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CHAPTER 3

Structure and Working of Viral Fusion Machinery

Aurélie Albertini, Stéphane Bressanelli, Jean Lepault and Yves Gaudin
Centre de Recherche de Gif, Laboratoire de Virologie Moléculaire et Structurale, CNRS (UPR 3296), Gif sur Yvette, France

I. OVERVIEW

The entry of enveloped viruses into cells requires the fusion of viral and cellular membranes, driven by conformational changes in viral glycoproteins. Structural studies have defined three classes of viral membrane fusion proteins. Despite their different structural organizations, all seem to have a common mechanism of action that generates the same lipid organizations during the fusion pathway. In this chapter, the structures of the best characterized viral fusion glycoproteins of the three classes will be presented. We will focus on the interactions between fusion glycoproteins and membranes, presenting

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evidences for the existence of an extended intermediate conformation exposing the fusion peptides at the top of the molecule. We will show that the fusion peptides cannot penetrate deeply inside the membrane and that their rather interfacial interaction with the lipid bilayer is probably sufficient to initiate the fusion mechanism by facilitating the formation of stalk precursors. We will also review the regulatory mechanisms that allow fusion to be triggered at the right time and right place. Finally, many studies have demonstrated that fusion involves the cooperative action of a large number of fusion glycoproteins. We will discuss new data that have shown that the glycoproteins located outside the contact zone between virions and liposomes reorganize into regular arrays that have been proposed to drive the late stages of the fusion reaction.

II. INTRODUCTION

Entry of enveloped viruses into host cells requires binding of the virus to one or more receptors present at the cell surface, followed by fusion of the viral envelope with a cellular membrane. These steps are mediated by virally encoded glycoproteins that promote both receptor recognition and membrane fusion. The membrane fusion process involves large structural rearrangements of the fusogenic glycoproteins upon interaction with specific triggers (e.g., low pH environment and cellular receptors). These conformational changes result in the exposure of hydrophobic motifs (the so-called fusion peptides or fusion loops) that then interact with one or both of the participating membranes, resulting in their destabilization and merger. Triggering of the conformational change in the absence of a target membrane leads to inactivation of the fusion properties of the fusogenic glycoprotein.

Three different classes of viral fusion proteins have been hitherto identified based on their common postfusion structural motifs (see below). However, experimental data suggest that the membrane fusion pathway is very similar for all the enveloped viruses studied so far whatever the organization of their fusion machinery (Chernomordik, Frolov, Leikina, Bronk, & Zimmerberg, 1998; Chernomordik, Leikina, Frolov, Bronk, & Zimmerberg, J., 1997; Gaudin, 2000; Zaitseva, Mittal, Griffin, & Chernomordik, 2005). It is generally assumed that fusion proceeds via the formation of an intermediate stalk that is a local lipidic connection between the outer leaflets of the fusing membranes. Radial expansion of the stalk would induce the formation of a transient hemifusion diaphragm (i.e., a local bilayer made by the two initial inner leaflets) in which the formation of a pore and its enlargement would lead to complete fusion (Chernomordik & Kozlov, 2005, 2008; Chernomordik, Kozlov, & Zimmerberg, 1995). Even if fusion of two lipid bilayers is thermodynamically favorable, there are energetic barriers that have to be overcome at different steps of the process,

the highest one being apparently encountered during the expansion of the initial fusion pore (Cohen & Melikyan, 2004). It has been proposed that the energy released during the favorable fusogenic structural transition is used to overcome these different barriers (Carr, Chaudhry, & Kim, 1997; Harrison, 2008).

In this chapter, the structures of the best characterized viral fusion glycoproteins of the three classes will be described, putting the emphasis on their similarities and their common mechanisms of action. Data that indicate the existence of an extended intermediate conformation, exposing the fusion peptides at the head of the molecule, will be presented. We will also discuss data from recent studies showing that fusion peptides cannot penetrate deeply inside the membrane and that their interfacial interaction with the lipid bilayer is probably sufficient to initiate the fusion mechanism by facilitating the formation of stalk precursors.

We will also review the regulatory mechanisms that allow fusion to be triggered at the right time and right place. We will particularly review the literature showing that viruses for which fusion is triggered by low pH have the ability to protect their fusion protein from undergoing irreversible conformational change and to avoid undesirable fusion reactions during their transport through the acidic compartments of the Golgi apparatus.

Finally, many studies have indicated that fusion involves the cooperative action of a large number of fusion glycoproteins. We will discuss new data showing that the glycoproteins located outside the contact zone between virion and liposomes reorganize into regular arrays that have been proposed to drive the late stages of the fusion reaction.

III. STRUCTURES OF FUSION PROTEINS

A. Class I Fusion Proteins

1. Influenza Hemagglutinin

The first crystal structure of a viral fusion protein ectodomain that has been determined is that of influenza virus hemagglutinin (HA) in its prefusion conformation (Wilson, Skehel, & Wiley, 1981) (Figure 1A, left). HA is synthesized as HA0, a precursor molecule cleaved by a cellular protease to produce HA1, a sialic acid binding domain, and HA2, a membrane-anchored fusion domain (Stieneke-Grober et al., 1992). HA0 cleavage primes the protein for the low pH-induced fusogenic conformational change.

HA is a homotrimer, each protomer consisting of an HA1 and an HA2 chain connected through a single disulfide bridge. Most of HA2 forms a fibrous triple coiled coil and HA1 is tethered to the top of this coiled coil and contains the major antigenic sites. The amino-terminal extremity of HA2, which has been generated by cleavage of HA0, is highly hydrophobic and constitutes the fusion

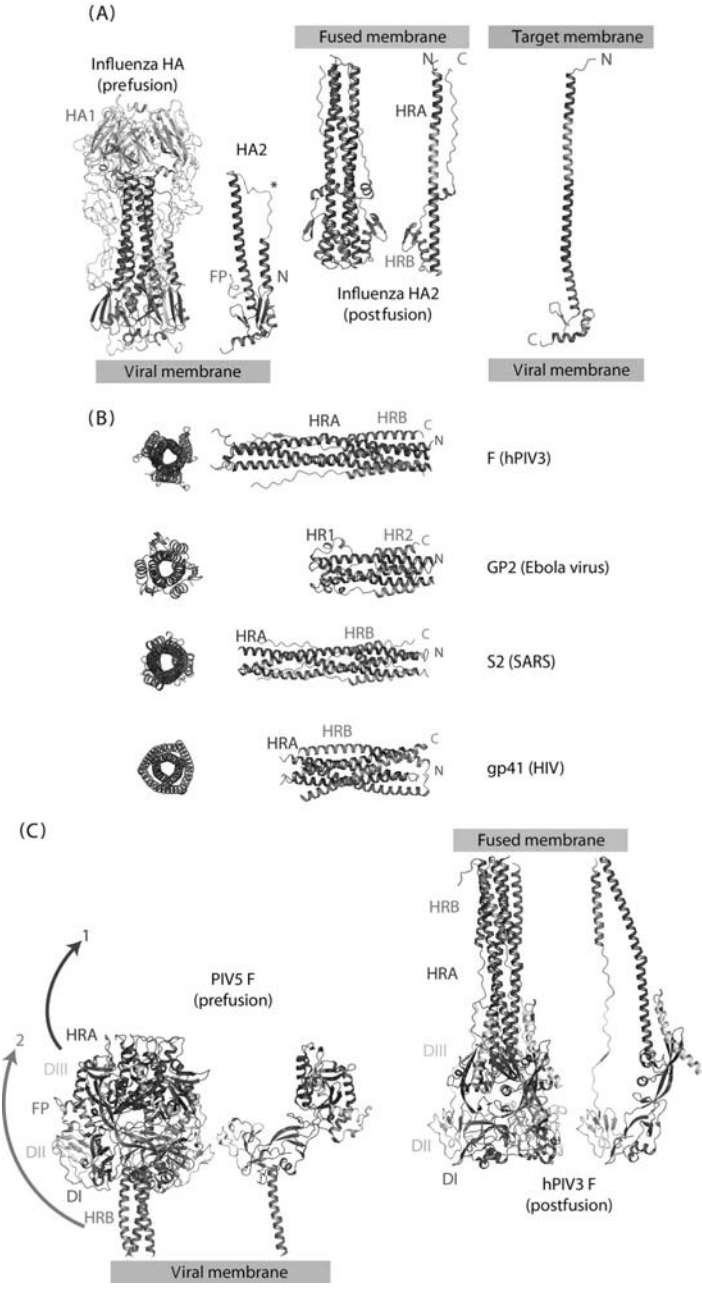

FIGURE 1 Class I fusion proteins. (A) Influenza virus hemagglutinin (HA) structures. *Left part:* Ribbon diagram of hemagglutinin trimer and HA2 protomer at neutral pH (pre-fusion

peptide. In the prefusion structure, it is buried along the threefold axis (Figure 1A, left).

When exposed to low pH (between pH 5 and 6, depending on the viral strain), HA undergoes a large-scale conformational change, the extent of which was visualized only in 1994 when the postfusion conformation of HA2 was crystallized (Bullough, Hughson, Skehel, & Wiley, 1994) (Figure 1A, middle). Low pH treatment results in the loss of HA1–HA2 interactions and dissociation of the HA1–HA1 interface stabilizing the prefusion structure. After the transition, the structure of HA1 is not significantly modified as it is still able to bind the viral receptor (Sauter et al., 1992) and is still recognized by monoclonal antibodies (Bizebard et al., 1995). In fact, HA low-pH-induced conformational change is essentially a dramatic refolding of HA2 (Figure 1A, middle). During this refolding, a long loop in native HA2 converts to a helix, resulting in length increase of the central coiled coil at its N-terminal end. This movement translocates the fusion peptide toward the head of the molecule, that is, toward the target membrane. At this stage, HA is in an extended intermediate conformation (Figure 1A, right). The structural transition is completed by a relocation of the carboxyterminal membrane anchor through a foldback mechanism to the amino-terminal end of the rod-shaped molecule. This results in the so-called

conformation) (Wilson et al., 1981). The receptor binding domain (HA1) subunits are colored in grey and the HA2 fusion proteins are colored in blue and red, the fusion peptide (FP) is depicted in green. *Middle part:* Ribbon diagram of HA2 trimer and protomer in the post-fusion conformation (Chen, Skehel, & Wiley, 1999). The central structure formed by HA2 folds into a long trimeric coiled-coil positioning the fusion peptides (absent in the crystalline structure) near the C-terminus of the molecule linked to the transmembrane anchor. *Right part:* Putative extended intermediate state adopted by HA2 during the structural transition. The cyan loop marked by an asterisk on the neutral HA2 protomer refolds into a helix resulting in a length increase of the central trimeric coiled-coil. This loop-helix transition translocates the fusion peptide toward the target membrane. (B) Trimeric hairpins structures of several class I viral fusion proteins. Side and top views of the trimeric core of the post-fusion structures from class I fusion proteins of four different viruses: hPIV3 F (Yin et al., 2006), Ebola virus GP2 (Weissenhorn et al., 1998), SRAS S2 (Supekar et al., 2004) and HIV gp41 (Weissenhorn et al., 1997). The N-terminal coiled-coil core (HRA or HR1 regions) is colored in blue and the C-terminal domain (HRB and HR2 regions) is in red. In all structures, the trimer-of-hairpins conformation brings the N- and C- termini (corresponding to the position of the fusion peptides and transmembrane domains) into close proximity. (C) Paramyxovirus F structures. Pre-fusion structure of parainfluenza virus 5 (PIV5) F (left part) and post-fusion structure of human parainfluenza virus 3 (hPIV3) F (right part) (Yin et al., 2005; Yin et al., 2006). For each conformation, trimer and protomer are presented. Domains I (DI), II (DII) and III (DIII), heptad repeats A (HRA) and B (HRB) are shown, hydrophobic fusion peptides (FP) are in green. The two conformations are related by flipping the fusion peptide and the C-terminal domain around a block made of DI and DII that do not change during the structural transition. HRA segments undergo a major reorganization that projects the hydrophobic fusion peptide toward the target membrane (movement illustrated by the blue arrow 1), then the HRB segments dissociate and turn around the globular head to form the post fusion structure (movement illustrated by red arrow 2). (See Color Insert.)

hairpin conformation in which the transmembrane segment and the fusion peptide are in close proximity at the same end of the elongated molecule (Figure 1A, middle).

2. Class I Structural Motif

The structural motif, found in the postfusion structure of HA2, is encountered in the postfusion structure of many other viral fusion proteins including those of retroviruses (Buzon et al., 2010; Weissenhorn, Dessen, Harrison, Skehel, & Wiley, 1997), paramyxoviruses (Baker, Dutch, Lamb, & Jardetzky, 1999; Yin, Paterson, Wen, Lamb, & Jardetzky, 2005), filoviruses (Weissenhorn, Carfi, Lee, Skehel, & Wiley, 1998), and coronaviruses (Duquerroy, Vigouroux, Rottier, Rey, & Bosch, 2005; Supekar et al., 2004; Xu et al., 2004) (Figure 1B). All these glycoproteins show a trimeric coiled coil at the N-termini of which are displayed the fusion peptides and against which are packed, in an antiparallel manner, the segments abutting the transmembrane (TM) domains. This motif has thus served to define the class I of fusion proteins. Most of the class I fusion proteins have also an internal, frequently furin-like, cleavage site located upstream a stretch of about 20 hydrophobic amino acids that will constitute the fusion peptide. Class I signature is the presence of a first heptad repeat region (named HRA or HR1) that is immediately downstream the fusion peptide and forms the central trimeric coiled coil. It is often (but not always) accompanied by a second one (named HRB or HR2) located in the C-terminal part of the ectodomain that constitutes the helices positioned, in an antiparallel manner, in the groove of the central coiled coil (Figure 1B).

Except for this common structural motif, the structure of the proteins are very different, as illustrated by the recent determination of the structure of the prefusion state of parainfluenza virus type 5 fusion protein (PIV5 F) (Yin, Wen, Paterson, Lamb, & Jardetzky, 2006) (Figure 1C) and that of GP of Ebola virus (Lee et al., 2008). The issue concerning whether there is a common origin to all these proteins or if they represent convergent solutions to developing a membrane fusion machinery is still an open question.

Furthermore, the mechanisms for activation of different class I viral fusion proteins are distinct. For many of them, activation is not dependent on low pH environment. Most of retroviruses env proteins and paramyxovirus F are activated by receptor binding events. This activation can be by direct interactions (HIV env) (Melikyan, 2008) or indirectly through a viral attachment protein (paramyxovirus F) (Lamb & Jardetzky, 2007). Finally, although Ebola virus enters cell by the endocytic pathway, this probably reflects a need for glycoprotein GP processing by lysosomal proteases, such as cathepsins, to trigger GP-mediated fusion, rather than simply low pH itself (Chandran, Sullivan, Felbor, Whelan, & Cunningham, 2005; Schornberg et al., 2006).

3. Structure of Paramyxovirus Fusion Protein

Structural studies of the paramyxovirus fusion proteins provided new insight into the molecular organization of a class I fusion machinery for which the folding and the mode of activation are very different from that of influenza virus. For paramyxoviruses, two viral glycoproteins are involved in cell entry. The first one, called HN, H, or G, depending on the virus, is an attachment protein. The second is the fusion protein F.

The structures of prefusion PIV5 F (Yin et al., 2006) and postfusion Newcastle disease virus (Swanson et al., 2010) and human PIV3 F proteins (Yin et al., 2005) have been solved (Figure 1C). F is a trimer that is proteolytically cleaved to liberate the N terminus of the fusion peptide (Lamb & Jardetzky, 2007). It is worth noting that in absence of its transmembrane segment, soluble F ectodomain can spontaneously convert into its postfusion conformation even in absence of the cleavage that primes the full-length glycoprotein for subsequent conformational change (Yin et al., 2005). Thus, to replace the missing transmembrane domain and the cytoplasmic tail and to stabilize the prefusion form, the trimeric GCN4 derivative was placed in register with C-terminal HRB of the secreted PIV5 F protein ectodomain (Yin et al., 2006).

After triggering, region HRA, adjacent to the fusion peptide and initially collapsed in a compact set of 11 small segments, undergoes a major conformational change that extends the fusion peptide outward for insertion into the target membrane (Figure 1C) (Yin et al., 2006). After this process, involving the refolding of the 11 segments into a single, extended α-helix, F forms an extended intermediate with the fusion peptide exposed at the top of the molecule. Region HRB, adjacent to the transmembrane domain, then translocates to bind to HRA, forming a six-helix bundle and the postfusion hairpin conformation.

How interaction of the attachment protein to the viral receptor promotes F conformational change is still a matter of debate. Two models have been proposed. On the one hand, the attachment protein may clamp and stabilize F in its prefusion state until the attachment protein binds the target cell (the ''clamp model'') (Aguilar et al., 2006; Bishop et al., 2007; Corey & Iorio, 2009; Plemper, Hammond, Gerlier, Fielding, & Cattaneo, 2002). On the other hand, the attachment protein itself may undergo a conformational change after receptor binding that destabilizes F and triggers its conformational change (the ''provocateur model'') (Connolly, Leser, Jardetzky, & Lamb, 2009).

The structures of several paramyxovirus receptor binding proteins have been resolved (Bowden, Aricescu, et al., 2008; Bowden, Crispin, et al., 2008; Bowden, Crispin, Harvey, Jones, & Stuart, 2010; Colf, Juo, & Garcia, 2007; Crennell, Takimoto, Portner, & Taylor, 2000; Lawrence et al., 2004; Hashiguchi et al., 2007; Xu et al., 2008; Yuan et al., 2005). Their structures are very similar,

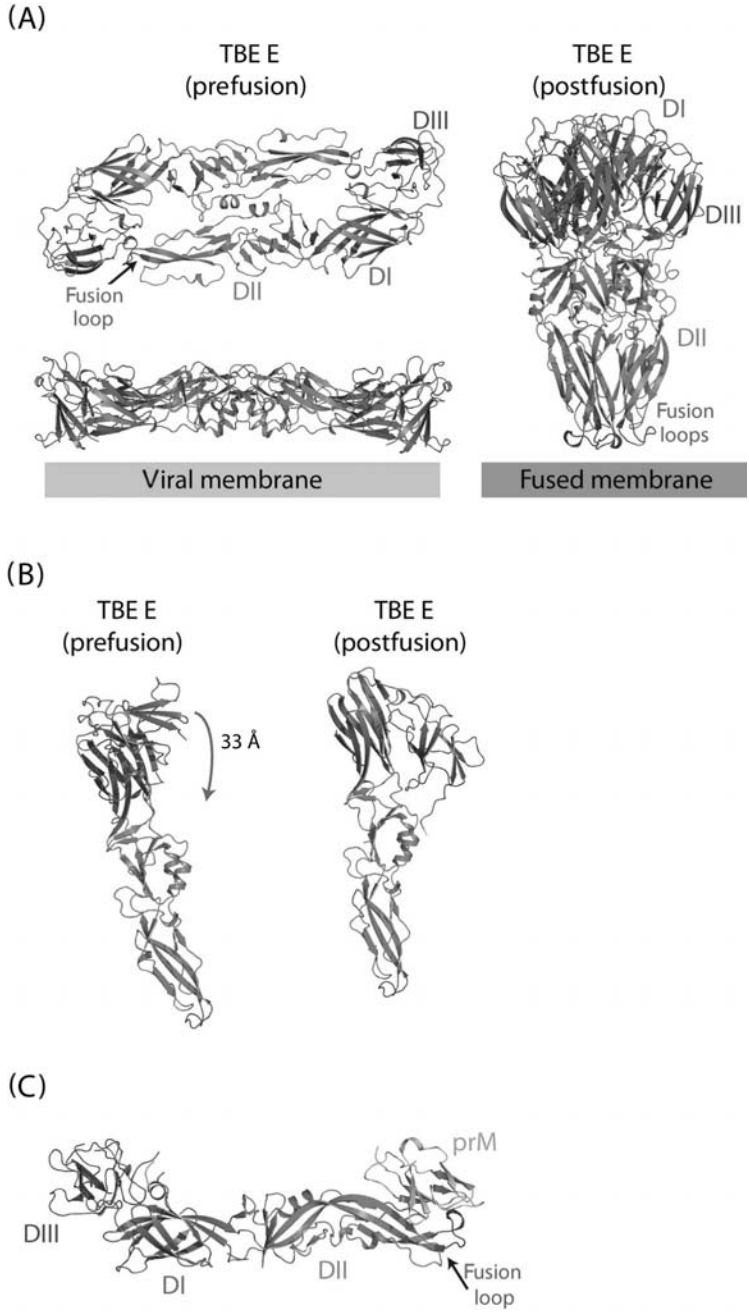

FIGURE 2 Class II fusion proteins. (A) TBEV glycoprotein E structures. Ribbon diagram of the pre-fusion E dimer (Rey et al., 1995) (left part) and post-fusion E trimer (Bressanelli et al., 2004)

suggesting that they trigger F conformational change through similar mechanisms. For some of these proteins, the structure of both the native free form and the receptor bound form has been solved without revealing any major change in the structure of the protein (Bowden, Aricescu, et al., 2008; Bowden, Crispin, et al., 2008; Xu et al., 2008; Yuan et al., 2005). It has to be noted that the stalk, connecting the head of the receptor binding protein to the transmembrane domain, was not resolved in any of the crystal structures. Multiple studies, however, have mapped the F-interacting region to the stalk of the receptor binding protein (Bishop et al., 2008; Bousse, Takimoto, Gorman, Takahashi, & Portner, 1994; Corey & Iorio, 2007; Porotto, Murrell, Greengard, & Moscona, 2003; Stone-Hulslander & Morrison, 1999).

B. Class II Fusion Proteins

The second class of fusion proteins was identified when the X-ray prefusion structure of the ectodomain of the fusion protein E1 of Semliki Forest virus (SFV) (Lescar et al., 2001), an alphavirus, was shown to have a remarkably similar secondary and tertiary structure to that of the fusion protein E of tick-borne encephalitis virus (TBEV) (Rey, Heinz, Mandl, Kunz, & Harrison, 1995), a flavivirus (Figure 2A). This indicated that the genes encoding these proteins were derived from a common ancestor. The alphaviruses and flaviviruses are members of the Togaviridae and Flaviviridae families, respectively, and other fusion proteins of viruses from these families, including West Nile virus E (Kanai et al., 2006; Nybakken, Nelson, Chen, Diamond, & Fremont, 2006), dengue virus E (Modis, Ogata, Clements, & Harrison, 2003), and hepatitis C virus E2 (Krey et al., 2010), have been demonstrated to belong to class II. It has also been proposed that the fusion glycoprotein of members of the Bunyaviridae family also belong to class II (Garry & Garry, 2004).

All class II fusion proteins identified so far fold cotranslationally with a chaperone protein, named p62 (or pE2) for alphaviruses (Wahlberg, Boere, & Garoff, 1989) and prM (promembrane protein) for flaviviruses (Heinz et al., 1994). This heterodimeric interaction is important for the correct folding and transport of the fusion protein (Sanchez-San Martin, Liu, & Kielian, 2009). The

(right part). Red yellow and blue part of the protein correspond to respectively the central domain (DI), the dimerization domain (DII) and the C-terminal domain (DIII); fusion loop is depicted in green and located at the tip of DII. (B) TBEV E protomer conforlmational change. Ribbon diagram of the pre- and post-fusion E protomer of TBEV. The arrow indicates the movement of domain III (connected to the transmembrane domain of the protein) toward the fusion loop. (C) Dengue virus precursor membrane-envelope protein complex (prM–E) structure. Ribbon diagram of the prM-E heterodimer (Li et al., 2008). The pr peptide, in light blue, protects the fusion loop in green. Domains of E are indicated and colored according to A. (See Color Insert.)

chaperone protein is cleaved by the cellular protease furin late in the secretory pathway (Stadler, Allison, Schalich, & Heinz, 1997; Zhang, Fugere, Day, & Kielian, 2003). The p62 is cleaved into E2 and E3 (Salminen, Wahlberg, Lobigs, Liljestrom, & Garoff, 1992; Wahlberg et al., 1989) and prM is cleaved into pr peptide and M protein (Heinz et al., 1994). This cleavage is crucial as it primes the protein, which is then in a metastable conformation, for subsequent low-pH-induced conformational change (Sanchez-San Martin, Liu, & Kielian, 2009). In their prefusion state anchored in the viral membrane, class II fusion proteins are organized following the icosahedral symmetry (Kuhn et al., 2002; Lescar et al., 2001).

The polypeptide chain of the class II proteins is folded into three domains essentially constituted by β-sheet (Lescar et al., 2001; Modis et al., 2003; Rey et al., 1995). The C terminus part of the ectodomain, which is linked to the transmembrane segment, and the internal fusion loop are found at the two ends of an extended molecule. The fusion loop is located at the tip of an elongated beta sheet. In the prefusion state, it is buried at the E1–E2 interface for alphaviruses and at the E homodimer interface for flaviviruses (Figure 2A).

Low pH exposure, in presence of a target membrane, triggers a complete rearrangement of the proteins (Bressanelli et al., 2004; Gibbons et al., 2004; Modis, Ogata, Clements, & Harrison, 2004). First, the alphavirus E2–E1 heterodimers or the flavivirus E–E homodimers dissociate and then reassociate to form E1 or E homotrimers (Figure 2A). The conformational change is less impressive than that observed for HA or for paramyxovirus F protein as there is essentially no change in secondary structure. Nevertheless, the interactions between the domains constituting the protomer are markedly different in the pre- and postfusion conformations. Particularly, domain III, which is connected to the C-terminal part of the molecule, moves by about 35 Å toward the fusion loop (Figure 2B). The movement of domain III redirects the polypeptide chain, so that the transmembrane segment is located near the fusion loop region in the postfusion trimeric form. Despite the different architecture of the proteins, the postfusion structure is thus reminiscent of the postfusion hairpin structure of class I fusion proteins.

A particularity of class II fusion proteins is that the presence of a target membrane catalyzes the formation of the postfusion trimer. In the absence of a membrane, soluble E ectodomain dimers dissociate at low pH but redimerize if the pH is returned to neutral (Stiasny, Allison, Marchler-Bauer, Kunz, & Heinz, 1996). Furthermore, several class II fusion proteins require the presence of specific lipids in the target membrane to achieve membrane fusion. SFV and Sindbis virus-induced fusion is strictly dependent on the presence of both cholesterol (Kielian & Helenius, 1984) and sphingolipids (Nieva, Bron, Corver, & Wilschut, 1994; Smit, Bittman, & Wilschut, 1999). Cholesterol does not seem to be absolutely required for flavivirus-induced membrane fusion (Umashankar et al., 2008) although its presence may facilitate the process for

the hepatitis C virus glycoprotein (Lavillette et al., 2006) and strongly promotes West Nile virus fusion activity (Moesker, Rodenhuis-Zybert, Meijerhof, Wilschut, & Smit, 2010). Finally, effective dengue virus fusion to plasma and intracellular membranes, as well as to protein-free liposomes, requires the target membrane to contain anionic lipids such as bis(monoacylglycero)phosphate and phosphatidylserine (Zaitseva, Yang, Melikov, Pourmal, & Chernomordik, 2010).

C. Class III Fusion Proteins

The third class of fusion proteins was identified when the structures of the ectodomains of the fusogenic proteins G (G) of vesicular stomatitis virus (VSV) (Roche, Bressanelli, Rey, & Gaudin, 2006) and glycoprotein B (gB) of herpes simplex virus 1 were determined (Heldwein et al., 2006) (Figure 3). This revealed an unanticipated homology between the two proteins that belong to a negative-strand RNA and a DNA virus, respectively, and for which no sequence similarity was previously identified. Epstein Barr virus (EBV) gB (Backovic, Longnecker, & Jardetzky, 2009) and baculovirus gp64 (Kadlec, Loureiro, Abrescia, Stuart, & Jones, 2008), of which the structures were further determined, have been also demonstrated to belong to this new class (Figure 3B). Alignment of the amino acid sequences of G proteins from animal rhabdoviruses belonging to different genera also reveals that all of them share the same fold (Roche et al., 2006). The glycoprotein of Borna disease virus, another member of the Mononegavirales order, has been also proposed to belong to class III (Garry & Garry, 2009).

VSV G is the only class III fusion protein for which the structures of both the prefusion (Roche, Rey, Gaudin, & Bressanelli, 2007) and postfusion states (Roche et al., 2006) have been determined. The structures determined for other class III members are presumptive postfusion conformations, based on their structural similarity with the postfusion conformation of G.

The soluble ectodomain (G_{th}) of VSV, which has been crystallized, has been generated by limited proteolysis with thermolysin directly on purified virus, whereas the soluble form of HSV1 gB, EBV gB, and baculovirus gp64 were obtained through heterologous expression system.

The polypeptide chain of G_{th} folds into four distinct domains: a β-sheet-rich lateral domain (domain I), a central domain that is involved in the trimerization of the molecule (domain II), a pleckstrin homology domain (domain III), and the fusion domain (domain IV) (Figure 3A). The organization of class III fusion domain is similar (although not homologous) to that of class II fusion proteins. It contains an extended β-sheet structure at the tip of which are located two loops that constitute the membrane-interacting motif of the G ectodomain.

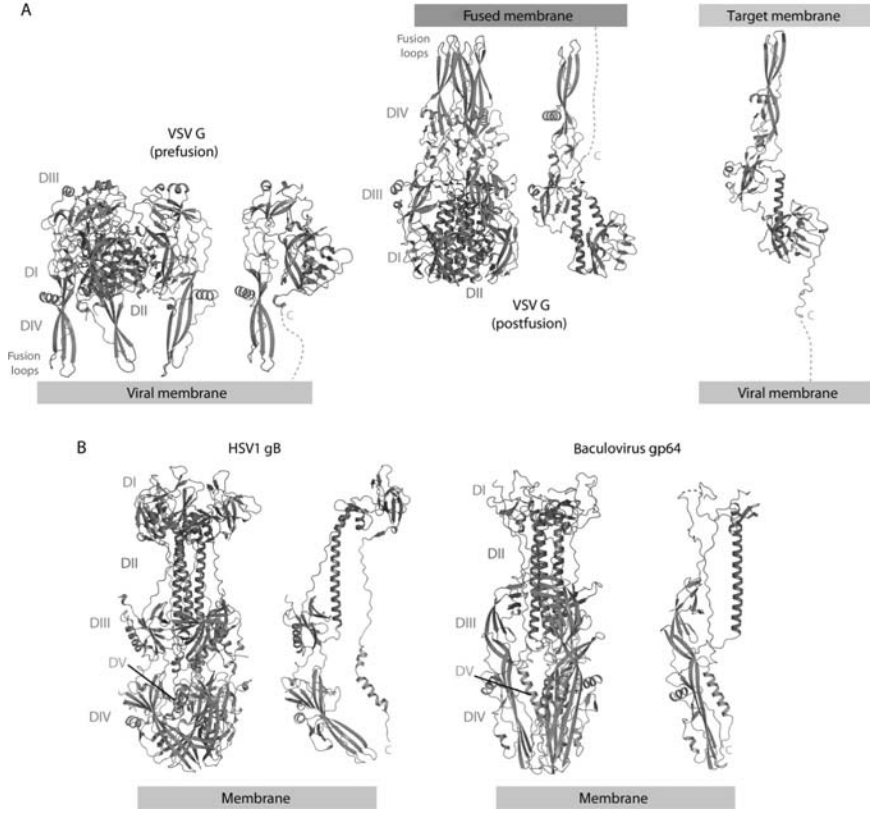

FIGURE 3 Class III fusion proteins. (A) Overall structures of the pre- and post-fusion forms of VSV glycoprotein. *Left part:* Ribbon diagram of the G pre-fusion trimer and protomer (Roche et al., 2007). *Middle part:* Ribbon diagrams of the G post-fusion trimer and protomer. During the structural transition from pre- to post-fusion conformation fusion domain and C-terminal domain turn around a rigid block made of DI and DII. The two conformations are thus related by flipping the fusion peptide and the C-terminal domain around this rigid block (Roche et al., 2007). *Right part:* Putative elongated monomeric conformation adopted by G during the structural transition. G is colored by domains: DI is the lateral domain (red), DII is the trimerization domain (blue), DIII is the pleckstrin homology domain (orange) and DIV is the fusion domain (yellow) fusion loops are in green. The C-terminus segment reaching the trans-membrane domain (not observed in the x-ray structures) is represented as a magenta dashed line. B. Overall structures of presumptive post-fusion states of HSV1-gb and baculovirus gp64. Left part: ribbon diagram of HSV1 trimer and protomer (Heldwein et al., 2006). Right part: ribbon diagram of baculovirus gp64 trimer and protomer (Kadlec et al., 2008). Domains on both proteins are colored according to their homologous counterparts of VSV G. The domain DV (not visible in the crystal structures of VSV G) is in magenta. (See Color Insert.)

In the prefusion structure, in striking contrast to class I and class II viral fusion proteins, the fusion loops are not buried at an oligomeric interface, but point toward the viral membrane (Figure 3A, left). It is therefore possible that in the prefusion conformation, the fusion loops also interact with the viral membrane.

After the end of the trimerization domain, there remain 40 amino acids for the polypeptide chain to reach G transmembrane domain, but the structure of this segment is unknown for both the pre- and the postfusion conformation. This missing part corresponds to the C-terminal segment of domain V of gB and gp64 that forms a α-helical structure that inserts between the other two protomers within the trimer (Backovic et al., 2009; Heldwein et al., 2006; Kadlec et al., 2008).

G_{th} structures revealed that the conformational change from pre- to postfusion state involves a dramatic reorganization of the glycoprotein (Figure 3A, middle). During the structural transition, domains I, III, and IV retain their tertiary structure but undergo large rearrangements in their relative orientation. This is due to both secondary structure modifications occurring in the hinge regions between the pleckstrin homology domain (domain III) and the fusion domain (domain IV) and complete refolding of the trimerization domain.

Global refolding of G exhibits striking similarities to that of class I proteins, such as paramyxovirus fusion protein F and influenza HA. The pre- and postfusion states of a protomer are related by flipping both the fusion domain and a C-terminal segment relative to a rigid block made by domain I and part of domain II. During this movement, both the fusion loops and the TM domain move $\sim$160 Å from one end of the molecule to the other. The fusion domain is projected toward the membrane through the combination of two movements: a rotation around the hinge between the pleckstrin homology domain and the fusion domain and the lengthening of the central helix (involved in the formation of the trimeric central core of the postfusion conformation). The movement of the transmembrane domain is due to the refolding of the C-terminal segment into an α-helix. In the trimeric postfusion state, the three C-terminal helices position themselves into the groove of the trimeric central core in an antiparallel manner to form a six-helix bundle.

The resulting overall organization of the postfusion state is reminiscent of that of class I protein. As in class I proteins, the fusion domains are N-terminal to the central helices and the transmembrane domains are located at the C-terminus of the antiparallel outer helices.

D. Other Viral Fusion Proteins

There are viral fusion proteins that cannot be classified in one of the three previously described classes. As an example, the fusion machinery of poxviruses

(dsDNA viruses, of which the prototype is vaccinia virus) is poorly characterized. Genetic and biochemical studies have provided evidence for an entry/fusion complex, made of at least eight viral proteins, that takes part in entry of vaccinia virus into cells (Senkevich, Ojeda, Townsley, Nelson, & Moss, 2005).

1. Fusion Machinery of Herpesviruses

The fusion machinery of herpesviruses is more complex than that of other enveloped viruses. Although gB has the fold of a class III viral fusion protein (Figure 3B), it does not function alone. The conserved core fusion machinery consists of glycoproteins gB and the gH–gL heterodimer. Furthermore, binding of additional glycoproteins, such as gD in HSV-1 and gp42 in EBV, to the cellular receptors is essential for fusion to occur (Backovic & Jardetzky, 2009; Heldwein & Krummenacher, 2008).

In HSV-1, gD interacts with both gB and gH/gL and triggers formation of the core fusion machinery made of gB and gH/gL (Atanasiu et al., 2007). Recent data have suggested that gB may interact with target membranes prior to an interaction with gH/gL (Atanasiu, Saw, Cohen, & Eisenberg, 2010). The gB–gH/gL interaction seems to be required for the progression of fusion.

There are some conflicting accounts regarding whether gH/gL is a fusion protein. The gH/gL has been reported to be sufficient for induction of hemifusion in the presence of gD, consistent with the idea that gH/gL is a fusion protein (Subramanian & Geraghty, 2007). Two recent reports suggest that it is not the case (Atanasiu et al., 2010; Jackson & Longnecker, 2010) and that binding of gH/gL to gB rather triggers the conformational change of gB from prefusion to postfusion conformation. Consistent with this view, the crystal structure of the gH ectodomain bound to gL from HSV-2 has recently been determined. This complex has a unique architecture that does not resemble any known viral fusogen (Chowdary et al., 2010).

2. Fusion Proteins of Reoviruses

The nonenveloped avian, reptilian, and baboon reoviruses encode small fusion proteins (molecular weight ranging from 10 to 15 kDa) called FAST proteins (fusion-associated small transmembrane proteins) (Shmulevitz & Duncan, 2000). They are nonstructural proteins and thus are not involved in viral entry. They promote only syncytia formation. The small size and the lack of any homologues in previously characterized membrane fusion proteins indicate that the FAST proteins of reovirus represent a new class of membrane fusion proteins. Within the ectodomain of FAST proteins, an 11-residue sequence of apolar residues, termed the hydrophobic patch (HP), flanked by two highly conserved cysteines that form a disulfide bridge and create a cystine loop, constitutes a new class of fusion peptide (Barry, Key, Haddad, & Duncan, 2010). Remarkably, membrane curvature agents that inhibit hemifusion or

promote pore formation mediated by other fusion proteins have no effect on FAST-induced cell–cell fusion, indicating that FAST-mediated fusion reaction may somehow differ from other virus-induced fusion reactions (Clancy, Barry, Ciechonska, & Duncan, 2010).

IV. REGULATION OF THE CONFORMATIONAL CHANGE

A. pH Sensitive Molecular Switches

The structures of viral fusion glycoproteins, of which the conformational change is triggered at low pH, has allowed the identification of amino acid residues that play the role of pH-sensitive molecular switches.

For TBEV, the initiation of fusion is crucially dependent on the protonation of one conserved histidine located at the interface between domains I and III of E. Its protonation results into the dissolution of domain interactions and to the exposure of the fusion peptide (Fritz, Stiasny, & Heinz, 2008). A similar study performed on SFV has also identified a histidine residue that acts to regulate the low-pH-dependent refolding of E1 (Qin, Zheng, & Kielian, 2009).

In the case of VSV, it was known that modification of G histidines with diethylpyrocarbonate inhibits its fusion properties (Carneiro et al., 2003). In the prefusion state, three conserved histidines (H60 and H162, both located in the fusion domain, and H407, located in the C-terminal segment of the protein) cluster together (Roche et al., 2007). Protonation of these residues at low pH is likely to destabilize the interaction between the C-terminal segment of G_{th} and the fusion domain in the prefusion conformation. This might trigger the movement of the fusion domain toward the target membrane.

Conversely, in the postfusion form, a large number of acidic amino acids are brought close together in the six-helix bundle (Roche et al., 2006). In the postfusion state, these residues are protonated and form hydrogen bonds. The deprotonation of these residues at higher pH induces strong repulsive forces that destabilize the trimer and trigger the conformational change back to the prefusion state. These residues are buried in the postfusion state, but are solvent-exposed in the prefusion state (Roche et al., 2006, 2007). As a consequence, they have a pK_a that is much higher in the postfusion than in the prefusion conformation. This stronger affinity of the postfusion state for the protons explains the cooperativity of rhabdoviral structural transition as a function of pH (Roche & Gaudin, 2002).

In the case of influenza HA, mutations at distinct positions affect the stability of trimer interfaces, that break during the rearrangement, and therefore alter the threshold pH for fusion (Daniels et al., 1985), and no specific amino acid residue has been identified, of which the protonation would trigger HA conformational change.

B. Dealing with Low pH During Transport Through the Golgi Apparatus

During their maturation and transport through the Golgi apparatus, fusion proteins encounter acidic compartments. It appears that viruses for which fusion is triggered by low pH have the ability to protect their fusion protein from undergoing irreversible conformational change and to avoid undesirable fusion reactions.

For influenza virus, the pH of the transport vesicles is regulated by the M2 protein (Sugrue et al., 1990), a proton channel (Pinto, Holsinger, & Lamb, 1992). Furthermore, the activating cleavage of HA0 into HA1 and HA2 occurs at a late stage of transport (Stieneke-Grober et al., 1992) or after viral budding in the extracellular medium (Skehel & Wiley, 2000).

As mentioned above, class II fusion proteins are transported in association with a chaperone protein. The cleavage of this accompanying protein is required to promote viral fusion under biological conditions. The recent structure of the immature dengue virus at low pH (Yu et al., 2008) has explained how this virus, which buds into the ER, avoids undesirable fusion in the acidic compartments of the Golgi. Immature particles bud into ER as spiky virions with 60 icosahedrally arranged protrusions, each consisting of a trimer of prM–E heterodimers (Y. Zhang et al., 2003b). The atomic structure of dengue virus prM–E heterodimer has been determined (Figure 2C) and fits well into the cryo-electron microscopy density of immature virus (Li et al., 2008). The immature particles are transported into the trans-Golgi network (TGN), where the low pH environment induces a conformational change in the virion, resulting in particles having a much smoother surface with E proteins arranged in a herringbone pattern. This structural transition exposes the furin cleavage site, and proteolysis of prM into pr peptide and M takes place in the TGN. After cleavage, pr peptide remains stably associated with the virion. The pr peptide stabilizes the metastable E dimer, preventing its dissociation and the formation of the postfusion homotrimers. It also covers the fusion loop in E, preventing interaction with host cell membranes. In fact, the dissociation of pr from the virion is pH dependent and pr dissociates only when the virion is released into the slightly alkaline extracellular medium. This definitively primes the metastable E dimer for subsequent low-pH-induced fusogenic conformational change.

For alphaviruses, the recently determined structures of E1 in complex with p62 (Voss et al., 2010), mature E1–E2–E3 glycoprotein complexes (Voss et al., 2010), and trimeric E1–E2 spikes crystallized at low pH (Li, Jose, Xiang, Kuhn, & Rossmann, 2010) have also provided a detailed picture of the maturation of the viral glycoproteins during transport and of the finely tuned regulation of their conformational changes.

For rhabdoviruses such as VSV or rabies virus, the native, prefusion conformation is not metastable, and thus the low-pH-induced structural transition is

reversible. In fact, there is a pH-dependent equilibrium between the different conformations of G (Roche & Gaudin, 2002). This allows G to be transported through the acidic compartment of the Golgi apparatus and to recover its native prefusion state at the viral surface (Gaudin, Tuffereau, Durrer, Flamand, & Ruigrok, 1995).

V. WORKING OF THE FUSION MACHINERY

A. Interaction Between the Fusion Peptides and Membranes

Interactions between not only fusion peptides and target membranes but also with viral membranes have been demonstrated for both rhabdoviruses and influenza virus using hydrophobic photolabeling (Durrer, Gaudin, Ruigrok, Graf, & Brunner, 1995; Stegmann, Delfino, Richards, & Helenius, 1991; Tsurudome et al., 1992).

The low pH structures of TBE virus E, dengue fever virus E and SFV E1 (class II), and VSV G (class III) indicate that only hydrophobic side chains of the loops insert into the hydrocarbon chains of the outer leaflet of a target membrane. Any deep penetration of the fusion domain inside the membrane is precluded by the presence of charged residues and polar groups in the vicinity of the fusion loops (Gibbons et al., 2004; Modis et al., 2004; Roche et al., 2006). Residues located in the loops have been shown to be essential to the fusion process by directed mutagenesis experiments on both VSV G (Fredericksen & Whitt, 1995; Sun, Belouzard, & Whittaker, 2007) and herpes virus gB (Backovic, Jardetzky, & Longnecker, 2007; Hannah, Heldwein, Bender, Cohen, & Eisenberg, 2007).

Remarkably, all these fusion proteins have at least one polar aromatic residue in their fusion loops, a feature that is conserved across the rhabdovirus family. Tyrosines and tryptophans are residues that often position themselves at the interface between the fatty acid chains and the polar head group layers of lipids (Wimley & White, 1992). Such an interfacial interaction involving only a few residues cannot create a strong point of anchoring that can be used to pull the target membrane toward the viral one. Most probably, by perturbating the outer leaflet of the target bilayer, it facilitates the formation of point-like protrusions (Campelo, McMahon, & Kozlov, 2008) that have been proposed to be stalk precursors (Efrat, Chernomordik, & Kozlov, 2007). In addition, membrane deformation is probably facilitated by the large number of glycoproteins at the viral surface and their oligomeric status, allowing multiple fusion loops to interact with the external leaflet of the target bilayer.

As for class I protein, the structure of the amino-terminal fusion peptide of HA2 has been determined in a membrane mimicking environment by

combining NMR and EPR spectroscopies (Han, Bushweller, Cafiso, & Tamm, 2001). At both pH 7.4 and 5, the domain is a kinked, predominantly helical amphipathic structure. Nevertheless, there are slight differences between both structures that may explain a deeper insertion of the fusion domain into the core of the lipid bilayer at low pH. Nevertheless, here again, in the proposed model of insertion, HA fusion peptides do not penetrate deep into the bilayer structure but rather induce local membrane curvature similar to class II and class III fusion loops. Finally, some class I proteins seem to have also internal fusion loops. This is the case for Ebola virus Gp2 whose fusion loop could expose a conserved WIPYF sequence at its tip (Weissenhorn et al., 1998).

Finally, as mentioned above, in G_{th} prefusion conformation, the fusion loops point toward the viral membrane. Whether they interact with it remains an intriguing question. Indeed, a direct destabilization of the viral bilayer could facilitate the initiation of the fusion process.

B. Other Putative Membrane Destabilizing Domains

Besides the fusion peptides or fusion loops, two other domains have been postulated to play a major role in the fusion process. The first one is the TM domain. Replacement of the TM domain of influenza HA or VSV G by a glycerophosphatidylinosotol (GPI) anchor results in the loss of the fusion properties (Kemble, Danieli, & White, 1994; Odell, Wanas, Yan, & Ghosh, 1997). In the case of GPI-anchored HA, fusion is blocked at an early stage (Kemble et al., 1994). In fact, it appears that GPI-anchored HA is able to open the initial fusion pore but is unable to expand it for complete membrane merger (Frolov, Cho, Bronk, Reese, & Zimmerberg 2000; Markosyan, Cohen, & Melikyan, 2000; Nüssler, Clague, Herrmann, 1997).

It has also been demonstrated that the transmembrane domain of influenza HA exhibits a stringent length requirement to support the hemifusion to fusion transition and that it must span the bilayer to promote full fusion (Armstrong, Kushnir, & White, 2000). Furthermore, mutations of some transmembrane residues of many fusion proteins have been shown to affect fusion (Cleverley & Lenard, 1998; Helseth et al., 1990; Melikyan, Markosyan, Roth, & Cohen, 2000). Mutagenesis studies performed on VSV revealed a crucial role for glycine residues in the TM domain. Mutations of these residues block fusion at a hemifusion stage (Cleverley & Lenard, 1998).

From all these observations, it has been proposed that the TM domain acts downstream of the formation of the initial fusion pore and contributes to its expansion. Nevertheless, the precise role played by the TM domain at a molecular level is still far from being understood.

The second domain is the membrane-proximal domain. For many fusion proteins, this rather hydrophobic and frequently tryptophan-rich domain has been shown to play a role in the fusion reaction (Howard et al., 2008; Vishwanathan & Hunter, 2008).

For VSV, a deletion of the 13 membrane-proximal amino acids dramatically reduces cell–cell fusion activity and reduces virus infectivity approximately 100-fold (Jeetendra et al., 2003). However, mutations of conserved aromatic residues (W441, F442, and W445) indicated that these residues are not important for fusion (Jeetendra et al., 2003). The structure of this segment, absent in the crystal structure, is not known but in the case of rabies virus G and in agreement with the structure of other class III proteins, it has been proposed to adopt an α-helical conformation having a strong amphipathic signature (Maillard et al., 2003).

Similar data have been obtained with HIV for which NMR experiments suggest that the membrane-proximal segment could form a bent amphipathic helix lying at the surface of the membrane bilayer (Schibli, Montelaro, & Vogel, 2001; Sun et al., 2008). Finally, the recent crystalline structure of a gp41 fragment including both fusion peptide and membrane-proximal external regions confirms that three aromatic side chains (Trp 678, Trp 680, and Tyr 681) are exposed toward the membrane and well positioned to insert their side chains into the bilayer (Buzon et al., 2010). Whether these residues are anchored in the viral membrane only in the postfusion conformation or in both the pre- and postfusion conformations remains an open question.

C. Structural Intermediates During the Conformational Changes

1. The Extended Intermediate Conformation

All the data obtained are consistent with the formation of an extended intermediate conformation (Figure 1A, right; Figure 3A, right). In this conformation, the fusion peptides or fusion loops are exposed at the top of the molecule and directed toward the target membrane. This intermediate is formed early during the fusion process as demonstrated by hydrophobic photolabeling for rhabdoviruses and influenza virus (Durrer et al., 1995; Stegmann et al., 1991; Tsurudome et al., 1992).

This extended intermediate has been particularly studied in the case of gp41. For this intermediate, it is proposed that the central coiled coil (made of HR1 segments) is already formed but that the relocation of HR2 segments against this central core has not occurred. Indeed, peptides corresponding to the HR2 segment can inhibit the fusion process (Furuta, Wild, Weng, & Weiss, 1998; Wild, Shugars, Greenwell, McDanal, & Matthews, 1994). A similar inhibition has been observed for paramyxoviruses with peptides derived from the

carboxyterminal HRB region of F protein (Russell, Jardetzky, & Lamb, 2001). Such inhibitors do not function in the case of influenza HA. This is probably due to the shorter lifetime of HA extended conformation compared to that of gp41 (several minutes) (Melikyan et al., 2000; Munoz-Barroso, Durell, Sakaguchi, Appella, & Blumenthal, 1998).

For class II fusion proteins, it has been shown that domain III is also acting as a dominant-negative inhibitor of virus membrane fusion (Liao & Kielian, 2005). For SFV, it has been shown to bind to an intermediate trimeric E1 conformation and is thus supposed to inhibit the folding back of the molecule into the hairpin conformation.

For TBEV, exposure of virions to alkaline instead of acidic pH traps the glycoprotein in a monomeric intermediate conformation that is perpendicular to the viral membrane and interacts with target membranes via the fusion loop without proceeding to the merger of the membranes. Further treatment at low pH, however, leads to fusion, suggesting that these monomers correspond to an intermediate required to convert the prefusion dimer into the postfusion trimer (Stiasny, Kossl, Lepault, Rey, & Heinz, 2007).

2. Monomerization During the Refolding Mechanism

Refolding of class II fusion proteins generates trimers assembled from intermediate monomer conformations. Whether monomerization also occurs during class I and class III proteins 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 to break the threefold symmetry of the molecule (Weissenhorn, Hinz, & Gaudin, 2007).

For both VSV G and paramyxovirus F proteins, there are large differences between the trimeric interfaces of the pre- and postfusion structures (Roche et al., 2006, 2007; Yin et al., 2005, 2006). A plausible scheme for the structural transition is that it goes through transient monomerization (Roche, Albertini, Lepault, Bressanelli, & Gaudin, 2008). Consistent with this hypothesis, it has been demonstrated that VSV G is a monomer at pH 7.4 (Doms, Keller, Helenius & Balch, 1987) after detergent solubilization. It is possible that this monomer is an intermediate state between the pre-and postfusion conformations.

D. Cooperativity

The activation energy of the fusion process has been estimated to be in the range of 40 kcal/mol (Lee & Lentz, 1998). For class I and class II fusion proteins, it has been proposed that the energy released during the irreversible transition from the metastable prefusion state to the stable postfusion state 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 (Kuzmin, Zimmerberg, Chizmadzhev, & Cohen, 2001) and experimental data have suggested that in the case of HIV, a single env glycoprotein trimer is sufficient for fusion (Yang, Kurteva, Ren, Lee, & Sodroski, 2005, 2006). However, work on many viruses (also including HIV) suggests that several fusion proteins are involved during the fusion process.

For HIV, another analysis of the data used by Yang and coworkers, with less restricted mathematical models, indicated that HIV fusion machinery is made of several env proteins (Klasse, 2007). This latter conclusion is consistent with other experimental data, indicating that both cellular receptor density and env density affect the kinetics of HIV-1 env-mediated fusion (Reeves et al., 2002) and that four to six coreceptor molecules are recruited into fusion complexes (Kuhmann, Platt, Kozak, & Kabat, 2000). Such interactions would require more than one Env trimer per virion.

Work on influenza virus hemagglutinin also indicates that HA surface density is important for fusion (Danieli, Pelletier, Henis, & White, 1996) and that its decrease arrests fusion at the hemifusion stage (Chernomordik et al., 1998). 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 (Chernomordik et al., 1998). Such a ring structure of HA around a lipid neck joining the viral and the liposome membranes has been recently observed by cryo-electron microscopy (Lee, 2010). Finally, it has been demonstrated that HA molecules located outside the direct contact zone between fusing membranes are involved in the late stages of the fusion process (Leikina et al., 2004).

In the case of rhabdoviruses, the pH-dependent equilibrium between pre- and postfusion conformations of G implies that the energy released during the structural transition of one trimer cannot be sufficient to overcome the activation energy barrier and drive the fusion reaction. Indeed, the minimal number of spikes involved in the formation of a rabies virus fusion complex has been estimated to be about 15 (Roche & Gaudin, 2002).

How proteins cooperate to facilitate the formation of the different intermediates on the fusion pathway is still not known. However, some recent data provide new insights into the organization of the fusion machinery and suggest that distinct assemblies of fusion glycoproteins are involved at distinct stages of the fusion process.

For rabies virus (another member of the rhabdovirus family), a local hexagonal lattice of glycoproteins was observed at the surface of some rabies virus mutants affected in the kinetics of their structural transition when briefly incubated under suboptimal fusion conditions (Gaudin, Raux, Flamand, & Ruigrok, 1996). Each hexagon was made up of six G trimers. Interestingly, a similar

organization of G_{th} was found in the crystals of the prefusion form (that had the P622 symmetry) (Roche et al., 2007).

Furthermore, an EM study performed on VSV revealed that the flat base of the virion (as other rhabdoviruses, VSV has a typical bullet shape with a flat base and a round tip) is a privileged site for fusion (Libersou et al., 2010). The plane nature of this part of the virion would help the protein to form the P6 lattice. Such a local organization could favor not only the formation of the initial lipid intermediates but also a concerted transition for the proteins making up the local network.

A second network, helical this time, of glycoproteins in their postfusion conformations can be observed on the cylindrical part of VSV particles incubated below pH 6 (Libersou et al., 2010). When formed in absence of a target membrane, this helical array disrupts the viral membrane, indicating that this network formation induces tension in the viral membranes. Furthermore, when G_{th} alone is incubated with target liposomes at low pH, this also leads to the formation of tubular structures at the surface of which G_{th} adopts a helical symmetry. This indicates that the postfusion state of G ectodomain is independently able to self-associate into a quasicrystalline array, thereby deforming membranes. It has been proposed that these interactions between glycoproteins G, in their postfusion conformation and located outside the contact zone, drive the enlargement of the initial fusion pore (Libersou et al., 2010).

A similar two-stage model can also be proposed for class II fusion glycoproteins. In their prefusion conformation, they adopt an icosahedral organization (Kuhn et al., 2002; Lescar et al., 2001). Here again, this organization could favor a concerted conformational change (at least locally) of the protein making the network. In their postfusion conformation, the spikes self-associate to form more or less regular networks, different from their initial organization observed before fusion (Gibbons et al., 2003; Sanchez-San Martin, Sosa, & Kielian, 2008; Stiasny, Bressanelli, Lepault, Rey, & Heinz, 2004).

Finally, for paramyxovirus PIV5, micrographs indicate that F proteins in their postfusion conformation tend to cluster at the viral surface (Ludwig et al., 2008). Thus, the tendency of fusion glycoproteins in their postfusion conformation to cluster and form closely packed arrays seems to be general. We propose that the glycoproteins located outside the contact zone between fusing membranes exert a role during the fusion process via the formation of these networks by inducing tension in the viral membrane.

An objection to this model can be raised that retroviruses, and particularly HIV, have very few spikes at their surface (Zhu et al., 2006). This is hardly reconcilable with network formation. For these viruses, it is possible that membrane tension needed at the late stages of the fusion process to drive the enlargement of the initial fusion pore is generated by cellular proteins in response to signalization induced by env binding to coreceptors. Interestingly,

recent data revealed that dynamin is required for efficient HIV-1 fusion (Miyauchi, Kim, Latinovic, Morozov, & Melikyan, 2009). The authors have proposed that the ability of dynamin to regulate actin remodeling and associate with membrane-bending proteins (Kruchten & McNiven, 2006) could provide a driving force for initial pore expansion.

VI. CONCLUDING REMARKS

The determination of an increasing number of structures of viral fusion proteins and recent electron microscopy studies have shed light on the molecular mechanisms of fusion machinery. This has revealed similar principles of action for machinery made up of proteins having very different shapes. Nevertheless, some new hypotheses remain to be challenged and many questions remain unanswered.

As mentioned above, it is known that transmembrane domains of viral fusion proteins play an important role at the late stages of the fusion process, but no structure of full-length viral fusion glycoprotein (i.e., with its TM domain) has been determined. Thus, further structural studies of complete pre- and postfusion conformations of viral glycoproteins containing both membrane anchors (TM and fusion peptides) are needed. This should indicate how both regions cooperate to regulate the transition from hemifusion to fusion pore opening and pore enlargement.

The structures of intermediate conformations of fusion proteins, how they cooperate, and how they interact with and deform the fusing membranes are still very elusive. Understanding the structure of full-length intermediates in associations with the membranes is now a major challenge in the field.

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