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The Many Mechanisms of Viral Membrane Fusion Proteins

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Abstract Every enveloped virus fuses its membrane with a host cell membrane, thereby releasing its genome into the cytoplasm and initiating the viral replication cycle. In each case, one or a small set of viral surface transmembrane glycoproteins mediates fusion. Viral fusion proteins vary in their mode of activation and in structural class. These features combine to yield many different fusion mechanisms. Despite their differences, common principles for how fusion proteins function are emerging: In response to an activating trigger, the metastable fusion protein converts to an extended, in some cases rodlike structure, which inserts into the target membrane via its fusion peptide. A subsequent conformational change causes the fusion protein to fold back upon itself, thereby bringing its fusion peptide and its transmembrane domain—and their attached target and viral membranes—into intimate contact. Fusion ensues as the initial lipid stalk progresses through local hemifusion, and then opening and enlargement of a fusion pore. Here we review recent advances in our understanding of how fusion proteins are activated, how fusion proteins change conformation during fusion, and what is happening to the lipids during fusion. We also briefly discuss the therapeutic potential of fusion inhibitors in treating viral infections.

Keywords Membrane fusion protein · Class I fusion protein · Class II fusion protein · Influenza HA · HIV Env · Low-pH activation · Receptor activation · Conformational changes · Membrane dynamics · Anti-fusion antivirals

1 Introduction

Fusion of enveloped viruses with host cells remains an important topic of research for two major reasons. First, it has recently become clear that fusion is a good target for therapeutic intervention (Kilby et al. 1998). Second, viral fusion reactions continue to serve as models for cellular fusion events. Although several viral fusion proteins, such as influenza hemagglutinin (HA) and the human immunodeficiency virus (HIV) envelope glycoprotein (Env), have emerged as paradigms, it is important to realize that there are many distinguishing features among viral fusion proteins (Table 1). Viral fusion proteins can be activated for fusion by different mechanisms. They have also been classified according to structural criteria. For some viruses, the viral receptor does not actively participate in fusion, whereas for others, one or more receptors are essential players. The location of the fusion peptide, critical for fusion, can vary. Finally, whereas some viruses require a single viral glycoprotein to mediate fusion, others require multiple viral glycoproteins. There are many excellent recent reviews on viral fusion and the glycoproteins that mediate this process (Durell et al. 1997; Eckert and Kim 2001; Heinz and

Table 1. Viral membrane fusion proteins

Family	Viral proteins needed	pH of fusion	Class	Fusion peptide
Orthomyxovirus	HA	Low	I	N-terminal
Alphavirus	E1	Low	II	Internal
Flavivirus	E	Low	II	Internal
Rhabdovirus	G	Low	?	Internal
Bunyavirus	G1/G2	Low	?	?
Arenavirus	GP	Low	?	?
Filovirus	GP	Low[a]	I	Internal
Retrovirus	Env	Neutral[b]	I	N-terminal, internal
Paramyxovirus	F,HN	Neutral	I	N-terminal
Herpesvirus	gB, gD, gH, gL	Neutral	?	?
Coronavirus	S	Neutral	I	Internal
Poxvirus	N.D.	Neutral	?	?
Hepadnavirus	S	Neutral?[d]	?	?[e]
Iridovirus	N.D.	N.D.	?	?

[a] Inferred from infectivity assays.

[b] Most retroviruses fuse at neutral pH. MMTV appears to require low pH [Ross et al. (2002) PNAS 99:12386–90] to fuse. Avian retroviruses require receptor priming at neutral pH followed by exposure to low pH [Mothes et al. (2000) Cell 103:679–89; see text for a discussion of this model].

[c] Coronaviruses possess heptad repeats [Chambers et al. (1990) J Gen Virol 71:3075–80] characteristic of class I viral fusion proteins. Recent work indicates that they are, indeed, class I fusion proteins [Bosch et al. (2003) J Virol 77:8801–11].

[d] With infectivity assays, hepadnavirus uptake was shown to be pH-independent [Hagelstein et al. (1997) Virology 229:292–4]. However, recent studies have shown that duck hepatitis B virus may require low pH [Grgacic et al. (2000) J Virol 74:5116–22].

[e] The S protein contains a stretch of amino acids predicted to be a fusion peptide but has not been further characterized.

Allison 2001; Skehel and Wiley 2000; Weissenhorn et al. 1999). The goal of this review is to give the reader an appreciation for the diversity of viral fusion mechanisms.

2
Activation of Viral Fusion Proteins

All fusion proteins exist on virion surfaces in a metastable state in which the fusion peptide, a critical hydrophobic sequence, is hidden or shielded within the glycoprotein oligomer (Carr et al. 1997; Hernandez et al.

1996; Rey et al. 1995; Skehel and Wiley 2000; Wilson et al. 1981). After activation, the fusion peptides are rendered accessible for interaction with a target membrane. A major distinction among viral fusion proteins is the "trigger" for activation. There are two well-recognized mechanisms: (1) exposure to low pH and (2) specific interactions with target cell receptors at neutral pH. A third mechanism involving receptor priming at neutral pH followed by further activation at low pH was recently proposed (Mothes et al. 2000).

2.1 Low pH Activation

Orthomyxoviruses, togaviruses, flaviviruses, rhabdoviruses, bunyaviruses, arenaviruses, and, apparently, filoviruses require low pH to fuse with target membranes (Table 1) (Doms et al. 1985; Gaudin et al. 1999b; Stegmann et al. 1987; White and Helenius 1980). These viruses are endocytosed after binding to the target cell surface. The low-pH environment of the endosome activates the viral fusion protein to convert from a metastable state to one that is capable of driving fusion. Although the presence of a receptor may modulate the rate or extent of fusion (Ohuchi et al. 2002; Stegmann et al. 1996; White et al. 1982), receptors are not essential for low-pH-dependent fusion. Low-pH-dependent fusion generally occurs within seconds to minutes at 37°C but can also occur, albeit more slowly, at T<22°C.

Four main techniques have been used to assess whether a virus requires low pH to fuse. The first technique is testing the effects of agents, such as bafilomycin, that inhibit endosomal acidification. In some studies of this type, fusion has been measured directly by assessing the transfer of fluorescent probes from the virus to the target cell (Earp et al. 2003; Irurzun et al. 1997; Zarkik et al. 1997). In others, fusion has been inferred by monitoring postfusion events, such as the synthesis of viral DNA (Mothes et al. 2000).

A second test is to assess whether fusion of bound virions can be induced by briefly warming virus-cell complexes in low-pH medium (Mothes et al. 2000; White et al. 1980). A third test is to assess whether pretreatment of virions at low pH (in the absence of target membranes) inactivates the virus for fusion. Some (Bron et al. 1993; Corver et al. 2000; Di Simone and Buchmeier 1995; Korte et al. 1999; Nir et al. 1990; Stegmann et al. 1987), but not all (Puri et al. 1988), viruses that fuse at low pH can be inactivated by this method. Viral fusion proteins that are inactivated by low pH undergo irreversible conformational changes. In

the case of X:31 HA, this results in insertion of the fusion peptide into the viral membrane (Korte et al. 1999; Weber et al. 1994).

The fourth test is to assess whether cells expressing the viral fusion protein can fuse. Cell-cell fusion can be observed by light or fluorescence microscopy (Frey et al. 1995; Melikyan et al. 1997b; Mothes et al. 2000), or it can be scored with gene reporter assays that monitor interactions of components from the fusing cells (Delos and White 2000; Earp et al. 2003; Feng et al. 1996; Nussbaum et al. 1994). Although cell-cell fusion assays are relatively simple to perform, the results do not always correlate with virus-cell fusion or infection (Earp et al. 2003; Lavillette et al. 1998; Schmid et al. 2000).

2.2 Receptor Activation at Neutral pH

Many enveloped viruses do not require low pH to fuse with target cells. This has generally been established in controlled experiments using the approaches described in Sect. 2.1. Viruses that can fuse at neutral pH include paramyxoviruses, herpesviruses, coronaviruses, poxviruses, and most retroviruses (Table 1) (Hernandez et al. 1997; McClure et al. 1990; Stein et al. 1987; Taguchi and Matsuyama 2002). The fusion proteins of these viruses are activated via specific interactions with one or more receptors in the target cell membrane (Hernandez et al. 1996; Hunter 1997; Stein et al. 1987). Viruses that can fuse at neutral pH are thought to do so at the plasma membrane. However, they may also be able to fuse with neutral-pH intracellular compartments (e.g., caveosomes) that can be accessed through newly recognized endocytic pathways (Pelkmans and Helenius 2003; Shin and Abraham 2001) (see also the chapter by Sieczkarski and Whittaker, this volume). It is important to note, however, that viruses that can fuse at neutral pH may also possess the ability to fuse at low pH (Earp et al. 2003; Fackler and Peterlin 2000). To date, neutral-pH fusion has been found to display a sharp temperature threshold, with little or no fusion occurring at $T<20°C$.

2.3 "Two-Step" Activation

Recently, a third model was proposed for the activation of alpharetroviruses. In this model, activation of the alpharetroviral Env begins with receptor binding at neutral pH (at $T>22°C$) but is only complete after exposure to low pH (Mothes et al. 2000). The role of low pH in this

"two-step" model is derived from two key observations: (1) The continuous presence of endosomal acidification inhibitors prevents production of alpharetroviral reverse transcripts, and (2) cells expressing Env and cells expressing the viral receptor only form large syncytia after exposure to low pH (Mothes et al. 2000). Our recent work indicates that alpharetrovirus fusion can proceed to the lipid mixing stage at neutral pH (Earp et al. 2003), and that receptor binding and low pH sequentially induce distinct conformational changes in the alpharetrovial Env (Matsuyama et al. 2004). Current work is now focused on determining the precise role of low pH in the fusion cascade.

3
Classification of Fusion Proteins Based on Structural Criteria

All viral fusion proteins contain a relatively large ectodomain, generally a single transmembrane domain, and all contain a cytoplasmic tail. So far, two major groups (class I and class II) have been defined based on structural criteria (Heinz and Allison 2001; Lescar et al. 2001) (Tables 1 and 2).

Class I fusion proteins are synthesized as precursors that are cleaved into two subunits by host cell proteases. In some cases (e.g., influenza HA), the two subunits remain associated through a disulfide bond; in others (e.g., HIV Env), the two subunits remain associated through noncovalent interactions. The proteolytic processing event that generates the two subunits is critical, as it creates the metastable state of the fusion protein (Chen et al. 1998). Class I fusion proteins exist as relatively long trimeric spikes in both their metastable and activated states. In their metastable states, they project perpendicularly to the viral membrane. The activated forms of the fusion subunits of known class I fusion proteins are highly α-helical (Skehel and Wiley 2000), and the final lowest-energy (which we will refer to as "postfusion") forms (Fig. 1) contain "six-helix bundles" (Bullough et al. 1994; Carr and Kim 1993). All six-helix bundles contain a relatively long (65–115 Å) central N-terminal trimeric coiled-coil. Some (e.g., HIV Env, SIV Env, and paramyxovirus F) form six-helix bundles that extend to their membrane proximal ends [i.e., three C-terminal helices (Fig. 1A, green) pack in the grooves of the central coiled-coil (Fig. 1A, blue)]. Others display a mixture of helical and nonhelical segments that pack into the grooves of the central coiled-coil. For example, the HA2 subunit of influenza HA contains a relatively small six-helix bundle (Fig. 1, green/blue) at its membrane distal end,

Table 2. Class I vs. class II viral membrane fusion proteins

Property	Class I	Class II
Type of integral membrane protein	Type I	Type I[a]
Synthesized as	Inactive precursor	Inactive precursor[b]
Exist on virion in	Metastable state	Metastable state
Orientation in virion (to membrane)	Perpendicular	Parallel
Converted to metastable state by	Proteolytic processing within fusion protein precursor	Proteolytic processing of an associated protein
No. of subunits in fusion protein	2	1
Major secondary structure of fusion subunit	α-Helix[c]	β-Sheet
Activated to fusogenic form by	Low pH or cell receptor(s)[d]	Low pH
Oligomeric state of metastable protein	Trimer	Dimer
Oligomeric state of fusion active protein	Trimer	Trimer
Location of fusion peptide	N-terminal or internal	Internal loop
Structure of final fusogenic form	Trimer of hairpins (coiled-coil)	Trimer of hairpins (non-coiled-coil)

[a] The TBE E glycoprotein has two membrane anchoring segments near its C-terminal end [Heinz and Allison (2001) Curr Opin Microbiol 4:450–5].

[b] Known class II fusion proteins are activated by proteolytic cleavage of an accessory protein.

[c] The postfusion forms of all known class I fusion proteins are α-helical. The fusion subunit of metastable influenza HA is also highly α-helical, and this appears to be the case for a paramyxovirus F protein [Chen et al. (2001a) Structure 9:255–66]. Comparable information is not available for the metastable forms of other class I fusion proteins.

[d] In the case of paramyxoviruses, the receptor binding protein relays the information of receptor binding to the fusion subunit [Lamb 1993; Colman and Lawrence 2003]

followed by an extended chain (Fig. 1, yellow) that packs in the groove and extends to the N-terminal (membrane proximal) end of its central coiled-coil (Fig. 1B). Because of these variations, the postfusion forms of class I fusion proteins are often referred to as "trimers of hairpins" (Eckert and Kim 2001).

The general structure of class II fusion proteins is quite different from that of class I fusion proteins. A well-characterized example is the envelope glycoprotein (E) of tick-borne encephalitis (TBE) virus. During biosynthesis, TBE E and a second viral membrane glycoprotein, the precursor to the membrane protein (prM), form heterodimers. As virions ma-

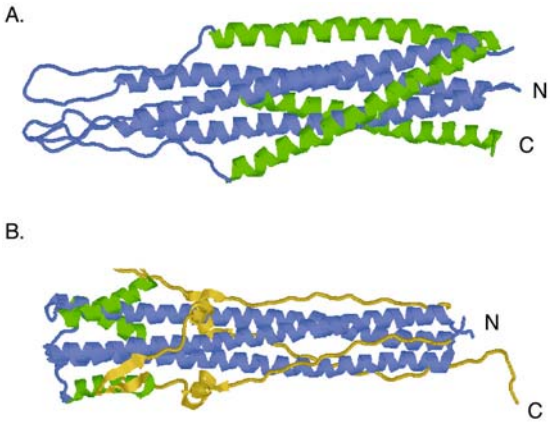

Fig. 1. Structures of the postfusion forms of SIV Env (**A**) and influenza HA (**B**). **A** NMR structure of the postfusion form of SIV Env gp41 subunit (PDB accession number 2EZO). **B** Crystal structure of the postfusion form of influenza HA2 subunit (PDB accession number 1QU1). Coiled-coil regions are *blue*. C-terminal helices are *green*. For influenza HA2, the C-terminal extended region is *yellow*. *N* and *C* indicate the points where the fusion peptide and the transmembrane domain, respectively, attach

ture, a host cell protease cleaves prM, resulting in reorganization of proteins on the viral surface (Allison et al. 1995). After prM cleavage, the E proteins exist as metastable homodimers. The ectodomains of the dimer are oriented antiparallel to one another. In further contrast to the trimeric class I fusion protein spikes, the ectodomains of the E homodimer lie parallel to the viral membrane and close to the surface. The TBE E protein is composed mostly of β-strand structure (Heinz and Allison 2001; Rey et al. 1995). The architecture of the Semliki Forest virus (SFV) spike, another well-characterized class II fusion protein, is similar to that of TBE E, but in this case, the metastable oligomer is a heterodimer of two membrane-anchored proteins, E1 and E2, with an associated small protein (E3).

4 Examples of Fusion Activation Mechanisms

In Sects. 4.1–4.4, we discuss a few examples of viral fusion proteins that employ different fusion mechanisms in more detail. These will include

examples of class I and class II fusion proteins, activated by low pH or by receptor interactions at neutral pH.

4.1 Influenza HA (Class I Fusion Protein, Low pH)

High-resolution structures are available for both the complete native (metastable) (Wilson et al. 1981) and activated (Bullough et al. 1994; Chen et al. 1999) forms of the influenza HA. On the viral surface, HA exists as a trimer of heterodimers (Fig. 2A). Each heterodimer consists of HA1, which contains the receptor binding domain (Fig. 2, gray), and HA2, which contains the fusion peptide (Fig. 2, red) and the transmembrane domain (located at the C-terminus). In the native (neutral pH) structure, the fusion peptide is buried within the HA oligomer. Three long helices, one from each monomer, come together to form the triple-stranded coiled-coil of the metastable trimer (Fig. 2A and B, blue and green).

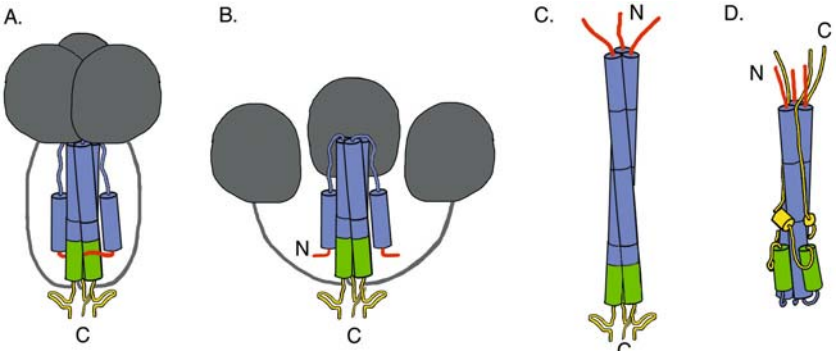

Fig. 2A–D. Low-pH-induced conformational changes within influenza HA. HA1 is depicted in *gray*. The fusion peptide is *red* (HA2 residues 1–24). The coiled-coil is *blue*, with the C-terminal helix colored *green*. The C-terminal extended region is *yellow*. The transmembrane domain (not shown) attaches to the C-terminal end, indicated by "*C*", of HA2. A model for conformational changes: **A** In the native, metastable, structure of HA, the fusion peptides are buried within the trimer interface. HA1 acts as a clamp to hold HA2 in a metastable state. HA2 is largely shielded by HA1. To illuminate the HA2 core, we have cartooned the portion of HA1 that covers HA2 as a simple (*gray*) line. **B** On exposure to low pH, the HA1 headgroups separate, allowing expulsion of the fusion peptide. **C** A loop-to-helix transition causes the fusion peptide to be repositioned to one end of HA2, where it can bind to the target membrane. **D** A helix-to-loop transition causes the C-terminal helix and the C-terminal extended region to reverse direction and bind to the grooves of the coiled-coil in an antiparallel orientation

On exposure to low pH, HA undergoes dramatic conformational changes. The globular head domains separate, releasing the clamp that holds HA2 in its metastable state (Fig. 2B). As a result, the fusion peptide is exposed (Fig. 2C, red) at the top of an extended triple-stranded coiled-coil, in a position where it can interact with the target membrane. A helix-to-loop transition causes a short helix (Fig. 2D, green) and the C-terminal extended region (yellow) to flip up and run antiparallel to the central coiled-coil (Bullough et al. 1994). As a result, the fusion peptide and transmembrane domain are brought into close proximity at the same end of the molecule (Fig. 2D).

Many regions of HA are important for fusion. The fusion peptide is critical for hydrophobic attachment of the virus to the target membrane (Sect. 6.1). Mutations that prevent (1) globular head domain separation (Godley et al. 1992; Kemble et al. 1992), (2) the "B-loop"-to helix transition (Gruenke et al. 2002; Qiao et al. 1998), or (3) the C-terminal extended region from packing into the grooves of the final coiled-coil (Borrego-Diaz et al. 2003; Park et al. 2003) ablate the ability of HA to reach the lipid mixing stage of fusion. In our model (Gruenke et al. 2002), conversion of HA to a prehairpin intermediate (Fig. 2C) allows HA to bind to the target membrane. Further conversion to the hairpin structure (Fig. 2D) then drives the formation and opening of a fusion pore.

4.2
HIV Env (Class I Fusion Protein, Neutral pH)

Like influenza HA, HIV Env is synthesized as a single-chain precursor and cleaved during biosynthesis to yield gp120 and gp41. Native (metastable) HIV Env is a trimer of the heterodimers of gp120 (the receptor binding subunit) and gp41 (the fusion subunit). Env is activated for fusion (at neutral pH) after sequential binding to CD4 and a coreceptor (a chemokine receptor). Binding of Env to CD4 causes conformational changes in Env that permit binding to the coreceptor. After coreceptor binding, additional conformational changes occur in Env that lead to fusion (Eckert and Kim 2001).

Crystal structures exist for the core of the gp120 subunit (Kwong et al. 1998) as well as for the postfusion (Fig. 3, Step 6) form of gp41 (Chan et al. 1997; Weissenhorn et al. 1997). However, there is not yet a crystal structure of the native (metastable) Env trimer. Therefore, a detailed picture of HIV Env activation via receptor interaction is not available. We presume that the first steps of Env activation are separation of the

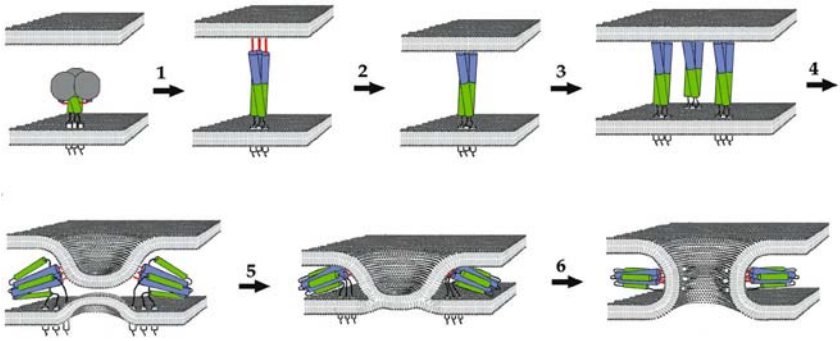

Fig. 3. Model of HIV fusion. Env exists as a trimer in the surface of the native viral membrane, with fusion peptides (*red*) presumably buried within the trimer interface. SU domains (pictured as *gray* globular domains at the top of the trimer) provide the receptor-binding function. For clarity, SU domains are omitted after *Step 1*. Target cell receptors are not pictured in this model. On exposure to receptor and coreceptor at T $\geq$22°C and neutral pH, Env undergoes conformational changes that result in exposure of the fusion peptides (*Step 1*), which then insert into the target membrane (*Step 2*). Multiple Envs may cluster (*Step 3*) to form a fusion site. Additional conformational changes (*Steps 4* and *5*) lead to the formation of a six-helix bundle, resulting in hemifusion (*Step 5*) (defined as mixing of the outer leaflets of the viral and cellular membranes). Eventually a fusion pore forms (*Step 6*) and enlarges (not shown)

globular head domains, expulsion of the fusion peptide, and extension of gp41 into a prehairpin intermediate (Fig. 3, Step 1). Several lines of evidence indicate the existence of the prehairpin intermediate. For example, peptide analogs of the C-terminal helix (Fig. 3, green) strongly inhibit HIV fusion and infection (Chan and Kim 1998; Kilby et al. 1998). Also, a synthetic peptide corresponding to the C-terminal helix coimmunoprecipitates with HIV Env after engagement of receptors (Furuta et al. 1998; He et al. 2003). The C-terminal helix then packs, in an antiparallel fashion, into the groove of the N-terminal coiled-coil (Fig. 3, Step 5). Because the C-terminal helices of gp41 extend along the entire length of the N-terminal coiled-coil, this packing would bring the fusion peptide and transmembrane domain very close together. The transition to the six-helix bundle drives membrane merger (Melikyan et al. 2000a). Moreover, complete six-helix bundles are needed to form "robust" fusion pores (Markosyan et al. 2003).

As mentioned above, HIV studies, primarily using epitope accessibility assays, have indicated that engagement of HIV receptors induces con-

formational changes in gp120 and gp41 (Eckert and Kim 2001; Xiang et al. 2002). A remaining issue for all receptor-activated viral fusion proteins is how information is transmitted (after receptor binding) through the receptor binding subunit to the fusion subunit. Such transmission is essential to allow rearrangements in the fusion subunit (e.g., six-helix bundle formation) that drive fusion. For HIV, part of the mechanism may involve reduction of one or more disulfide bonds in gp120 (Abrahamyan et al. 2003; Barbouche et al. 2003; Fenouillet et al. 2001; Gallina et al. 2002). In murine retroviral Envs, a proline-rich hinge region appears to relay receptor binding information from the N-terminal to the C-terminal region of the receptor binding subunits (SU) (Barnett and Cunningham 2001; Lavillette et al. 2001). Because the proline-rich region of SU is linked to TM by a disulfide bond (Pinter et al. 1997), this may provide a relay system to trigger conformational changes in the fusion subunit. Clearly, the molecular pathways by which receptor-activated fusion proteins change from their metastable to their activated forms need to be defined.

In Fig. 3, we show a working model for HIV Env-mediated fusion. It is derived in part from studies with influenza HA, and it is similar to other HIV fusion models (Eckert and Kim 2001). Our hypothesis is that all class I fusion proteins will employ similar mechanisms. We note, however, that even in the case of influenza HA, alternate models are still entertained (see Fig. 2 in Jahn et al. 2003). Furthermore, others have suggested that different class I fusion proteins may use fundamentally different mechanisms (Chen et al. 2001a).

The features that we predict will be common to the fusion mechanisms of all class I fusion proteins (Fig. 3) include: (1) conversion from a metastable state to an activated state, (2) exposure and repositioning of the fusion peptide for binding to the target bilayer, (3) recruitment of several activated fusion proteins to a fusion site (Blumenthal et al. 1996; Danieli et al. 1996; Markovic et al. 2001; Markovic et al. 1998), and (4) subsequent conformational changes that result in close apposition of the fusion peptide and the transmembrane domain.

4.3 Paramyxovirus F Proteins (Class I Fusion Protein, Neutral pH, Attachment Protein Assisted)

The viral fusion proteins that have thus far been discussed in detail contain a receptor binding domain (e.g., the gp120 subunit of HIV Env) within the fusion protein spike. In other cases, the receptor binding do-

main resides in a separate viral spike. Paramyxoviruses have an attachment protein spike and a separate fusion (F) protein spike. Most, but not all, paramyxoviruses require both the attachment protein and the F protein for fusion (Bagai and Lamb 1995; Paterson et al. 2000). In most cases, the attachment protein must come from the same paramyxovirus as the fusion protein (Bossart et al. 2002). In the few cases in which the F protein is sufficient, fusion is enhanced if the attachment protein is also expressed (Bagai and Lamb 1995). The need for the attachment protein can be overcome by mutations in the F protein (Paterson et al. 2000; Seth et al. 2003) or by conducting fusion reactions at T>37°C (Paterson et al. 2000; Wharton et al. 2000). Paramyxovirus fusion proteins thus represent special cases of receptor-activated fusion proteins, in which receptor activation is communicated from one viral spike glycoprotein to another.

F proteins are proteolytically cleaved during biosynthesis to generate two disulfide-bonded subunits, F_1 and F_2 (Begona Ruiz-Arguello et al. 2002; Gonzalez-Reyes et al. 2001; Lamb 1993), found as metastable trimers of dimers (Baker et al. 1999) on virions. It has been suggested that binding of the attachment protein to a host cell receptor causes conformational changes in this protein, which in turn cause activating conformational changes in the metastable F protein (Lamb 1993; Russell et al. 2001; Takimoto et al. 2002). The exact mechanism by which attachment proteins activate F proteins is not known, but several groups have provided evidence for cross talk between attachment and F proteins (Bossart et al. 2002; Deng et al. 1999; McGinnes et al. 2002; Stone-Hulslander and Morrison 1997; Takimoto et al. 2002; Yao et al. 1997).

The post-fusion form of the F protein from the paramyxovirus SV5 contains a six-helix bundle (Baker et al. 1999). Similar to HIV Env (He et al. 2003; Kilby et al. 1998; Munoz-Barroso et al. 1998) and other retroviral fusion proteins (Earp et al. 2003; Netter 2002), peptide analogs of the N- and C-terminal helices of paramyxovirus six-helix bundles are potent inhibitors of fusion and infection (Bossart et al. 2002; Joshi et al. 1998; Lambert et al. 1996; Young et al. 1999). As is also the case for HIV Env (Markosyan et al. 2003; Melikyan et al. 2000a), a recent study showed that conversion of the SV5 F protein to a six-helix bundle drives membrane fusion (Russell et al. 2001).

Issues yet to be addressed for paramyxoviruses are the structure of the complete native (metastable) F trimer and how it is converted to its activated form. The first glimpses at the metastable and postfusion states of the F trimer came from EM observations of the respiratory syncytial virus (RSV) F protein. Preparations of purified recombinant F protein

contained both cone-shaped rods and "lollipop"-shaped structures. On storage, there appeared to be a shift from the cone-shaped to the "lollipop"-shaped structures (Calder et al. 2000). Examination of F complexed with specific monoclonal antibodies suggested that the "lollipop" structures contained six-helix bundles composed of N- and C-terminal heptad repeats (Calder et al. 2000).

A high-resolution structure of an F protein ectodomain was recently presented (Chen et al. 2001a). The protein used for the analysis contained a mixture of precursor F_0 and proteolytically cleaved F. It also apparently lacked the second heptad repeat, which forms the C-helix in the postfusion form. This trimeric F protein structure is fundamentally different from that of influenza HA; the N-terminal end of its coiled-coil is positioned near the viral membrane end of the molecule (i.e., opposite the orientation of the coiled-coil in the metastable HA trimer). If this F protein structure represents the native metastable F trimer, then it suggests a mechanism of fusion activation for F fundamentally different from that for HA (Chen et al. 2001a). Additional work is needed to test this idea.

4.4
TBE E and SFV E1 (Class II Fusion Proteins, Low pH)

All known class II fusion proteins are activated by low pH. However, the mechanism by which class II fusion proteins are initially activated is quite different than the mechanism by which class I fusion proteins are initially activated. For example, the ectodomain of the TBE glycoprotein forms an antiparallel dimer that lies parallel and close to the viral membrane (Fig. 4B). At low pH, the TBE E homodimer converts to an E homotrimer (Allison et al. 1995; Heinz and Allison 2001; Stiasny et al. 2001). This transformation is thought to occur in two steps: dissociation of the E homodimer, followed by reassociation of E trimers (Stiasny et al. 1996). Membrane binding occurs after dimer dissociation and promotes the formation of E homotrimers (Stiasny et al. 2002). Homotrimer formation may involve interactions between α-helices in the stem region of the E protein (Allison et al. 1999).

The SFV fusion protein also converts from a dimer to a trimer during fusion activation. On native virions, E1 exists as a tight heterodimeric complex with a second membrane protein, E2. On exposure to low pH, E1 dissociates from E2, changes conformation, and forms highly stable E1 homotrimers (Ahn et al. 1999; Kielian 1995; Wahlberg et al. 1992; Wahlberg and Garoff 1992). During this process, E1 binds hydrophobically through its fusion peptide to target membranes and mediates fu-

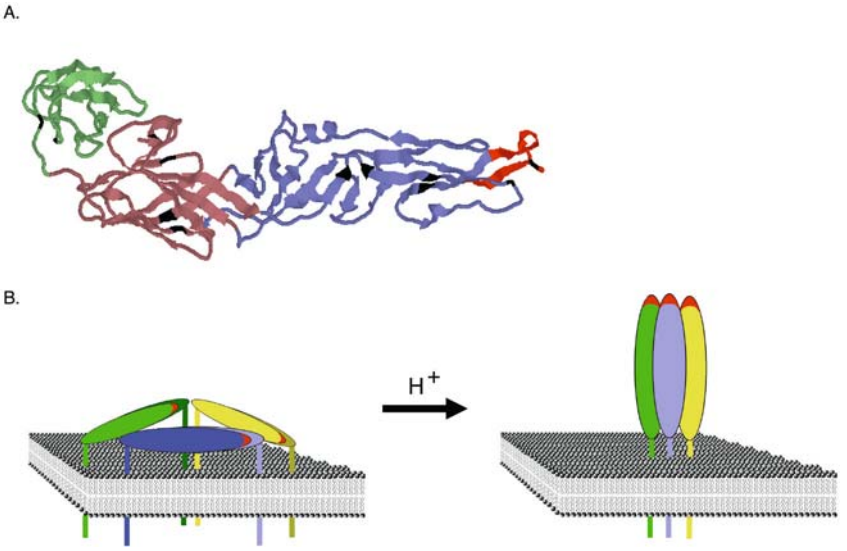

Fig. 4. Structure and cartoon of conformational changes of the TBE E protein. **A** Crystal structure of TBE E (PDB accession number 1SVB). The fusion peptide is *red*. Domains I, II, and III are *pink*, *blue*, and *green*, respectively. Disulfide bonds are *black*. **B** Cartoon depicting possible rearrangements during the dimer to trimer transition upon exposure to low pH (Allison et al. 1995). Each of the three dimers (*blue*, *green, yellow*; *left*) supplies one monomer (*light shaded subunits*) to the homotrimer (*right*). The organization of the dimers is as found in TBE recombinant subviral particles (Ferlenghi et al. 2001); it may represent an intermediate arrangement (from that on native virions) found during fusion activation (Kuhm et al. 2002). Note that other possibilities for the dimer to trimer transition exist (for example involving relative movements of domains about hinge regions). For very recent developments regarding the fusion mechanism of class II fusion proteins, see Bressanelli et al. (2004), Gibbons et al. (2004) and Modis et al. (2004)

sion. Similar to TBE E, it appears that binding to the target bilayer fosters formation of the activated E1 homotrimer (Kielian 1995; Kielian et al. 2000); the fusion peptide and the transmembrane domain of E1 appear to be important for E1 homotrimer formation (Kielian et al. 1996, 2000; Sjoberg and Garoff 2003). Thus both class I and class II viral fusion proteins appear to function as trimers during fusion. It has been proposed that activated TBE E (Helenius 1995) and SFV E1 stand up as trimeric spikes and present their fusion peptides to the target membrane (Fig. 4B, right). This would be analogous to Fig. 3, Step 1. If this occurs,

then the spike would have to refold to bring the viral and cellular membranes together (e.g., analogous to Steps 4 and 5 in Fig. 3). Very recent evidence indicates that this is, indeed, the case (Bressanelli et al. 2004; Gibbons et al. 2004; Modis et al. 2004).

5
Membrane Dynamics During Fusion

Thus far we have focused on the conditions that elicit viral fusion reactions and the conformational changes in viral fusion proteins necessary for fusion. However, it is the viral and cellular bilayer membranes that merge during fusion. Lipid bilayers are stable structures that do not fuse spontaneously. Fusion proteins have evolved to catalyze the necessary lipid rearrangements. We now review a lipid rearrangement model and focus on the roles of different regions of viral fusion proteins in choreographing the structural changes that the membranes undergo throughout the fusion cascade (Fig. 3).

The favored model for the lipid transition state during membrane fusion is the stalk model. In this model, two opposing membranes bend toward each other, creating "dimples" (when viewed from the *trans* surface) or "nipples" (when viewed from the *cis* surface) (Fig. 3, Step 4). Nipples continue to bend until they meet. The two *cis* leaflets then merge, creating a lipid stalk (see Fig. 2 in Kozlovsky and Kozlov 2002) that proceeds to a state of local hemifusion (Fig. 3, Step 5). In a second step, transient fusion pores form, which give rise to stable pores (Fig. 3, Step 6).

The first direct visualization of a lipid stalk intermediate was achieved by electron diffraction studies of the effect of sequential dehydration on lipid bilayers composed of a lipid that has negative spontaneous curvature (Yang and Huang 2002). The stalk intermediate was stable at intermediate relative humidities. The results suggested that both the formation of a lipid stalk and its transition to a conformation that can be equated with pore formation require external forces.

Cellular membranes do not have spontaneous negative curvature and are highly hydrated. Membrane curvature can be promoted by introducing defects into the contacting bilayers. Thus roles for the fusion protein include pulling the fusing bilayers toward one another (dimpling), dehydrating the membranes, and creating membrane defects that lower the energy barrier for stalk and pore formation. Two intermediates in HIV fusion have been trapped: one in which the two membranes are joined

by activated Envs, but are not yet fused (Melikyan et al. 2000a), and one in which small, "labile" pores have formed that can either expand into stable, "robust" pores or return to the prefusion state (Markosyan et al. 2003). These observations suggest a role for the fusion protein in formation and stabilization of both the fusion stalk and the fusion pore.

The mechanism by which a small pore enlarges is not known. However, several possibilities have been proposed. One is that the initial fusion pore is formed by a small number of activated fusion proteins. Additional activated fusion proteins then move into the fusion site to buttress and stabilize the pore, thereby allowing it to expand (Kozlov and Chernomordik 2002). Another possibility is that multiple small fusion pores coalesce to form larger ones. This was supported by EM visualization of HA-mediated fusion, in which multiple dimples/nipples were arranged circularly and lipid fragments were seen at the center of a fusion ring (Kanaseki et al. 1997).

6
Membrane-Interacting Regions of Viral Fusion Proteins

As discussed above, roles for the fusion protein in the fusion cascade (Fig. 3) include pulling the fusing bilayers toward one another (dimpling) and creating membrane defects that lower the energy barriers for stalk formation and fusion pore opening/enlargement. The fusion peptide and the transmembrane domain must remain stably associated with the target and viral membranes, respectively, for fusion to occur. Once the fusion peptide is stably associated with the target bilayer (Fig. 3, Step 2), we envision that rearrangements in the fusion protein ectodomain that bring the fusion peptide and transmembrane domains close together (Fig. 3, Step 4) result in dimpling of membranes toward one another. In addition to serving as critical membrane anchors, the fusion peptide and the transmembrane domain likely create membrane defects that facilitate the next stages of fusion. Here, we review information about the structure and function of the fusion peptide and the transmembrane domain during fusion. We also review evidence that juxtamembrane sequences, on both sides of the transmembrane domain, participate in fusion.

6.1 The Fusion Peptide

Fusion peptides are relatively apolar sequences that interact with membranes and are central to viral fusion reactions (Martin and Ruysschaert 2000; Martin et al. 1999; Skehel et al. 2001; White 1990). They have been

A.

N-terminal		
Class I	Influenza HA2:	GLFGAIAGFIENGWEG
	Sendai F1:	FFGAVIGTIALGVATA
	Resp. Syn. F1:	FLGFLLGVGSAIASGV
	HIV gp41:	AAIGALFLFGLGAAGSTMGAA
Internal		
Class I	Ebola GP:	GAAIGLAWIPYFGPAA
	ASLV gp37:	IFASILAPGVAAAQAL
Class II	SFV E1:	DYQCKVYTGVYPFMWGGAYCFCD
	TBE E:	DRGWGNHCGLFGKGSIVA
unclassified	VSV G:	QGTWLNPGFPPQSCGYATV

B.

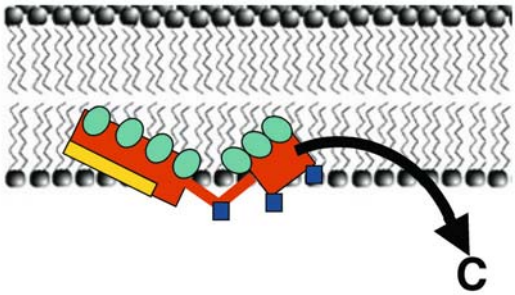

Fig. 5A, B. Characteristics of viral fusion peptides. **A** Selected viral fusion peptide sequences. N-terminal (Skehel et al. 2001) and internal (Delos et al. 2000) fusion peptide sequences are aligned according to their first noncharged residue. **B** Model of HA fusion peptide structure in target membrane at pH 5 (adapted from Tamm et al. 2002). The fusion peptide (*red*) resides in the target membrane in a kinked structure composed of two α-helices, each penetrating the outer leaflet. The glycine ridge is depicted by a *yellow box*, the hydrophobic interior face by *cyan ovals*, and the surface charged residues by *blue squares*. "*C*" *denotes the direction of the HA2 ectodomain*

classified as N-terminal or internal depending on their location within the fusion subunit (Table 1). Although fusion peptides are highly conserved within each virus family, there is little sequence similarity between fusion peptides of different families (Fig. 5A). Generally, however, fusion peptides contain a high percentage of glycines and/or alanines, as well as several critical bulky hydrophobic residues (Martin and Ruysschaert 2000; Martin et al. 1999; Skehel et al. 2001; Tamm and Han 2000; Tamm et al. 2002).

6.1.1 Structure of N-terminal Fusion Peptides

A significant body of work has emerged on the structure and function of synthetic fusion peptides (Martin and Ruysschaert 2000; Martin et al. 1999; Skehel et al. 2001; Tamm and Han 2000; Tamm et al. 2002). Synthetic fusion peptides are disordered in solution but ordered (α-helix and/or β-sheet) when they associate with membranes. The N-terminal fusion peptides that have been studied insert into membranes at oblique angles and do not penetrate the inner leaflet of the membrane. In general, mutations that abrogate fusion reduce the ability of synthetic fusion peptides to insert at oblique angles and to disrupt membranes (Martin et al. 1999). Contradictory conclusions on the precise structure of synthetic fusion peptides in membranes likely stem from the general low solubility of the peptides in aqueous solution and the different experimental methods employed (Tamm et al. 2002).

To circumvent solubility problems, a polar sequence was added to the C-terminal end of the influenza HA fusion peptide, rendering it soluble in both aqueous and hydrophobic environments (Han et al. 2001). At pH 5, the HA fusion peptide consists of an N-terminal helix, a kink, and a short C-terminal helix (Fig. 5B). Both the N- and C-terminal helices penetrate the outer leaflet of the target bilayer. The kink remains at the phospholipid surface; the interior (lipid-facing surface) of the kink is lined with hydrophobic residues. The conserved glycines form a ridge along the outer face of the N-terminal helix. Three charged residues are also found on the outer face (Fig. 5B). An HA in which the conserved glycine at the beginning of the fusion peptide (Gly1) has been changed to valine cannot mediate fusion. If Gly1 is changed to serine, HA mediates only hemifusion or only forms small nonexpanding fusion pores (Qiao et al. 1999; Skehel et al. 2001). Interestingly, these mutant fusion peptides have membrane-associated structures and orientations significantly different from those of the wild-type fusion peptide (Li et al.

2003). Simulations suggested similar membrane penetrating orientations for the HIV fusion peptide and two fusion-defective mutants (Kamath and Wong 2002).

6.1.2 Structure of Internal Fusion Peptides

In addition to a significant number of apolar residues, many internal fusion peptides contain a conserved proline at or near their centers (Fig. 5A). Mutagenesis of this proline in the avian sarcoma/leukosis virus (ASLV) EnvA fusion peptide suggested that it stabilizes a β-turn (Delos et al. 2000). This, coupled with the observation that mutating two cysteines that flank the fusion peptide abolishes fusion activity (Delos and White 2000), suggested that the internal EnvA fusion peptide exists as a looped structure stabilized by a disulfide bond. The ability of the Ebola virus fusion protein, which also contains an internal fusion peptide, to support infection was similarly inhibited when its central proline and flanking cysteines were mutated (Ito et al. 1999; Jeffers et al. 2002). A similar mutation of a proline within the predicted turn segment of the candidate fusion peptide of VSV G also significantly decreased fusion and abolished infectivity (Fredericksen and Whitt 1995). The idea of loop structures for internal fusion peptides is further supported by the known looped structure of the TBE E and SFV EI fusion peptides (Rey et al. 1995; Allison et al. 2001; Lescar et al. 2001). In some cases, two or more noncontiguous sequence loops may function as a collective fusion peptide (Gaudin et al. 1999a; Li et al. 1993).

Like N-terminal fusion peptides, internal fusion peptides contain a significant number of glycines and hydrophobic residues (Fig. 5A). Changing either of two glycines within the SFV E1 fusion peptide to alanines altered the pH threshold for fusion, and changing one of the glycines to aspartic acid abolished fusion (Duffus et al. 1995; Kielian et al. 1996). Alteration of hydrophobic residues at the beginning, middle, or end of the (internal) ASLV EnvA fusion peptide to charged residues impaired the ability of EnvA to mediate fusion (Hernandez and White 1998). Similarly, a tryptophan and a glycine are critical for Ebola GP-mediated infection (Ito et al. 1999). Also, a bulky hydrophobic residue is needed at the tip of the TBE E fusion peptide loop (Rey et al. 1995) (Fig. 4A, red). Collectively, these results suggest that internal fusion peptides function as loops that require a mixture of hydrophobic and flexible residues, similar to those found in N-terminal fusion peptides.

6.1.3
Roles of Fusion Peptides

Fusion peptides appear to act at several steps along the fusion pathway. As demonstrated by mutants in which apolar fusion peptide residues were changed to charged residues (Freed et al. 1992; Gething et al. 1986; Hernandez and White 1998; Schoch and Blumenthal 1993), fusion peptides clearly play an important role in anchoring the fusion protein to the target membrane (Fig. 3, Step 2). The energy provided by inserting the fusion peptides of a single HA trimer into a membrane would be sufficient to initiate stalk formation (Gunther-Ausborn et al. 2000). The fusion peptide may also assist in creating the stalk by displacing water from the lipid-water interface, thus decreasing the repulsive force between the two fusing membranes (Tamm and Han 2000). Fusion peptides may also function in fusion pore opening. In support of this possibility is the observation that an HA mutant in which Gly1 was changed to serine mediates extensive lipid, but not content mixing (Qiao et al. 1999). Furthermore, defects in syncytium formation and infectivity were observed for HIV Env harboring the mutation V2E in its fusion peptide (Freed et al. 1992). Biophysical studies comparing a synthetic fusion peptide harboring this mutation with the wild-type peptide suggested a requirement for fusion peptide aggregation in the creation of the HIV fusion pore (Kliger et al. 1997; Pereira et al. 1995).

6.2
The Transmembrane Domain

Studies with chimeric fusion proteins have suggested that the transmembrane domains of some viral fusion proteins do not require a specific sequence to support fusion (Armstrong et al. 2000 and references therein). In contrast, studies with glycosylphosphatidylinositol (GPI)-anchored fusion proteins have demonstrated that there is a strict requirement for a proteinaceous membrane anchor for fusion proteins to efficiently mediate the transition from hemifusion to full fusion (Kemble et al. 1994; Melikyan et al. 1997a; Tong and Compans 2000). There also appears to be a minimum length for the fusion protein transmembrane domain to be able to support this transition (Armstrong et al. 2000; West et al. 2001). Therefore, it has been suggested that fusion protein transmembrane domains must span both leaflets of the viral bilayer to mediate fusion pore opening (Armstrong et al. 2000).

The transmembrane domains of some fusion proteins appear to have specific amino acid requirements for fusion function. For example, a conserved positively charged residue in the middle of the transmembrane domains of certain retroviral Envs appears to be important for the ability to mediate fusion and infection (Einfeld and Hunter 1994; Owens et al. 1994; Pietschmann et al. 2000; West et al. 2001). Two glycine residues in the transmembrane domain of VSV-G appear to be important for the transition from hemifusion to full fusion (Cleverley and Lenard 1998). Studies using a synthetic peptide corresponding to the mutant VSV-G transmembrane domain (Dennison et al. 2002) suggested that the VSV-G transmembrane domain lowers the energy barrier for fusion and stabilizes the transient fusion pore, thereby promoting its conversion to a stable fusion pore. Two glycines may allow the VSV-G transmembrane domain to adopt alternative conformations under different conditions, and such flexibility may be important for function. The transmembrane domain of HA from the Japan (Melikyan et al. 2000b), but not the X:31 (Armstrong et al. 2000), strain of influenza appears to require a glycine near the middle. An ability to adopt alternative conformations was also invoked to explain the requirement for a proline near the middle of the transmembrane domain of the murine leukemia virus (MLV) Env glycoprotein (Taylor and Sanders 1999).

The observations that mutations in fusion peptides or transmembrane domains (Armstrong et al. 2000; Baker et al. 1999; Tamm et al. 2002) can impair the ability to mediate full fusion (Fig. 3, Step 6) have suggested that both of these apolar domains function in the transition from hemifusion to full fusion. Initially, the fusion peptide appears to insert only into the outer leaflet of the target membrane (Tamm and Han 2000). It has been proposed that the transmembrane domain and the fusion peptide, which are close to each other after membrane merger, may interact to stabilize the fusion pore (Tamm et al. 2002; Zhou et al. 1997). If this is the case, the fusion peptide might span both leaflets of the fused membrane in its final conformation (Tamm et al. 2002).

6.3 The Juxtamembrane Region of the Ectodomain

Several lines of evidence suggest that ectodomain sequences that lie just before the transmembrane domains of certain viral fusion proteins may be important for fusion. These sequences tend to have a high proportion of tryptophans or other aromatic residues and are predicted to partition into the interfacial regions of membranes (Suarez et al. 2000). Indeed,

synthetic peptides containing juxtamembrane ectodomain sequences from HIV Env and Ebola GP partition into the interfacial region of target membranes (Saez-Cirion et al. 2003; Saez-Cirion et al. 2002; Schibli et al. 2001; Suarez et al. 2000). Mutation of three tryptophans within this region of HIV gp41 abrogated infection (Salzwedel et al. 1999), apparently by inhibiting fusion pore enlargement (Munoz-Barroso et al. 1999). Extending the HIV gp41 C-terminal heptad repeat peptide to include the tryptophan-rich juxtamembrane ectodomain sequence appeared to increase the potency of the peptide as an inhibitor of fusion (Kliger et al. 2001). It was suggested that the extended heptad repeat peptide was more potent because it could bind to two sites on HIV Env (the N-terminal coiled-coil and a second, as yet unidentified site) (Kliger et al. 2001). A likely effect of these peptides on late stages of fusion is to prevent formation of a required structure in Env that provides additional membrane destabilization. In this manner, partitioning of juxtamembrane sequences into the interfacial region of membranes may promote the transition from a stalk intermediate to a fusion pore (see Sect. 5).

6.4 The Cytoplasmic Tail

A specific cytoplasmic tail sequence does not appear to be essential, but it can modulate late stages of fusion. The cytoplasmic tail has been shown to influence the transition from hemifusion to full fusion (Sakai et al. 2002) or fusion pore enlargement (Dutch and Lamb 2001; Kozerski et al. 2000) in some viruses. The mechanism by which cytoplasmic tails may influence these later stages of fusion is not known. Some studies using synthetic peptides have suggested a direct interaction between the cytoplasmic tail and the viral membrane (Chen et al. 2001b; Fujii et al. 1992; Gawrisch et al. 1993; Haffar et al. 1991; Kliger and Shai 1997). Others have shown that the cytoplasmic tail can influence the structure of the ectodomain of the fusion protein (Aguilar et al. 2003; Edwards et al. 2002).

The ability of the cytoplasmic tail to affect ectodomain structure is most clearly manifested for those viral fusion proteins that harbor fusion-suppressing sequences. These sequences have been found in the fusion proteins of MLV (Ragheb and Anderson 1994), other type C retroviruses (Bobkova et al. 2002), some lentiviruses (Kim et al. 2003; Luciw et al. 1998), and a paramyxovirus F protein (Tong et al. 2002). The cytoplasmic tail of MLV Env is cleaved during virus budding (Schultz and Rein 1985). MLV Envs harboring uncleaved cytoplasmic tails do not

induce fusion (Yang and Compans 1996). Although viruses lacking fusion-suppressing sequences display increased cell-cell fusion, they are more susceptible to neutralizing antibodies (Januszeski et al. 1997; Li et al. 2001; Rein et al. 1994; Yang and Compans 1996) and are impaired in their ability to sustain multiple rounds of infection (Cathomen et al. 1998; Freed and Martin 1996; Piller et al. 2000).

Acylation of cytoplasmic tails can also affect fusion, apparently at a late stage. For example, a mutant HA from the Japan strain of influenza in which three (normally palmitoylated) cysteine residues were mutated appeared to fuse normally when monitored by dye redistribution assays (Melikyan et al. 1997b). However, electrophysiological measurements revealed that fusion pores formed by the mutant HA did not flicker like those formed by wt-HA (Melikyan et al. 1997b). Similar mutations in HA from the A/USSR/77 (H1N1) and A/FPV/Rostock/34 (H7N1) influenza subtypes were shown, respectively, to inhibit syncytia formation (Fischer et al. 1998) and the transition from hemifusion to full fusion (Sakai et al. 2002). Palmitoylation of HIV Env was also shown to be important for Env incorporation into virions and for infectivity (Rousso et al. 2000). Thus acylation of cytoplasmic tails appears to have multiple effects on viral fusion reactions, the details of which are not completely understood.

7
Rafts in Viral Membrane Fusion

Lipid rafts are plasma membrane microdomains that are enriched in cholesterol and glycosphingolipids with saturated acyl chains. They are organizational platforms for a variety of cellular functions including sorting of membrane proteins and signaling (Brown and London 2000). Although there is growing evidence that certain viruses employ rafts, or raftlike membrane microdomains, during virus assembly (Suomalainen 2002), the question of whether these structures are required at the site of fusion in the target cell is less clear. Here, we consider the role of rafts in the fusion of two enveloped viruses, SFV and HIV. It is important to consider whether cholesterol and/or sphingolipids are required for fusion because they are found in lipid rafts, or if they serve some other purpose. For example, cholesterol may interact directly with the fusion protein, thereby facilitating its insertion into the target membrane. Alternatively, a need for cholesterol and sphingolipids could reflect an ability of raft structures to concentrate viral receptors. A third possibility is that

the cholesterol imparts the membrane fluidity (or other biophysical properties) needed to lower the energy barrier for fusion.

SFV requires cholesterol and sphingolipids in the target membrane for fusion. These moieties enable the SFV spike protein to undergo conformational changes and bind to the target membrane (Ahn et al. 2002; Kielian et al. 2000). In a recent study, it was shown that after hydrophobic association with target bilayers, the SFV glycoprotein ectodomain associates with membrane structures with properties similar to rafts. However, careful studies using liposomes prepared with specific cholesterol and sphingolipid analogs demonstrated that the cholesterol and sphingolipid requirements in the target membrane did *not* correlate with their ability to form lipid rafts (Ahn et al. 2002). A related conclusion was drawn based on the fusion activities of both SFV and Sindbis virus with liposomes (Waarts et al. 2002). For both viruses, the requirement for cholesterol and sphingolipids in the target membrane appears to be for insertion of the fusion peptide (Vashishtha et al. 1998).

In the case of HIV, several studies have suggested a need for raftlike membrane microdomains for virus entry (Kozak et al. 2002; Popik et al. 2002). Depleting plasma membrane cholesterol from target cells resulted in reduced levels of virus infectivity or cell-cell fusion. Other studies have concluded that rafts are not necessary for HIV entry (Percherancier et al. 2003; Viard et al. 2002). In one study, depleting cholesterol from cells that express low levels of virus receptors inhibited HIV Env-mediated cell-cell fusion, but depleting cholesterol from cells that express high levels of virus receptors did not (Viard et al. 2002). Therefore, it was concluded that rafts per se are not needed for fusion. Rather, the presence of raftlike structures in the plasma membrane may concentrate virus receptors. Previous work has shown that a critical density of HIV receptors is required for fusion and infection (Reeves et al. 2002). Clearly more work is needed to clarify the role of rafts in virus-cell fusion and entry.

8
Inhibitors of Viral Fusion

It has recently become apparent, largely because of the success of T-20 in the inhibition of HIV infection in patients (Jiang et al. 2002; Kilby et al. 1998), that fusion is a good target for antiviral intervention. This was originally conceptualized because fusion is an essential early step in the virus infectious cycle, it happens in an exoplasmic space, and strategies

can be designed to inhibit fusion without interfering with host cell proteins. Some fusion inhibitors function by inhibiting six-helix bundle formation. Others function by preventing earlier conformational changes in viral fusion proteins.

8.1 Inhibition of Helix Bundle Formation

The peptide T-20 (also known as Fuzeon) corresponds to the C-terminal helix of HIV Env (Fig. 3, green). T-20 works by preventing six-helix bundle formation. T-20 is a potent inhibitor of infections in tissue culture. Peptides corresponding to equivalent regions of other retroviruses as well as several paramyxoviruses function similarly (Earp et al. 2003; Russell et al. 2001). Notably, all of the viruses that have been shown to be highly susceptible to "C-helix" peptide inhibitors function at neutral pH, at least up to the lipid interacting stage of virus-cell fusion (Earp et al. 2003). Peptides corresponding to the N-terminal helices of HIV Env and the SV5 F protein also inhibit fusion, although with lower potency (Lu et al. 1995; Russell et al. 2001). The mechanism of inhibition by N-terminal peptides is still under consideration (He et al. 2003). In the case of the SV5 F, the N-peptide appears to target an earlier intermediate than the C-peptide (Russell et al. 2001). Other strategies are being considered to stabilize the prehairpin intermediate (Fig. 3, Step 1) and thereby prevent six-helix bundle formation. One strategy is the development of antibodies that recognize the prehairpin intermediate (Golding et al. 2002). Another, exemplified in three studies, is the development of small molecules that prevent six-helix bundle formation (Debnath et al. 1999; Eckert et al. 1999; Ferrer et al. 1999). All three studies targeted a hydrophobic pocket in the groove of the central coiled-coil of HIV gp41 that is important for interaction with the C-terminal helix in the post-fusion form. In the first approach, two organic compounds were identified from a screening effort conducted in conjunction with molecular docking, a method to identify small molecules that fit in a target site (Debnath et al. 1999). The second approach replaced three residues of the C-terminal helix that bind to the hydrophobic pocket with organic moieties, generated by combinatorial chemistry (Ferrer et al. 1999). The third approach used a mirror image phage display library to identify small, D-amino acid containing peptides that bind to the pocket (Eckert et al. 1999). Although none of the small molecules identified to date is as potent as T-20, the precedent has been set for attaining this goal.

8.2
Inhibition of Other Steps in Fusion

An effort to block the fusion activity of influenza was based on the idea that maintaining HA in its native metastable state should prevent fusion and infection. The first trial targeted a site in X:31 HA that includes part of the fusion peptide. With the use of an antibody-based assay to monitor fusion peptide exposure, a compound, *tert*-butylhydroquinone (TBHQ), that prevents the first stages of the HA conformational change and inhibits infectivity was discovered (Bodian et al. 1993). A follow-up study, targeting a site near the B-loop in HA, yielded additional inhibitors. Whereas some functioned like TBHQ, a second class was identified that appeared to push HA to an inactive state (Hoffman et al. 1997). A random screen against an H1 influenza virus identified an inhibitor that appears to function similarly to TBHQ. In the latter case, the binding site for the inhibitor was mapped to the vicinity of the fusion peptide (Cianci et al. 1999). Other small molecules that inhibit conformational changes in HA have been identified (Staschke et al. 1998). To date, none of the HA inhibitors has blocked all HA subtypes and none has an IC_{50} value in the submicromolar range. It is not yet clear whether the latter limitation represents a fundamental difficulty in inhibiting viral fusion proteins that function at low pH.

In addition to the small molecule approaches described above, antibodies that prevent fusion-inducing changes in viral glycoproteins have been described. The first example was an antibody that prevents low-pH-induced fusion of West Nile virus with model liposomes (Gollins and Porterfield 1986). Recently, a Fab fragment that binds to two HA1 monomers was shown to prevent an early conformational change in influenza HA (Barbey-Martin et al. 2002), separation of the globular head domains (Fig. 2B). As described above, antibodies have been developed that likely block six-helix bundle formation in the case of HIV Env (Golding et al. 2002).

9
Perspectives

The goal of this review was to give the reader an appreciation for the diversity of viral fusion mechanisms while recognizing their common underlying principles. We also summarized what is known about the lipid dynamics and lipid structures involved in fusion, and we also briefly

overviewed recent developments in targeting viral fusion as an antiviral strategy. There is clearly much more we need to know about viral fusion proteins, viral fusion reactions, and the design of antifusion agents.

We end this review by enumerating some pressing issues and questions that remain about viral fusion. A major goal is to determine high-resolution structures for the complete ectodomains of the metastable trimers of class I viral fusion proteins in addition to the influenza HA. Structures of a complete paramyxovirus F and a complete retroviral Env ectodomain will be highly informative because we currently lack a detailed molecular description of how a receptor activates any viral fusion protein at neutral pH. A second goal will be to further delineate the mechanisms of class II viral fusion proteins. Do the transitions to their recently described low pH forms (Bressanelli et al. 2004; Gibbons et al. 2004; Modis et al. 2004) mediate hemifusion or fusion pore opening? What about the mechanisms of the as yet unclassified viral fusion proteins? These include viruses such as rhabdoviruses (e.g., VSV) that need only one protein to promote fusion, as well as more complicated viruses such as herpesviruses and poxviruses that require multiple viral glycoproteins.

The ensuing years should also bring a more complete understanding of how viral fusion proteins interact with target membrane bilayers. Class II fusion proteins insert their internal fusion peptides into target membranes as loops (Bressanelli et al. 2004; Gibbons et al. 2004; Modis et al. 2004). It has been predicted that the internal fusion proteins of the class I fusion proteins from Ebola and avian retroviruses form disulfide-bonded loop structures (Weisenhorn et al. 1998), and mutagenesis work has supported this prediction (Delos et al. 2000; Delos and White 2000; Jeffers et al. 2002). It remains to be seen, from high resolution structural studies, whether all internal fusion peptides, be they from class I, class II, or other classes of fusion proteins, interact with target bilayers as (disulfide bond) stabilized loops. Finally, we expect that there will be major developments in furthering the concept of targeting fusion as a weapon against pathogenic enveloped viruses. Particular emphasis will likely be on the development of small molecule inhibitors through the use of combinatorial chemistry in conjunction with high-throughput screens. It will be interesting to learn whether small molecule fusion inhibitors can be identified that block the entry of viruses that fuse in endosomes in response to low pH. This is a challenge for low-pH-activated class I fusion proteins such as influenza HA as well as for all known class II fusion proteins. Stay tuned. There are likely to be exciting develop-

ments in our understanding of viral fusion mechanisms as well as in the development of antifusion antivirals in the years ahead.

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