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Review

Fusion of Enveloped Viruses in Endosomes

Judith M. White[1,*] and Gary R. Whittaker[2]

[1]Department of Cell Biology, University of Virginia, Charlottesville, VA USA
[2]Department of Microbiology & Immunology, Cornell University, Ithaca, NY USA
*Corresponding author: Judith M. White, jw7g@virginia.edu

Abstract

Ari Helenius launched the field of enveloped virus fusion in endosomes with a seminal paper in the *Journal of Cell Biology* in 1980. In the intervening years, a great deal has been learned about the structures and mechanisms of viral membrane fusion proteins as well as about the endosomes in which different enveloped viruses fuse and the endosomal cues that trigger fusion. We now recognize three classes of viral membrane fusion proteins based on structural criteria and four mechanisms of fusion triggering. After reviewing general features of viral membrane fusion proteins and viral fusion in endosomes, we delve into three characterized mechanisms for viral fusion triggering in endosomes: by low pH, by receptor binding plus low pH and by receptor binding plus the action of a protease. We end with a discussion of viruses that may employ novel endosomal fusion-triggering mechanisms. A key take-home message is that enveloped viruses that enter cells by fusing in endosomes traverse the endocytic pathway until they reach an endosome that has all of the environmental conditions (pH, proteases, ions, intracellular receptors and lipid composition) to (if needed) prime and (in all cases) trigger the fusion protein and to support membrane fusion.

Keywords enveloped virus, fuse, low pH, membrane, prime, proteases, trigger, viral fusion protein, virus entry, virus receptors

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All enveloped viruses deliver their genomes into the cytoplasm of their host cell by fusing with a cellular membrane. Ari Helenius inaugurated and has had a continual impact on this field in three major ways. He and his coworkers provided the first evidence that viruses can productively enter cells following endocytosis and transport to endosomes (1–5). His group was among the first to demonstrate that low pH is necessary and sufficient to trigger the fusion activity of certain enveloped viruses that enter cells through endosomes (1,2,6–10). And, extensive work led by Helenius demonstrated that not only can enveloped viruses productively enter cells through endosomes (Figure 1) but that most do so [for recent reviews, see (14–16)]. The focus of this review is on enveloped virus fusion in endosomes, in particular on the diversity of endosomal cues that trigger virus fusion.

Virus Fusion in Endosomes: General Considerations

Enveloped viruses that enter cells through endosomes begin their journey after binding to attachment factors and/or receptors on the cell surface followed by internalization through a variety of endocytic processes (14–18) (see Table 1 for definitions of terms). The major routes of virus internalization are clathrin-mediated endocytosis, used by Semliki Forest virus (SFV) (1) and vesicular stomatitis virus (VSV) (19), and macropinocytosis, used by vaccinia virus (20,21), Ebola virus (EBOV) (22–24) and others (25,26). Some viruses, notably influenza, can use either mode of internalization depending on which pathway is functional in a given cell under given conditions (27–30). Other means of internalization are caveolar endocytosis,

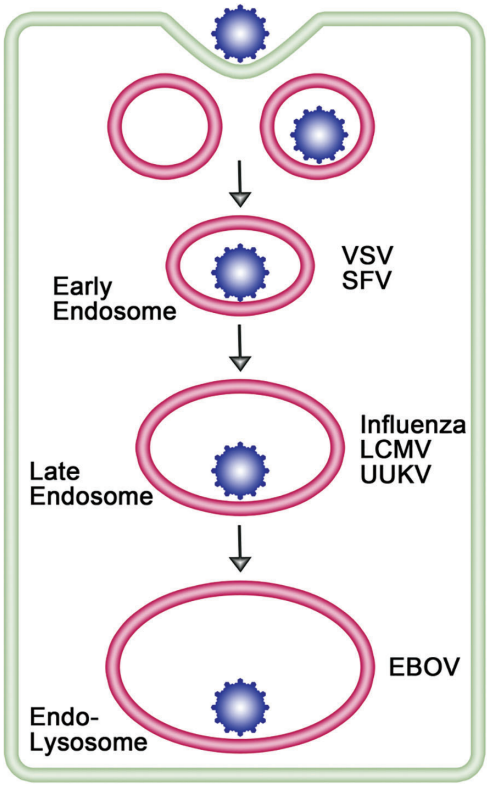

Figure 1: Enveloped virus entry through different endosomal compartments. Most enveloped viruses that enter the cell via endocytosis traverse the normal endocytic pathway (early endosome to late endosomes to endolysosome) and exit, by membrane fusion, where the conditions are sufficient to trigger the viral fusion protein; in some cases, the viral fusion protein is also proteolytically primed in the endocytic pathway as a prerequisite to fusion. [LCMV particles were found, however, to bypass early endosomes and traffic directly to, and fuse in, late endosomes (11,12).] Examples of enveloped viruses that exit through early endosomes, late endosomes and endolysosomes are indicated. Viruses that enter through late endosomes or endolysosomes are termed 'late penetrating viruses' (13). See text and table legends for abbreviations.

used by certain non-enveloped viruses, as well as clathrin- and caveolin-independent endocytosis.

Despite the various modes of internalization, most endocytosed enveloped viruses traverse the canonical endocytic pathway and enter the cytoplasm through either early endosomes, late endosomes or endolysosomes depending on which compartment has the proper environmental cue(s) to trigger and support fusion (Figure 1). While entry through endosomes does not necessarily imply a requirement for low pH for fusion (31–33), for most endocytosed enveloped viruses a major determinant of the entry site is the pH dependence of the viral fusion reaction (Table 2). The pH dependence of fusion varies among enveloped viruses (7), and these differences can correlate with the endosomal site of fusion (41). Hence, viruses with relatively high (~pH 6) pH dependencies, such as SFV (41) and VSV (42), generally fuse in early endosomes, whereas those with lower pH dependencies (~pH 5), including most strains of influenza, generally fuse in late endosomes (13). The pH dependence for influenza virus fusion varies by ~0.7 pH units among different strains, with human influenza viruses generally requiring lower pH than avian ones. This variation in pH triggering has been proposed as an 'acid stability marker' as part of risk-assessment analyses designed to predict the human transmissibility of influenza virus (43–48). However, single-particle tracking analyses of several clinical and laboratory-adapted H3 influenza viruses show that the typical acid stability assay (pH onset of syncytia formation) may not provide enough information about the rates of fusion and fusion inactivation to predict viral tropism in all cases (49).

SFV, VSV and influenza represent relatively simple cases for which low pH is sufficient to trigger fusion. For these, the pH of fusion correlates with the pH needed to induce fusion-activating conformational changes in the viral fusion protein, which in turn generally correlates with fusion in an early or a late endosome. However, even these simple cases have modifiers, such as special target membrane lipid requirements (6,50–54). In other cases, low pH may be necessary, but not sufficient, to trigger fusion. This applies for endosomal entry of certain retroviruses and coronaviruses.

Viral Membrane Fusion Proteins: General Considerations

Enveloped viruses vary in the number of different types of glycoproteins that protrude from their membranes. For example, retroviruses display a single transmembrane glycoprotein (Env), while most influenza viruses display two: a hemagglutinin (HA) and a neuraminidase.

Table 1: Definition of terms pertinent to viral membrane fusion proteins

Fusion protein	The transmembrane protein on the surface of an enveloped virus that engages the target bilayer to mediate virus–cell membrane fusion. Examples: influenza HA, HIV Env, Dengue E. All characterized viral fusion proteins contain both a fusion peptide (or fusion loop) that engages the target membrane and a transmembrane domain that anchors the protein in the viral membrane.
Fusion subunit	Certain viral fusion proteins (e.g. influenza HA and HIV Env) are trimers of heterodimers that consist of a receptor binding and a fusion subunit, held together by either a disulfide bond (influenza HA) or non-covalent interactions (HIV Env). In all of these cases the fusion subunit contains both the fusion peptide (or fusion loop) and a transmembrane domain.
Class I, II or III fusion proteins	All characterized viral fusion proteins fall into one of three classes based on the structure of their fusion protein/subunit: class I, largely α-helical; class II, largely β-structures; class III, contains both α-helical and β-structures. See Table 3.
Fusion peptide	A fusion peptide is a relatively hydrophobic sequence found at the N-terminal end of a fusion subunit. It is the portion of the fusion protein that engages the target membrane. See Table 3.
Fusion loop	A fusion loop is a relatively hydrophobic sequence found internal to the fusion protein/subunit. Like a fusion peptide, it is the region of the fusion protein that engages the target membrane. See Table 3.
Prefusion conformation	The conformation of the viral fusion protein as it appears on the viral membrane after priming, but before fusion triggering. See Figure 2, panel ii.
Priming	All characterized class I and class II viral fusion proteins are primed to a state capable of responding to a fusion trigger. This involves a proteolytic cleavage event in the fusion protein precursor or in a companion protein. See Table 3.
Triggering	All viral fusion proteins must be triggered for fusion. Triggering converts the prefusion conformation to a postfusion conformation through a series of structural changes (Figure 2), and is induced by an environmental cue (Figure 3) at the cellular fusion site (e.g. low pH in endosomes). In most cases, a single trigger is sufficient, but in some cases (e.g. for ASLV Env), two triggers (receptor and low pH) are required: for ASLV Env, one to convert the protein to a prehairpin and a second to convert the prehairpin to a hairpin.
Refolding	Refolding encompasses all of the conformational changes in the fusion protein/subunit during fusion. The fold-back stage of refolding encompasses the changes that convert the prehairpin to the hairpin (see Figure 2).
Postfusion conformation	The conformation of the fusion protein/subunit after the fusion reaction has been executed. For all characterized fusion proteins, this state is a trimer-of-hairpins (Figure 2).
Fusion cascade	The fusion cascade encompasses all changes in the viral fusion protein as well as in the target and viral membranes during the fusion reaction (panels ii to vi in Figure 2).

Other enveloped viruses display more than two surface proteins. Nonetheless, for all characterized enveloped viruses one glycoprotein is the fusion protein, the protein that actually merges the viral and cellular membranes (Table 3). For reviews on the structures and mechanisms of viral fusion proteins, see references (60,61,65–69) and primary citations within. For viruses that encode a single transmembrane glycoprotein (e.g. retroviruses and filoviruses) that glycoprotein is the fusion protein. For viruses with two transmembrane glycoproteins (e.g. orthomyxoviruses and paramyxoviruses), the fusion protein is one of the two (e.g. HA of influenza virus). As reviewed in references (59,60,70,71), large DNA viruses such as herpesviruses and poxviruses employ fusion machines, which consist of four (herpesviruses) or more (poxviruses) proteins associated with the virus membrane. However, at least for herpesviruses, one transmembrane glycoprotein, gB, is the fusion protein. Another important point is that despite their differing prefusion and postfusion structures, all characterized viral fusion proteins share a common architecture in their postfusion forms, a trimer-of-hairpins.

Priming and Triggering Viral Membrane Fusion Proteins

The field has converged on a model for how viral fusion proteins function [Figure 2; also see (60,61,65–70)]. All

Table 2: Site and fusion-triggering mechanism for representative enveloped viruses

Family	Virus	Site	Trigger
Retroviridae	MLV	Plasma membrane	Receptor
Paramyxoviridae	PIV5	Plasma membrane	Receptor
Herpesviridae	HSV-1	Plasma membrane	Receptor
Coronaviridae	SARS	Plasma membrane or late endosome	Receptor + protease
Rhabdoviridae	VSV	Early endosome	Low pH
Togaviridae	SFV	Early endosome	Low pH
Bornaviridae	BDV	Early endosome	Low pH
Flaviviridae	TBE	Endosome	Low pH
Orthomyxoviridae	Influenza	Late endosome	Low pH
Arenaviridae	LCMV	Late endosome	Low pH
Bunyaviridae	UUKV	Late endosome	Low pH
Filoviridae	EBOV	Endolysosome	Low pH + additional cue(s)
Asfarviridae	ASFV	Late endosome	Low pH + additional cue(s)
Poxviridae	VV	Late endosome	Low pH [+ additional cue(s)]
Arteriviridae	PRRSV	Early endosome	Low pH [+ additional cue(s)]
Hepadnaviridae	HBV	Late endosome	

Information is for the specific virus listed (viruses of invertebrates only not included). Variations on entry sites and triggers exist for different family members (see main text). 'Endosome' denotes that the specific endosomal entry site is not yet known. 'Low pH + additional cue(s)' denotes that low pH is necessary but not sufficient; 'low pH [+ additional cue(s)]' indicates that low pH is needed, but it is unclear if it is sufficient. Blank indicates insufficient information.

PIV5, parainfluenza virus 5; TBE, tick-borne encephalitis virus; LCMV, lymphocytic choriomenengitis virus; UUKV, Uukuniemi virus; VV, vaccinia virus (data are for the mature form, WR strain); ASFV, African swine fever virus; SHFV, simian hemorrhagic fever virus; BDV, Borna disease virus; HBV, hepatitis B virus. Additional information for the table can be found in references (34–40). For HBV, entry appears independent of late endosomal pH, but may require redox potential (38).

characterized class I and II viral fusion proteins must be *primed* before they can be *triggered* to induce fusion. Priming entails a proteolytic event that converts the fusion protein from a fusion-incompetent to a fusion-competent state (Figure 2). For class I fusion proteins the cleavage occurs in the fusion protein (precursor), whereas for class II fusion proteins, it occurs in a companion protein (Table 3). Depending on the viral fusion protein and the cells infected, priming can occur in the Golgi, at a cell surface or in an endosome. For example, for most strains of influenza virus, cleavage of the HA precursor (HA0) occurs in the extracellular space or at the cell surface by trypsin-like proteases, but highly pathogenic avian influenza viruses such as H5N1 are cleaved in the Golgi by furin-like proteases based on the presence of a multibasic cleavage site (73). Proteolytic priming separates HA0 into a receptor binding (HA1) and a fusion (HA2) subunit. Consequently, the fusion peptide is found at the amino terminus of HA2. Aspects of HA priming, for example accessibility of the cleavage site within HA0 (including obstruction by carbohydrates) as well as the host cells and tissues where priming occurs and the proteases involved, are thought to be critical determinants of influenza pathogenesis (43,74,75). Other priming events, as for the EBOV glycoprotein (GP) (76,77) and the F protein of henipaviruses (78–80), occur in endosomes, mediated by cathepsins or other proteases. Most importantly, irrespective of the cellular site where it occurs, the protein (fusion or companion) cleaved or the proteases involved, priming converts the fusion protein to a fusion-competent state (Figure 2).

The next stage of fusion is triggering, which for viruses that enter through endosomes is induced by an endosomal cue(s). An early consequence of triggering is exposure and repositioning of the fusion peptide (or loop) to engage the target bilayer; before triggering, the fusion peptide/loop is buried or tacked down in the fusion protein structure (or, for VSV G, points to the viral membrane) and therefore inaccessible to the target membrane. Upon triggering the fusion peptide/loop is exposed and inserts into the target membrane forming an intermediate termed a prehairpin (Figure 2). The prehairpin is a unique biological structure in which a single protein, the viral fusion protein,

Table 3: Examples of viral membrane fusion proteins

Family	Virus example	Fusion protein (subunit)	Fusion protein class	Protein primed	Priming protease(s)[a]	Metastable	Fusion peptide or fusion loop	Fusion trigger
Orthomyxoviridae	Influenza	HA (HA2)	I	HA0	Furin, trypsin	Yes	Peptide	Low pH
Paramyxoviridae	PIV5	F (F1)	I	F0	Furin, trypsin	Yes	Peptide	Receptor
Retroviridae	HIV	Env (gp41)	I	Env (gp160)	Furin	Yes	Peptide	Receptors
Coronaviridae	SARS	S (S2)	I	S0	Trypsin, cathepsin	Yes	Peptide	Receptor + protease
Arenaviridae	LCMV	GP (GP2 + SSP)	I	GPC	SKI/S1P	Yes	Peptide and loop	Low pH
Filoviridae	EBOV	GP (GP2)	I	GP	Cathepsins[b]	Yes	Loop	Low pH + additional cue(s)[c]
Togaviridae	SFV	E1	II	pE2	Furin	Yes	Loop	Low pH
Flaviviridae	TBE	E	II	prM	Furin	Yes	Loop	Low pH
Bunyaviridae	UUKV	Gc	II	GPC	Signal peptidase	Yes	Loop	Low pH
Rhabdoviridae	VSV	G	III	N/A	N/A	No[d]	Loops	Low pH
Herpesviridae	HSV-1	gB	III	N/A	N/A	(Yes)[e]	Loops	Receptor
Bornaviridae	BDV	GP (GP2)[f]		GP	Furin			Low pH + [additional cue(s)][c]
Poxviridae	VV	EFC						Low pH [+ additional cue(s)][c]
Asfarviridae	ASFV							Low pH + additional cue(s)[c]
Arteriviridae	PRRSV							Low pH [+ additional cue(s)][c]
Hepadnaviridae	HBV[g]							

Information is for the specific virus listed. Fusion proteins from most family members share structural class, metastability and presence of a fusion peptide or loops (but see text: pesti- and hepaciviruses). Within families, differences exist in the priming proteases and triggering mechanisms (see text). Information is not included for viruses that only infect invertebrates [but note that Baculovirus gp64 is a class III fusion protein (55)], nor for cell–cell fusion proteins from J paramyxovirus (56) or encoded by reoviruses (57). Blank indicates insufficient information.

HIV, human immunodeficiency virus; N/A, not applicable; SSP, stable signal peptide (58); EFC, entry fusion complex (59).

[a]Denotes furin- and trypsin-like proteases; SKI/S1P is a furin-family member.

[b]EBOV GP is cleaved to GP1 and GP2 by furin, but requires cathepsins for priming (see text).

[c]See Table 2.

[d]VSV G undergoes pH-reversible conformational changes..

[e]gB has been described as metastable (60), possibly due to interactions with gH/gL (60–63)

[f]The fusion mechanism of BDV is not clear. Its single glycoprotein (GP) is cleaved by furin (to GP1 and GP2), this cleavage is important for fusion (34) and GP2 is postulated to possess fusion activity (35). However, high-resolution structural information is not available for either GP1 or GP2.

[g]HBV encodes a preS surface protein that is processed to large (L), medium (M) and small (S) proteins, but which is/are the major player(s) in fusion and how HBV fusion is activated remain to be clarified (64).

is simultaneously anchored in, and therefore bridges, two distinct membranes: the target membrane via the fusion peptide/loop and its own (viral) membrane through its transmembrane domain (TMD). Class I fusion proteins are trimers in their prefusion and postfusion states. Interestingly, while all characterized class II fusion proteins are dimers (that sit low on the viral surface) in their prefusion states, they convert, through a monomeric intermediate, to homotrimers once their fusion loops lodge in the target membrane (68,81,82). After prehairpin formation, the fusion protein folds back upon itself, drawing the attached target and viral membranes closer and closer together. The final postfusion state of all characterized viral fusion proteins is, therefore, a trimer-of-hairpins. Although there is debate (83), it is commonly thought that multiple fusion protein trimers act in concert (84–91) to induce the later stages of fusion: close membrane apposition, formation of a lipid stalk, opening of a small fusion pore and expansion of the pore (92–95), to create a passage for the viral nucleocapsid.

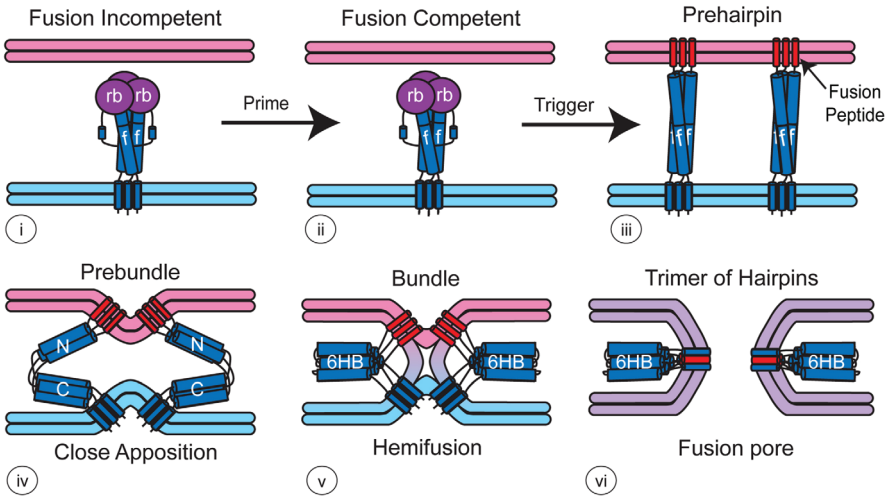

Figure 2: Model for how viral fusion proteins function. The model shown is for a class I fusion protein, but related models apply to class II and III fusion proteins. The term for the state of the protein is given above each image. For most class I fusion proteins [see (67) for paramyxovirus F proteins], prior to triggering (i and ii), the receptor-binding subunit (deep purple, rb) clamps the fusion subunit (dark blue, f). Upon triggering, the receptor-binding subunit moves out of the way unclamping the fusion subunit so that it can form a prehairpin embedded in the target membrane via the fusion peptide (red). The prehairpin then folds back causing the N- and C-α-helical heptad repeats to form a six-helix bundle (6HB) and progressively pulling the target (pink) and viral (light blue) membranes through stages of close apposition (iv), hemifusion (v) and fusion pore formation (vi). In some cases (e.g. for influenza HA), membrane coalescence is aided by further packing of sequences C-terminal to the C-heptad in the grooves of the central N-heptad coiled coil (72). Importantly, for all characterized viral fusion proteins, the final (postfusion) conformation (vi) is a trimer-of-hairpins.

Classes of Viral Fusion Proteins

The diagram in Figure 2 is for a class I viral fusion protein (Table 3) such as the influenza HA (96,97). For these, the fusion subunit is largely α-helical, containing an N- and a C-helical heptad repeat. In the prehairpin, the N-heptad is thought to sit atop the C-heptad in contiguous alignment. During the fold-back stages, the three C-heptads bind in the grooves of the trimeric N-heptad coiled coil creating a six-helix bundle-containing trimer-of-hairpins (Figure 2). Since the N- and C-heptads connect, respectively, to the fusion peptide and the TMD, hairpin formation pulls the attached membranes (cell and viral) together. Final interactions between the fusion peptide and TMD are thought to complete the event (98–101), possibly aided in some cases by a 'membrane proximal external region', located upstream of the TMD (102–106).

Based on structural criteria there are two other recognized classes of viral fusion proteins (60,61,65–70). Class II fusion proteins are largely composed of β-strands and β-sheets, with a fusion loop(s) at the tip of an extended β-sheet domain (68). Class III fusion proteins contain both α-helical and β-sheet regions, with fusion loops at the tips of an extended β-sheet (55,60,61,70,107). Most importantly, as for class I fusion proteins, the final 'postfusion' structure for characterized class II and III proteins is a trimer-of-hairpins (in which previously separated fusion peptides/loops and TMDs, and their attached target and viral membranes, have been brought together).

Fusion Peptides and Fusion Loops

The segments that engage the target membrane are termed 'fusion peptides' if at the N-terminus of the fusion subunit and 'fusion loops' if internal to the polypeptide chain. Most class I fusion proteins contain fusion peptides, but those of avian sarcoma leukosis virus (ASLV) and filoviruses contain a fusion loop. Arenavirus GPs may contain both a fusion peptide and a fusion loop (58). The situation for coronaviruses is not fully resolved, with most evidence for a fusion peptide (108–110) (see below).

In membranes or membrane mimetics, the fusion peptide of influenza HA forms a kinked or hairpin α-helical structure (111,112) while that of HIV Env forms α-helical or β-structures depending on the target lipid composition (113–115). These structures maximize interactions of hydrophobic side chains with the hydrocarbon portion of the bilayer. The EBOV GP, a class I fusion protein, contains an internal disulfide-bonded fusion loop. At neutral pH it adopts a relatively flat structure, as seen in the prefusion trimer (116,117). At low pH the structure bends to more firmly grasp the target membrane (117); low pH-induced changes also occur in the influenza HA fusion peptide (118,119). Where studied, specific residues in fusion peptides and loops affect their structure, membrane insertion and function [for example, see (119–121)]. Hence, the sequences and structures of fusion peptides/loops are important; their noted structural plasticity may be relevant at different stages of fusion.

Class II and III fusion proteins contain fusion loops at the tips of extended β-sheet domains. While some class II fusion proteins have a single fusion loop (87,122–125), others, as well as all characterized class III fusion proteins, possess two fusion loops (per monomer) at the tip of a β-sheet domain (126–132).

Fusion Triggering: General Considerations

There are four known ways by which a viral fusion protein can be triggered: by binding to a receptor(s), by exposure to low pH, by binding to a receptor followed by exposure to low pH and by a binding to a receptor followed by proteolytic cleavage (Figure 3). Interaction(s) with receptors is sufficient to trigger fusion for most retroviruses, paramyxoviruses and herpesviruses (Table 2), reflecting their predominant fusion at the cell surface at neutral pH [for reviews, see (60,67,70,133,134)]. Most enveloped viruses, however, fuse in endosomes reflecting a requirement for low pH. Low pH may be sufficient, or it may work in concert with, or after binding to, a receptor. Alternatively, following receptor engagement, low pH may be needed for the action of an endosomal protease (Table 4).

For some virus families, the fusion trigger is common to all members. For examples, low pH is sufficient to trigger

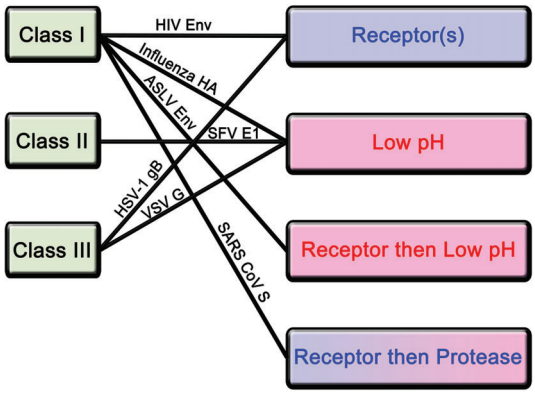

Figure 3: Different mechanisms by which class I, II and III fusion proteins are triggered. The three known classes of viral fusion proteins and the four confirmed mechanisms for fusion protein activation are shown on the left and right sides, respectively. Among fusion-triggering mechanisms (right), blue denotes events that occur at neutral, and pink denotes ones that require low, pH. Some receptor + protease mechanisms do, whereas others do not, require low pH. Lines join ways in which specific viral fusion proteins, from different structural classes, are triggered. See text for details and abbreviations.

the fusion proteins of influenza, alpha-, flavi- and rhabdoviruses. For other families, different members employ different triggers. For example, the coronavirus infectious bronchitis virus (IBV) is triggered by simple exposure to low pH (135), while others, including severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and feline coronavirus (FCoV), are activated by binding to a receptor followed by protease action. Similarly, while paramyxoviruses of the paramyxovirinae subfamily (e.g. measles and mumps) fuse at neutral pH in response to receptor binding (67), some metapneumoviruses (pneumovirinae subfamily) enter the cytoplasm following endocytosis, and for some this correlates with low pH enhancement of fusion activity (32,136,137). For respiratory syncytial virus (pneumovirus genus; pneumovirinae subfamily), endocytosis appears to be followed by pH-independent proteolytic activation of the fusion protein (25). Differences in neutral pH/cell surface versus low pH-dependent/endosomal entry apply among retroviruses (133,134,138–142) and to herpesviruses in some cell types (143).

Table 4: Examples of endosomal viral fusion triggers

Trigger	Virus	Fusion protein	Additional facilitating factors/other comments
Low pH	Influenza	HA	One study suggests a role for cathepsin W for influenza entry, but the substrate (viral or cell) is not known. Events initiating nucleocapsid uncoating require K^+.
	SFV	E1	SFV fusion requires cholesterol and sphingomyelin in the target membrane.
	Rubella	E1	Rubella virus fusion is enhanced by Ca^{++} ions.
	VSV	G	VSV fusion is enhanced by anionic lipids such as LBPA.
	Dengue	E	Dengue virus fusion is enhanced by anionic lipids such as LBPA.
	Andes	Gc	Andes virus fusion requires high levels of cholesterol in the target membrane.
	UUKV	Gc	UUKV fusion is enhanced by anionic lipids such as LBPA.
	LCMV	GP	Low pH appears sufficient to trigger LCMV fusion (no known enhancing factors).
Receptor + low pH	ASLV	Env	ASLV Env is the best-characterized fusion protein activated in two sequential steps by receptor (Tva) binding followed by exposure to low pH.
	JSRV	Env	JSRV Env appears to require both interaction with its receptor (Hyal2) and low pH, but details remain to be clarified.
	HCV	E1/E2	Fusion and entry mediated by HCV E1/E2 appears to require both binding to cell surface receptor(s) and low pH, but details remain to be clarified.
	LASV	GP	LASV fusion is reported to require its intracellular receptor (LAMP1) and low pH. The exact roles of LAMP1 and low pH remain to be clarified.
	EBOV	GP	After priming GP1 to ~19 kDa, EBOV GP requires its intracellular receptor (NPC1) and low pH for fusion, but the exact roles of NPC1, low pH and additional factor(s) remain to be clarified.
Receptor + endosomal protease	SARS	S	SARS requires binding to its surface receptor (ACE2) plus cathepsin L for endosomal entry. Low pH is not needed for fusion *per se* (rather for cathepsin activity); trypsin can trigger ACE2-bound SARS at the cell surface at neutral pH.
	MERS	S	MERS fusion requires binding to its cell surface receptor (DPP4) followed by proteolytic activation at the cell surface (by trypsin-like proteases) or in endosomes (by furin or cathepsins). Fusion may be triggered at neutral pH or under low pH conditions.
	RSV	F	RSV fusion is triggered in a pH-independent manner in early endosomes following two distinct furin-mediated cleavage events.

See text for references and details.
JSRV, Jaagsiekte sheep retrovirus. See text and other tables for other abbreviations.

An analysis of the ways in which different viral fusion proteins are activated also reveals that the mechanism of fusion triggering does not necessarily correlate with the structural class of the fusion protein (Table 3, Figure 3). Different class I fusion proteins can be activated by each of the four characterized fusion-triggering mechanisms (Table 3, Figure 3), while all characterized class II fusion proteins are activated by exposure to low pH alone. And, different class III fusion proteins can be activated either by low pH alone (VSV G), by binding to a receptor at neutral pH (e.g. most herpesviruses gB proteins in most cells) or by binding to a receptor followed by exposure to low pH (e.g. certain herpesviruses gB proteins in certain cells).

A major, perhaps the major, consideration about enveloped virus fusion in endosomes is that the specific endosome that serves as the fusion site (Figure 1, Table 2) is the endosome that possesses all of the necessary environmental cues to trigger the fusion protein. In some cases, this, or an upstream, endosome must also possess prefusion triggering priming factors. The endosomal fusion site will therefore be dictated by where the pH, receptors, ions, lipid

composition, proteases and other factors are all present to (prime), trigger and support fusion.

Triggering by Simple Exposure to Low pH

Details of the fusion process were first elaborated for the influenza HA (Figure 2), for which low pH is sufficient to trigger all of the steps in the fusion cascade (7–9,96,97,144–148). Unsurprisingly then, the pH dependence for key conformational changes in HA correlates with the pH dependence for fusion (43,149), which, as mentioned previously, varies for different influenza strains. Low pH is also sufficient to trigger alpha-, arena-, bunya-, flavi- and rhabdovirus fusion proteins (Table 3). For each of these, encompassing class I (150–153), class II (36,154–159) and class III (55,126,160,161) fusion proteins, protonation of multiple residues (147,149,162,163), in particular histidines (p*K*a ~ 6), is known or thought to be involved (164). For viruses activated solely by low pH, fusion in early or late endosomes is generally dictated by the pH dependence for key conformational changes in the viral fusion protein; those with higher pH thresholds generally fuse in earlier endosomes than ones requiring lower pH (7,41). Exposure of certain enveloped viruses to low pH can also elicit changes to the matrix layer underlying the virus membrane. For influenza, protons can access the matrix layer through the M2 channel in the viral envelope (165,166). Exposure of the matrix to H^+ and K^+, which can also enter via the M2 channel, likely aids later stages of fusion and/or uncoating (167–169). A related process may occur for other endosomally entering enveloped viruses (170,171).

Among viruses whose fusion activity is activated solely by low pH, several have been shown to have specific lipid or ionic requirements that can influence the specific endosomal site of fusion (Table 4). Where studied, these requirements reside at the level of either the initial interaction between the exposed fusion peptide/loop and the target membrane or at a later step in the fusion process. For example, SFV requires ~33 mol % cholesterol and ~1–2% sphingomyelin in the target membrane for optimal fusion (6,50,51,172). The cholesterol requirement is for stable insertion of the fusion loop (within its fusion protein, E1) into the target membrane prior to the fold-back steps (68,173). Similarly, hantaviruses such as Andes virus require high levels of cholesterol in the target membrane (in addition to low pH) for fusion (52).

Another intriguing case is dengue virus, which requires anionic lipids such as lysobisphosphatidic acid (LBPA) in the target membrane for fusion. Although dengue virus E protein displays conformational changes at a pH compatible with fusion in early endosomes, it traffics to late endosomes for fusion and entry (53,174), which was attributed to higher concentration of anionic lipids in later endocytic organelles (53). Other flaviviruses (175,176) as well as the rhabdovirus VSV (54,177,178) and the bunyavirus Uukuniemi (UUKV) (179) show higher rates and/or efficiencies of fusion when anionic lipids (including LBPA) are present in the target membrane. However, the physiological roles of specific anionic lipids in specific endosomal compartments for specific stages of fusion and/or capsid release are not fully understood. Some have argued that LBPA is needed for back-fusion of intraluminal vesicles laden with already fused virus particles, and hence capsid release (175,178).

In another twist, a recent study showed that in addition to low pH, rubella virus (RV) requires Ca^{++} for fusion (128). The RV fusion protein (E1) contains two fusion loops, which are closely associated through a metal ion-binding site in the postfusion structure (129). Ca^{++} appears to be needed to properly position the two fusion loops for coordinated stable target membrane interaction (Figure 2) and hence for RV fusion (128).

Triggering by Binding to a Receptor Followed by Exposure to Low pH or the Action of a Protease

Below, we will discuss how engagement of a host cell receptor can play an active, albeit not exclusive, role in triggering certain viral fusion proteins that function in endosomes, As a prelude, we review how viruses that fuse at neutral pH are activated by their host cell receptors.

Paradigms from viruses that fuse at neutral pH following receptor binding

Most enveloped viruses that fuse at neutral pH are triggered solely by binding to a host cell receptor(s) (Figure 3, Tables 2 and 3). Some neutral pH fusing viruses (most

retroviruses) possess a single glycoprotein (Env) whereas others (e.g. herpesviruses) have more. Retroviral Envs are class I fusion proteins. Binding of the receptor(s), to the receptor-binding subunit of Env, unclamps the fusion subunit, which then proceeds through conformational changes that mediate fusion (Figure 2). An interesting case is Env from murine leukemia virus (MLV), a γ-retrovirus. MLV Env contains a thiol exchange motif (CXXC) in its receptor-binding subunit. Engagement of the host cell surface receptor activates this motif, which then isomerizes a disulfide bond between the CXXC motif and a CX_6CC motif in the fusion subunit. By breaking this critical disulfide bond, the fusion subunit is unclamped and executes the fusion cascade (180). A similar isomerization occurs in Env of human T-cell leukemia virus, a δ-retrovirus (181). Human immunodeficiency virus (HIV), a lentivirus within the retrovirus family, presents a different case. The two subunits of HIV Env are not disulfide-bonded and HIV Env does not contain a thiol exchange motif. For most HIV strains Env engagement with its primary receptor (CD4) elicits a change in its receptor-binding subunit that reveals a binding site for a co-receptor (CXCR4 or CCR5). When the co-receptor binds it unclamps the fusion subunit allowing the fusion cascade to follow (138,182–184). While it had been thought that all of these events happen at the plasma membrane, some findings suggest that the (posthemifusion) content mixing stage of HIV fusion requires endocytosis and dynamin (but not low pH) (31). However, the exact site of fusion for specific strains of HIV in specific (physiologically relevant) cell types is currently under study (133,134).

Most, but not all, paramyxoviruses and herpesviruses fuse at neutral pH at the plasma membrane. In most of these cases, binding of a host cell receptor to a viral receptor-binding protein induces conformational changes (in the viral receptor-binding protein) that are relayed to the fusion protein, thereby sparking the fusion cascade. For paramyxoviruses, the relay is generally from the binding protein (HN, H or G) to the fusion protein (F) (67), with findings converging on a (receptor-binding protein) 'stalk exposure' model for fusion triggering (67,185,186). For herpesviruses, the relay goes from the binding subunit (gD for HSV-1) to an intermediate complex (gH/gL for HSV-1) to the fusion protein (gB) (60,70).

Triggering by binding to receptors followed by exposure to low pH

Unlike the cases described so far where either low pH alone or binding of a receptor(s) alone triggers the viral fusion protein, some viruses, notably the retrovirus ASLV, utilize a hybrid, two-step process. The fusion protein of ASLV (Env) is a class I fusion protein. Unlike MLV Env, ASLV Env does not contain a thiol exchange motif in its receptor-binding subunit (SU). In this case, binding of the ASLV receptor (Tva) to SU triggers conformational changes in SU (187,188) that release the clamp on the fusion subunit, thereby allowing Env to form a prehairpin embedded in the target membrane via its fusion loop (189–191). Subsequent exposure to low pH induces the fold-back events that generate a six helix bundle-containing trimer-of-hairpins (Figure 2) and hence fusion and viral entry (139–142,152,192–195). A His residue in the chain reversal region (between the N- and C-heptads) of the fusion subunit influences the pH dependence of fusion (152). This and a nearby His also influence the stability of the ASLV six-helix bundle at low pH (196). Interestingly, differences among six-helix bundles in terms of their dipole moments, stability at low versus neutral pH as well as the types of stabilizing interactions employed correlate with the pH dependence of fusion for certain retroviral (and filoviral) fusion proteins (196). ASLV Env is the only viral fusion protein currently known to use a clear-cut two-step mechanism where receptor binding triggers prehairpin formation and low pH triggers the fold-back steps leading to hemifusion and fusion pore formation. However, the Env glycoprotein of the Jaagsiekte sheep retrovirus appears to use a two-step process involving its receptor and low pH (197).

EBOV (discussed below) and Lassa virus (LASV) have recently been shown to employ intracellular receptors, located in late endocytic compartments, for productive entry. For EBOV the intracellular receptor is Niemann-Pick C1 (NPC1); for LASV it is LAMP1 (197–200). Both viruses require low pH for entry. In the case of LASV, both LAMP1 and low pH appear to be needed to induce LASV GP-mediated cell–cell fusion (199). A recent study employing electron cryotomography reported small changes in the membrane distal region of LASV GP1 at pH 5.0 and upon binding LAMP1 at pH 5.0, as well as shedding of GP1 at pH 3.0 (201). However, future

work is needed to determine if LAMP1 and physiological low pH elicit clear-cut sequential conformational changes in LASV GP akin to those induced by Tva and low pH (pH 5.0) in ASLV Env.

Triggering by binding to receptors followed by the action of a (low pH-dependent) protease

In some cases, viral fusion proteins are triggered by binding to a receptor followed by the action of a protease, which may or may not be low pH-dependent. Such triggering mechanisms are employed by some coronaviruses and also by the paramyxovirus respiratory syncytial virus (RSV). SARS-CoV has been studied extensively in this regard. Following its interaction with the ACE2 receptor, SARS-CoV is activated by proteolytic cleavage; cathepsin L activates the virus in late endosomes in a pH-dependent manner (202–204), but the virus can also be activated by trypsin-like proteases at the cell surface in a pH-independent manner (205–207). As first identified with SARS-CoV (208), coronaviruses are somewhat unusual in that they have two distinct cleavage sites within their spike proteins (209) termed S1/S2 and S2′. In the case of SARS-CoV either cathepsin L or trypsin-like enzymes cleave at both positions but likely in a sequential manner (S1/S2 followed by S2′) (208). The use of each protease may be different in different cell types (e.g. Vero cells versus respiratory epithelial cells).

The currently emerging MERS-CoV shares many features with SARS-CoV with regard to membrane fusion (210–212), but has wider cell tropism. While both cathepsin L and trypsin-like enzymes can activate MERS-CoV following binding to its receptor (DPP4), there is an additional ability to use furin as an activating enzyme (213), which may explain its broader tropism. As with SARS-CoV, the use of a given activating protease may be different in distinct cell types. Notably, MERS-CoV is activated at the S2′ site by endosomal furin in a pH-dependent manner (213,214). It is unclear if the effect of low pH reflects a role in activation of furin or for conformational changes in the spike protein. Given that coronavirus fusion can function under neutral pH conditions, it is possible that the pH dependency is related to the level of proteolytic cleavage, with decreased cleavage of S compensated by fusion at low(er) pH. If so, this would be reminiscent of findings with a p62 cleavage site-defective mutant of SFV (215).

While SARS-CoV does not appear to be able to use furin for cleavage activation, furin is commonly used by many other coronaviruses. In most cases, furin appears to prime the S precursor (S0) at the S1/S2 location likely during biosynthesis, as first shown with mouse hepatitis virus (MHV) (216). In general, coronaviruses appear to be quite flexible with regard to their postreceptor means of fusion activation, and this can profoundly affect viral pathogenesis. For instance, feline coronavirus and the human coronavirus HCoV-OC43 show distinct modulation of their pathogenesis based on sequence alterations in their furin cleavage sites (217–219). The second (endosomal or cell surface) protease likely acts at S2′, in close proximity to the fusion peptide (109,110), to drive the fusion reaction. While many details remain to be resolved, studies of MHV have provided firm evidence for an endosomal activation pathway (214) driving membrane fusion following proteolytic activation at a fusion peptide-proximal position, i.e. S2′ (214,220). Another coronavirus that has been used to demonstrate postreceptor triggering of fusion is porcine epidemic diarrhea virus (PEDV), in this case in a trypsin-dependent manner at S2′ (221) following interaction with the APN receptor. The action of trypsin on PEDV is considered to be pH-independent.

As mentioned previously, the avian coronavirus IBV appears to be an exception, with fusion based solely on low pH triggering (135). In this case, S may be cleaved at S2′ during biosynthesis (208,222), but any role of a yet-to-be-identified receptor is unknown.

RSV is a paramyxovirus whose fusion protein (F) has two cleavage activation sites. RSV F shares many features with MERS-CoV S, with furin activating at both cleavage positions and with the fusion peptide-proximal cleavage event occurring in early endosomes (25). However, the second RSV cleavage event appears to be pH-independent and is selective for furin, unlike for MERS-CoV.

Other endosomal proteases that have been implicated in virus entry include cathepsin E for porcine reproductive and respiratory syndrome virus (PRRSV) (37) and cathepsin W for influenza virus (223). In these cases, it is not yet known if the proteases act on the virus or on a host factor within the endosomal network.

Cases for Which the Triggering Mechanism Is Not Yet Clear

There are several viruses that enter cells through endosomes for which the mechanism of fusion triggering is unclear. Three, touched on above, are why certain herpesviruses require endocytosis and low pH for entry into certain cell types, whether an endosomal factor plays an active role in HIV fusion in all cell types and the precise roles of LAMP1 and low pH in triggering LASV fusion. Here, we elaborate on two important viruses that may use novel fusion-triggering mechanisms: EBOV and hepatitis C virus (HCV).

Triggering EBOV GP for fusion

EBOV fusion and entry are mediated by its sole GP, a trimer of a heterodimer containing a receptor-binding subunit (GP1) disulfide bonded to a fusion subunit (GP2) (224–227). Following internalization by macropinocytosis (22,23), EBOV traffics to and fuses in endolysosomes (Figure 1) that contain its intracellular receptor, NPC1, and two-pore channel 2 (TPC2), which is also required for EBOV entry (198,228–233). Before or upon arrival in endolysosomes, low pH-dependent cathepsins remove the mucin-like domain and glycan cap from GP1 (76,77,116,234,235). This *priming step* converts EBOV GP1 (formerly 130 kDa) to a 19-kDa species. Importantly, the fusion subunit (GP2) is still clamped in 19 kDa-GP1-S-S-GP2 (236,237). Priming (to 19-kDa GP1) has two important consequences: it exposes residues in GP1 critical for binding to NPC1 (228,235) and it appears to potentiate 19 kDa-GP1-S-S-GP2 for fusion triggering (236). An important observation about primed 19 kDa-GP1-S-S-GP2 is that it still requires low pH and a factor(s) thwarted by the cysteine protease inhibitor E64d to mediate entry (77,230,233,238). Concordantly, low pH and binding to NPC1 are necessary, but apparently not sufficient, to trigger productive 19 kDa-GP1-S-S-GP2-mediated virus–cell fusion (228).

Three lines of evidence indicate that low pH plays a role in triggering 19 kDa-GP1-S-S-GP2 beyond its role for optimal cathepsin activity. The first is that the fusion loop (in GP2) undergoes a low pH-dependent conformational change contingent on residues important for GP-mediated entry (117,121). The second is that low pH stabilizes the postfusion (six-helix bundle) form of EBOV GP2 (239–241). The third is that *in vitro*-induced conformational changes in primed EBOV GP occur more readily at low pH (236). While a recent study detected cell–cell fusion mediated by 19 kDa-GP1-S-S-GP2 at neutral pH, the fusion pores formed were small and non-expanding (242), likely not large enough to pass a viral nucleocapsid. Moreover, the latter findings (242) are difficult to reconcile with the observations that 19 kDa-GP1-S-S-GP2-mediated entry and infection are potently inhibited by both bafilomycin and E64d (77,230,238), and that only small structural changes were observed in the 19 kDa-GP1-S-S-GP2 ectodomain bound to the NPC1 C-loop and crystallized at pH 5.0 (243). Collectively, these findings suggest that in addition to NPC1 and low pH, another factor(s) is needed to mediate EBOV fusion in a manner that will lead to productive infection.

The sensitivity of 19 kDa-GP1-S-S-GP2-mediated fusion and entry to E64d (77,229,230,233,238) suggests that further action of a cysteine protease may be required. However, neither the factor(s) responsible for the E64 sensitivity nor its target (19 kDa-GP1-S-S-GP2 or a host constituent) has yet been identified. The roles of other potential triggering factors also remain unclear. Interestingly, the fusion subunits of EBOV GP and ASLV Env share structural and functional similarities (152,196). Both contain a CX6CC motif, but neither of their receptor-binding subunits contains a thiol exchange motif (CXXC), as seen in the MLV and HTLV Envs, which use disulfide bond isomerization for fusion (see above). Hence, an analogous fusion-triggering mechanism for EBOV GP would require exogenous reducing activity. While disulfide reducing agents (at low pH) can induce the 19 kDa-GP1-S-S-GP2 ectodomain to bind to target membranes (236), there is no evidence for a physiological role for disulfide bond reduction for EBOV entry. Even the role of NPC1 is still unclear. As for LAMP1 in LASV entry, does NPC1 play an active role in fusion triggering (i.e. induce significant conformational changes in 19 kDa-GP1-S-S-GP2 at physiological temperature) or is a main role, for example, to direct fusion to the limiting membrane of the endolysosome (i.e. away from the numerous small vesicles found within endolysosomes)? The role of TPC2 also remains to be elucidated (230,232). Hence, triggering of primed 19 kDa-GP1-S-S-GP2 to form a productive fusion pore

is complex, and it may represent a novel mode of fusion triggering.

Triggering HCV E1/E2 for fusion

HCV is a member of the Flaviviridae (*Hepacivirus* genus). Within the family, it groups most closely with pestiviruses (*Pestivirus* genus). Evidence suggests that the fusion mechanism of these viruses is novel. Both express two type I transmembrane glycoproteins (E1 and E2) required for fusion (244–250), but which is the *bona fide* fusion protein has been the subject of debate. While it was originally predicted that pestivirus and hepacivirus E2 proteins were class II fusion proteins, new evidence suggests this is not the case. First, the structures of the pestivirus (251,252) and HCV (253,254) E2 ectodomains do not conform to the class II fusion protein fold. Second, at low pH, the N-terminal domain of pestivirus E2 is disordered (252), as seen for the E2 protein (the receptor binding, not fusion, protein) of Sindbis virus. Third, a consensus is emerging for a fusion loop (residues 264–293) in HCV E1 (255–257). Fourth, a structure of the 79-residue N-terminal domain of HCV E1 (which does not include the predicted fusion loop) reveals a novel fold (258).

Additional findings point to a novel fusion mechanism for pestiviruses and hepaciviruses. Fusion for both viruses requires low pH (247,259,260), likely protonation of one or more histidines (251,261), but simple low pH treatment of cells with prebound viruses does not lead to infection. While the combined action of low pH and a disulfide bond reducing agent induced a low level of pestivirus infection via fusion at the plasma membrane (262), similar treatments did not induce HCV infection (260). Instead, for HCV, a 60-min incubation at neutral pH and 37°C followed by a low pH pulse induced some infection (259,260). One study (263) suggested a role for binding of HCV E2 to CD81, one of the HCV receptors (264,265), during the 60-min preparatory period. However, details of the mechanism of HCV fusion remain to be resolved.

Concluding Remarks

The field of enveloped virus fusion in endosomes has come a long way since the inaugural paper by Helenius and coworkers (1). As elaborated above, detailed fusion mechanisms – encompassing key structural elements and key structural changes in the fusion protein, and key environmental triggering cues (low pH, receptors and proteases) – are now known for representatives of class I, II and III fusion proteins. Many questions remain. For examples: Are there additional classes of enveloped viral fusion proteins? Are there additional fusion-triggering mechanisms? What are the common principles by which proteases trigger fusion proteins post receptor binding? How is fusion triggering orchestrated in multicomponent fusion machines? In another vein, recent work suggests that enveloped virus-membrane fusion can spark signaling responses that trigger innate immune responses (266,267), and that cells can use interferon-inducible transmembrane proteins to block virus–cell fusion (268). For their part, some viruses, notably HIV, have evolved means to counter attempts by cells to thwart virus–cell fusion (269,270). How exactly are these battles between enveloped viruses and cells enacted? And lastly, while there are drugs in use, in development or under consideration that block enveloped virus fusion (271), can we develop antifusion antiviral strategies that are broadly applicable, cost-effective, non-toxic and potent?

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