable postfusion conformations is considered to supply the energy that is necessary to overcome the elastic resistance of the lipid bilayers and generate the highly bent intermediate formations. Estimates of the number of individual viral fusion complexes required for productive fusion pore formation vary from a single trimer to over a dozen complexes [6,20]. While the hypothesis that a single HIV Env protein trimer is capable of inducing membrane fusion has been challenged recently [21,22], currently available data do not support a direct calculation of the free energy associated with the refolding of viral fusion complexes. Stoichiometric estimates are therefore based on indirect, functional assays [3,6,23], but typically assume cooperation of several complexes for productive fusion. Definitive estimates are further challenged by the possibility that the stoichiometric requirements for a given viral fusion protein may not be strictly defined, resulting in pore formation and enlargement mediated by different stoichiometric complex assemblies with variable degrees of efficiency. Examining fusion events on a single molecule level as exemplified by the analysis of cellular SNARE proteins [15] will likely be required to address these questions.

Class I fusion proteins

Of all fusogenic viral envelope glycoproteins, representatives of class I proteins were the first to be described and crystallized in both the pre- and postfusion conformations. This class comprises, amongst others, influenza HA, paramyxovirus F, HIV Env and Ebola virus GP proteins. All class I fusion proteins form homo-trimers. They are first synthesized as fusion-inactive precursor molecules that require proteolytic activation to liberate the N-terminus of the fusion peptide and gain fusion competence. Often, cleavage is mediated by the ubiquitous subtilisin-like cellular furin endoproteases in the host secretory system that

are most active at acidic pH in the Golgi and/or trans-Golgi network compartments [24], ensuring that proteolytically matured complexes reach the cell surface.

Not surprisingly, the efficiency of fusion protein cleavage can directly affect viral pathogenesis. In the case of influenza virus, for instance, the HA_0 precursor proteins of highly pathogenic H5 and H7 viruses are recognized and cleaved by furin and furin-like proteases, enabling systemic infections in the avian host [25]. In contrast, HA proteins of non-avian influenza A and avirulent avian strains are cleaved mostly by trypsin-like proteases such as, amongst others, tryptase Clara, plasmin or miniplasmin [26,27]. Infection by these strains typically remains localized to the respiratory and intestinal tract, lacking a systemic component. While most paramyxovirus fusion (F) proteins are cleaved by furin in transit to the plasma membrane, F proteins of paramyxoviruses of the henipavirus genus are internalized after initial transport to the cell surface for cathepsin L-mediated maturation, followed by re-routing to the plasma membrane [28,29].

Depending on the viral family, refolding of matured class I proteins can be initiated through a) acidic pH (i.e. influenza virus HA, b) direct receptor binding, possibly in conjunction with acidic pH (i.e. paramyxoviruses of the *Pneumovirinae* subfamily [30]), or c) receptor binding by a non-covalently associated attachment envelope glycoprotein that then stimulates refolding of the fusion protein at neutral pH (i.e. most paramyxoviruses of the *Paramyxovirinae* subfamily). For the latter, receptor binding appears to trigger separation of preformed hetero-oligomers of some *Paramyxovirinae* such as measles virus and the henipaviruses (fusion triggering through dissociation), whereas receptor binding-induced glycoprotein association was postulated for Newcastle disease virus and the parainfluenzaviruses (fusion triggering through association) [31]. Independent of the mechanism of fusion triggering, characteristic for all class I fusion proteins are two 4-3 heptad repeat (HR) domains, positioned adjacent to the fusion peptide and the transmembrane domain, respectively. Figures 2A and 3A illustrate major structural features of the class I fusion process by example of the paramyxovirus fusion (F) protein.

In the prefusion conformation, an F protein ectodomain was crystallized in a lollipop-like structure consisting of a globular head domain that is linked to the transmembrane domains (TMDs) and short luminal tails through the TMD-proximal HR sequences [32]. In this metastable prefusion conformation, the fusion peptide-proximal HR domains are broken up into 11 distinct segments forming part of the head structure. Upon fusion activation, major structural rearrangements take place that first result in assembly of head-domain HR segments into a central, trimeric α-helical coiled coil structure, propelling the fusion peptides towards the target membrane [33,34]. Subsequent hairpin-like refolding then positions the individual TMD-proximal HR domains in the grooves of the central triple helix, ultimately resulting in the formation of a thermodynamically extraordinarily stable six-helix bundle (6HB) structure [35,36,37,38]. The final arrangement of a fusion peptide-proximal core coiled-coil structure surrounded by the three TMD-proximal HR helices is a defining feature of class I fusion proteins.

6HB formation appears closely linked to the opening of a fusion pore [39,40] and is considered to provide, possibly augmented by neighboring sequences as in the case of influenza HA [41], a major driving force for pore formation and expansion. Consequently, peptides [36,42] or small molecules [43,44] that prevent final 6HB formation by interfering with refolding of the fusion protein function as potent viral entry inhibitors. Clearly, refolding of the fusion protein trimer as shown schematically in figure 3A cannot proceed symmetrically, as this would wrap the protein into the lipid of the viral envelope. Instead of an umbrella-like folding of the protein "through itself", an asymmetric process must be assumed in which individual monomers of the trimer progress through the conformational

rearrangements at different rates and individual TMD-proximal HR domains possibly engage the grooves in the central triple helix coiled-coil consecutively rather than in a concerted action.

Close confinement of the TMDs and the fusion peptides in the same lipid space after 6HB formation makes conceivable short-range proteinaceous contacts between these membrane-inserted domains in addition to protein-lipid interactions. While still understudied, direct peptidic relations between these domains have been proposed [45,46,47] and may well serve as important driving forces for stabilization and expansion of nascent fusion pores, thus moving beyond fusion flickering through pore closure and reversion to the hemifusion stage. Association studies of isolated TMDs and fusion peptides in artificial lipid environments complemented with mutational analysis of these domains in the context of functional fusion proteins should further test these possibilities.

Class II fusion proteins

Of all fusion protein classes identified thus far, only class II proteins such as flavivirus E show a dimeric stoichiometry in the prefusion conformation. First major structural insight came from the crystallization of Dengue virus E proteins under both neutral (prefusion) and low pH conditions [48,49]. Flavivirus E mediates both receptor binding and membrane fusion, but depends on association with chaperone proteins to stabilize the prefusion fold. Presumably in the endoplasmic reticulum, newly synthesized E forms hetero-dimers with the viral prM glycoprotein. Rearrangement of E into head-to-tail homo-dimers under the low pH conditions of the trans-Golgi network then renders prM accessible for proteolytic cleavage by cellular furin or furin-like endoproteases, and E homo-dimers are present on the surface of mature particles at neutral pH [50,51,52]. Unique to this class, these primed prefusion class II proteins assume an elongated, plate-like shape running parallel to the viral envelope (figure 2B). The E protein ectodomain has a high β-sheet content and is organized into three sections, forming a β-barrel proximal to the TMDs (domain I), generating the rod-like extensions (domain II), and, with an immunoglobulin-like fold, mediating receptor binding (domain III). The internal fusion loops are positioned at the tip of each domain II rod, distal to the TMDs and buried within the dimer interface [53]. Upon triggering through acidification of the endosome, the complex transiently dissociates and domains adjacent to TMDs and fusion loops are rotated into a position perpendicular to the viral envelope and target membrane. Reorganization into homo-trimers in this pre-hairpin intermediate sets the stage for refolding of the complex onto itself through a second rotation step, which again brings TMDs and fusion loops into close proximity (figure 3B and [49,54]. The final E protein structure remains rich in β-sheets in contrast to the high α-helical content that is characteristic for the class I proteins.

Alphavirus E1 proteins present a variation of the theme outlined above. Initially, the E1 protein, resembling the domain architecture of flavivirus E [55,56], is associated with the viral E2 protein, which mediates receptor binding. These hetero-dimer pairs form an organized lattice of 80 spikes on the virion surface [57,58]. Upon low pH triggering, the E1/E2 hetero-dimers dissociate, E1 reorganizes into homo-trimers, and refolds into the class II postfusion hairpin structure.

Class III fusion proteins

The group of class III fusion proteins currently comprises rhabdovirus G protein, herpesvirus gB and baculovirus gp64. In addition to postfusion conformations of gB [59,60] and gp64 [61], our structural understanding of this class was greatly advanced by crystallization of the vesicular stomatitis virus (VSV) G protein trimer in both the pre- and postfusion conformations (figure 2C and [62,63]). Similar to class II proteins, also G

mediates both receptor binding and, triggered by the low pH conditions of the endosome, membrane merger. Major conformational stages, a native (neutral pH) prefusion conformation, an extended conformation that engages both viral envelope and the target membrane, and a hairpin-like postfusion conformation likewise resemble key stages of class I and II fusion protein refolding (figure 3C). In striking contrast to the other fusion protein classes, however, G refolding is reversible, adhering to a pH-driven equilibrium. Consequently, the native prefusion conformation is not metastable [64], enabling the trimer to re-assume its native configuration subsequent to transport through the low-pH environment of the Golgi apparatus [65].

Native G trimers revealed a tripod-like arrangement consisting of four defined sections, a lateral domain (DI) with high β-sheet content, a trimerization domain (DII), a PH domain (DIII) located towards the top of the tripod and, forming the tripod legs, a fusion domain (DIV). The fusion loops are positioned at the tip of each leg, pointing towards the viral envelope [62]. While domains I, III and IV maintain their fold during G transition from pre- to postfusion conformation, the trimerization domain becomes subject to major refolding. Protonation of a cluster of histidine residues located at the intersection between domains at low pH was proposed to have a major effect on destabilizing the native conformation, priming the initiation of refolding [62]. Upon triggering, the tripod legs are proposed to swing upwards, driving the fusion loops towards the target membrane. Repositioning of the domains with conserved tertiary structure relative to each other through secondary structure changes in hinge regions and major reorganization of the trimerization domain then ultimately result in a classic hairpin postfusion conformation, in which fusion loops and TMDs are positioned in proximity in the same lipid space [63].

Despite the absence of heptad repeat domains in VSV G, two helices of each postfusion domain II engage in formation of an antiparallel 6HB in the postfusion trimer in strong reminiscence of the class I protein fusion core structure. A set of acidic amino acids, brought into close proximity in the central 6HB in the postfusion conformation, appear to serve as a second major molecular switch to initiate transition back to the native state upon deprotonation at elevated pH [63].

It was proposed that approximately 15 VSV G trimers are required to mediate membrane fusion [6]. Based on the observation that the prefusion structure of the G trimer supports organization into a hexagonal lattice, the local organization of the G protein spikes on the viral surface may set the stage for concerted conformational changes of multiple G spikes [62], thus optimizing the efficiency of the fusion process.

Conclusions

The basic principles of envelope glycoprotein-mediated membrane fusion, engagement of viral and target membranes followed by hairpin formation that juxtaposes TMDs and fusion peptides/loops in the same lipid environment, have emerged as well conserved in all three classes of enveloped virus fusion proteins identified to date. While X-ray structures of fusion protein ectodomains in pre- and postfusion conformations have resulted in a breakthrough in our general understanding of viral infection, much less is known about the molecular details of lipid merger, the spatial arrangement on the surface of infectious particles, and the nature of short-lived intermediate conformations of the refolding fusion proteins. Addressing these questions will likely require interfacing novel imaging approaches such as cryo-electron tomography reconstructions of emerging fusion pores [12] and refolding complexes arrested in intermediate conformations through addition of peptidic or small-molecule fusion inhibitors [36,43,66,67] with single molecule analyses in analogy to the observation of reconstituted cellular SNAREs [15] and new functional assays

[68,69,70,71]. Ultimately, a detailed, high-resolution structural and mechanistic understanding of the viral fusion process has high potential to aid the development of novel virus entry inhibitors through structure-based drug design.

Acknowledgments

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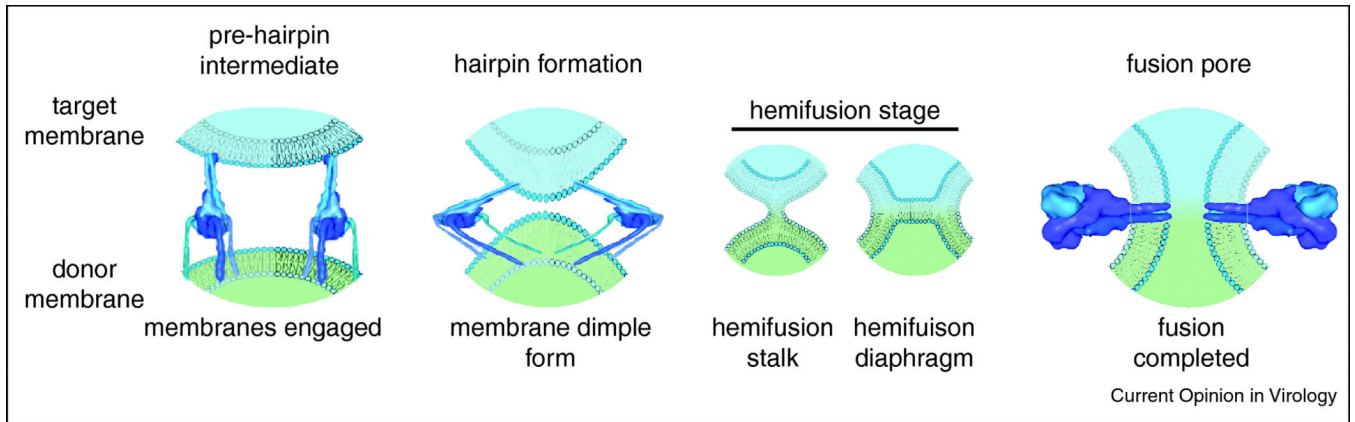
Figure 1.
Schematic of viral membrane merger, exemplified by the paramyxovirus F protein. Upon fusion triggering, conformational changes of the native F glycoprotein result in an extended conformation, in which both viral envelope and target membrane are engaged (left panel). Through refolding of several F trimers, a fusion funnel with high local lipid curvature is thought to form. Merger of the outer monolayers of the approaching membranes creates a lipid stalk, which, according to the common stalk-diaphragm model, expands to hemifusion and formation of a hemifusion diaphragm (center panel). Completion of the F refolding into a thermodynamically stable hairpin structure is considered to advance merger to fusion pore formation and subsequent pore expansion (right panel).
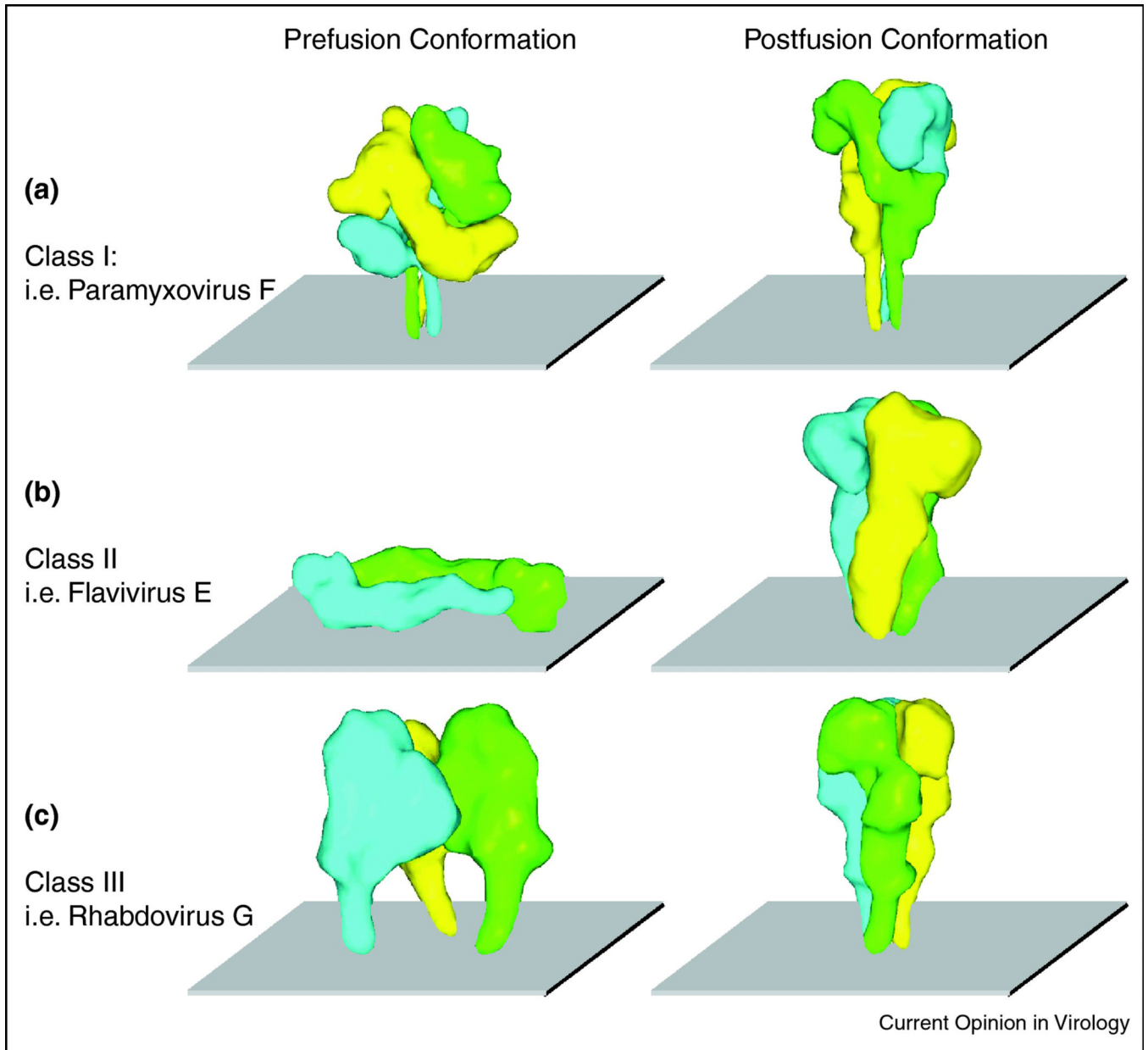
Prefusion Conformation   Postfusion Conformation

(a)

Class I:
i.e. Paramyxovirus F

(b)

Class II
i.e. Flavivirus E

(c)

Class III
i.e. Rhabdovirus G

Current Opinion in Virology

Figure 2.
Renderings of class I (paramyxovirus F (**A**)), class II (flavivirus E (**B**)) and class III
(rhabdovirus G (**C**)) viral fusion proteins in the pre- and postfusion conformations. For
clarity, high-resolution structural models were colored by monomer and then morphed into
lower resolution images using the Sculptor (resolution 12, voxel size 3) software package
[83]. Structures are based on PDB coordinates 2B9B and 1ZTM ([32,33] (A)), 1OAN and
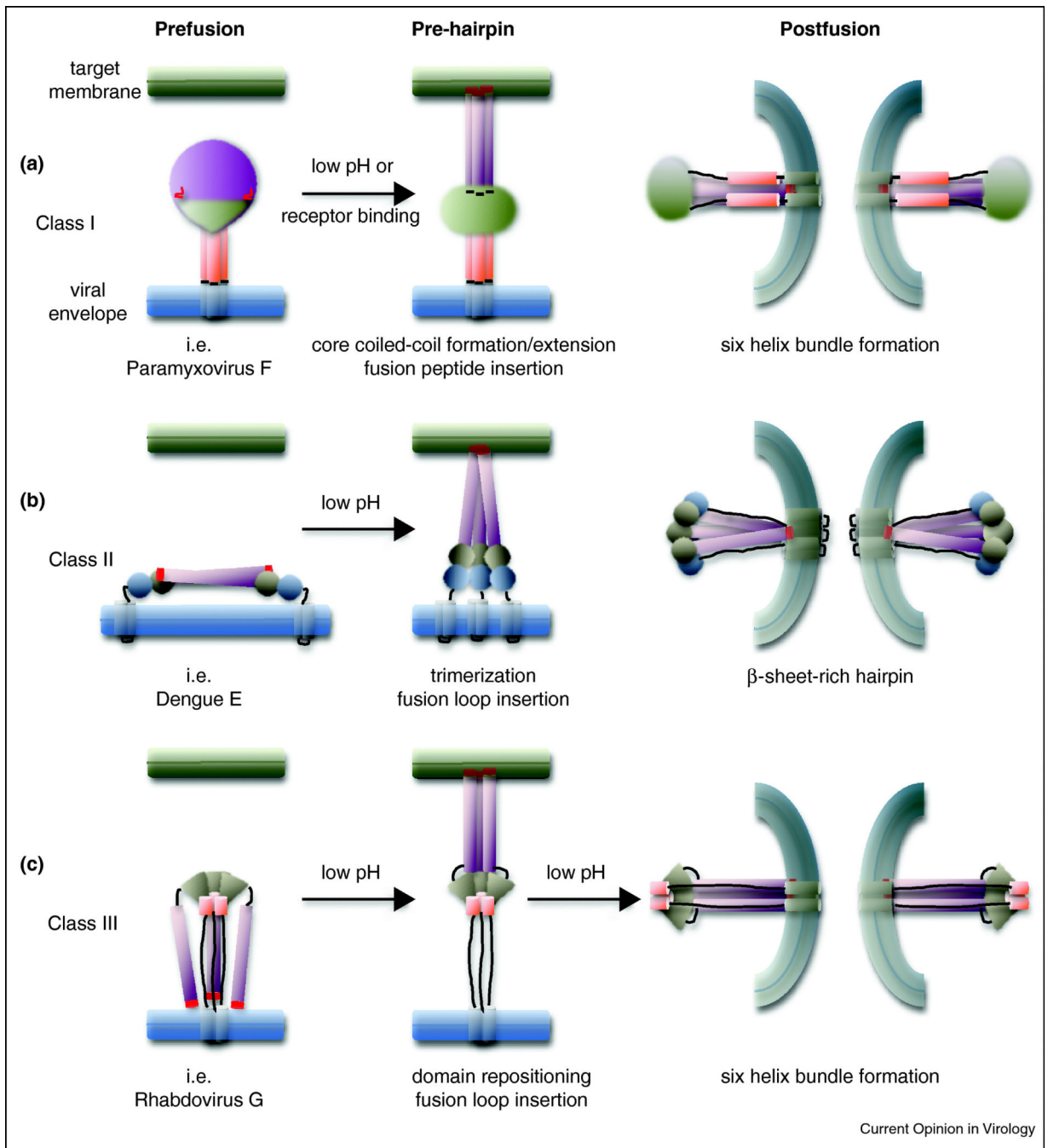1OK8 ([48,49] (B)), and 2J6J and 2CMZ ([62,63] (C)), respectively.

Figure 3.
Schematic representation of the general domain organization and conformational reorganization of the three viral fusion protein classes. Fusion peptides/loops are highlighted in red. **A)** Paramyxovirus F: fusion peptide-proximal HR domains forming the central trimeric coiled coil (purple) and TMD-proximal HR domains (orange) are highlighted. **B)** Flavivirus E: the extended domains II harboring the fusion loops are highlighted (purple). **C)** Rhabdovirus G: the fusion domains (DIV, purple) and the trimerization domains (DII) refolding into the postfusion 6HB (orange) are highlighted.

Table 1

Currently known distinct classes of viral fusiogenic membrane glycoproteins.

Fusion Protein	Characteristic Features	Examples
Class I	• trimeric in pre-and postfusion conformation • synthesized as inactive precursor, proteolytic cleavage required for fusogenic activity • cleavage liberates the N-terminus of the fusion peptide • fusion triggered by low pH or receptor binding of an associated attachment protein/subunit • formation/extension of an α-helical coiled-coil propels the fusion peptide towards the target membrane • postfusion conformation contains a heptad repeat-derived six helix bundle core structure	Influenza HA [72,73,74] HIV Env [75,76,77,78,79,80] Paramyxovirus F [32,33,34,81] Ebola virus GP [82]
Class II	• dimeric prefusion conformation, forming an elongated, plate-like structure parallel to the viral envelope • prefusion structure stabilized by chaperone proteins • fusion loop buried in the dimer interface • fusion triggered by low pH, presenting the fusion loops towards the target membrane • reorganization into trimeric complexes	Flavivirus E [48,49,51] Alphavirus E1 [55,56,57,58]
Class III	• trimeric in pre- and postfusion conformation • tripod-shape organization, fusion loops positioned towards the viral envelope • fusion triggered by low pH, inducing swinging of the tripod legs towards the target membrane • postfusion structure resembles six-helix bundle of class I proteins, but lacks heptad repeat domains	Rhabdovirus G [62,63] Herpes virus gB [59,60] Baculovirus gp64 [61]