MECHANISM OF MEMBRANE FUSION BY VIRAL ENVELOPE PROTEINS

Stephen C. Harrison

Children's Hospital, Harvard Medical School, and Howard Hughes Medical Institute
Boston, Massachusetts 02115

I. INTRODUCTION

Enveloped viruses enter cells by fusing their lipid bilayer membrane with a cellular membrane. They bear on their surface oligomers of a fusion protein, often part of a polypeptide that performs other functions, such as receptor binding. Most viral fusion proteins require priming by proteolytic processing, either of the fusion protein itself or of an accompanying protein. The priming step, which often occurs during transport of the fusion protein to the cell surface but may also occur extracellularly, then prepares the fusion protein for triggering by events that accompany attachment and uptake. For example, proton binding is frequently such a trigger, which provides the virus with a mechanism for detecting that it has arrived in the low-pH milieu of an endosome.

Two classes of viral fusion proteins have been identified so far by structural studies. Later, discuss what is known about fusion by members of each of those two classes. The fusion of two bilayers that these proteins catalyze is likely to proceed by the same pathway in both
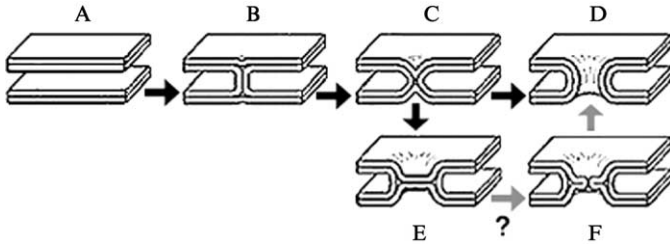
FIG 1. Fusion of two lipid bilayers. (A) Two parallel bilayer membranes. There is a substantial barrier to close approach. (B) Hemifusion stalk. (C) Proposed transition structure. (D) Fusion pore (before lateral expansion). (E) Hemifusion diaphragm. (F) Some models include perforation of the hemifusion diaphragm as a productive step toward fusion pore formation. Adapted from Jahn *et al*. (2003); see also Cohen *et al*. (2002).

cases. That is, these proteins are like enzymes that have different structures but that still catalyze the same chemical reaction.

The bilayer fusion reaction common to all the enveloped viral entry pathways is shown schematically in Fig. 1 (for a review, see Cohen *et al*., 2002). It is believed to pass through an intermediate known as a "hemifusion stalk" (Fig. 1B) (Markin *et al*., 1984; Siegel, 1993). In this intermediate, the two apposed leaflets have fused, but not the distal ones. Hemifused bilayers can proceed to form a "fusion pore" (Fig. 1D) or form a structure in which the two distal leaflets create a single bilayer. This state, which can spread laterally, is called a "hemifusion diaphragm" (Fig. 1E). Bilayers do not fuse spontaneously (e.g., concentrated liposomes are quite stable), because the reaction in Fig. 1 has a high activation barrier, both at the step between the precursor bilayers and the hemifusion stalk and at the step between the hemifusion stalk and the fusion pore. A newly opened pore appears to revert frequently to a hemifusion structure ("flickering"), and the largest kinetic barrier may be for the step in which the pore dilates rather than reverts. Once the fusion pore has dilated, the fused stucture is stable with respect to the initial, unfused structure.

II. CLASS I VIRAL FUSION PROTEINS

The fusion proteins of myxo- and paramyxoviruses, retroviruses, filoviruses, and at least some coronaviruses have sufficient common characteristics to classify and describe them together (Skehel and

Wiley, 1998). They are trimers, with a large, N-terminal ectodomain and a C-proximal transmembrane anchor. The subunit is synthesized as a precursor chain (e.g., HA_0, gp160), which is processed, usually late in the secretory pathway, by a proteolytic cleavage, which generates two associated chains (e.g., HA_1 and HA_2, gp120 and gp41). The carboxy-terminal fragment is the actual fusion effector, and it bears a relatively hydrophobic, glycine-rich "fusion peptide" at or near the cleavage site.

Cleavage of the precursor chain primes the fusion protein, but in HA and gp160 it does not produce major conformational alterations. By contrast, molecular interactions associated with attachment and uptake trigger massive rearrangements. Only for the influenza virus hemagglutinin (HA) do we currently have pre- and postfusion structures, so the extent of rearrangement must be extrapolated from that one example (Skehel and Wiley, 2000). But indirect lines of evidence suggest that the extrapolation is reasonable. The detailed descriptions that follow are restricted to influenza HA and human immunodeficiency virus/simian immunodeficiency virus (HIV/SIV) Env—the two for which the most extensive structural and biochemical data are available at the time of this writing.

A. Influenza A Hemagglutinin

The precursor trimer, HA_0, and the primed HA_1–HA_2 are similar in structure (Fig. 2A,B). The receptor-binding domain, the core of HA_1, is borne on a stalk formed not only by HA_2 but also by the N- and C-terminal segments of HA_1. The loop of HA_0 destined to be cleaved by furin is exposed and partly disordered. After cleavage, the N terminus of HA_2 (the fusion peptide) tucks between the long helices that cluster around the threefold axis (for many references on influenza virus HA before 2000, see Skehel and Wiley, 2000).

Sialic acid, linked to complex glycans on either glycoproteins or glycolipids, is the receptor for influenza virus. Receptor binding does not induce any significant conformational changes in HA. It merely attaches the virus to the cell surface and allows capture by endocytic vesicles. The trigger for a fusion-inducing conformational change is the binding of one or more protons, as the pH of the endosome becomes progressively lower. Most strains of influenza A have a critical pH of about 5–5.5, corresponding to the pH of a relatively late endosomal compartment. No particular titrating residue accounts for the transition; rather, when the charge on the protein destabilizes it sufficiently,
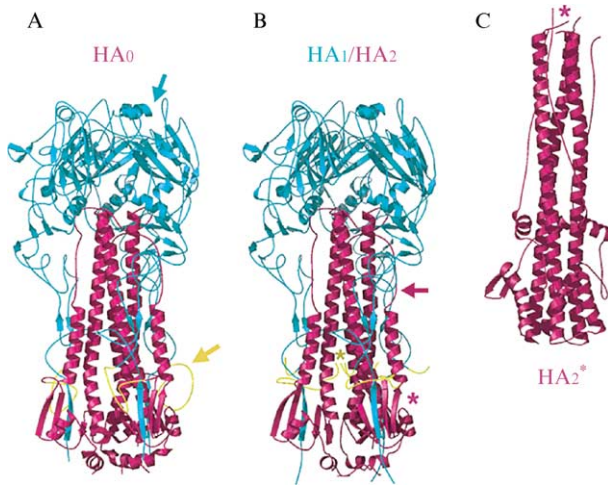
FIG 2. The influenza virus hemagglutinin. (A) HA$_0$, before cleavage between HA$_1$ and HA$_2$. The HA$_1$ part of the protein is blue; the HA$_2$ part, is purple. The fusion peptide, looped out before cleavage, is yellow. The sialic acid-binding site and the cleavage point are shown by blue and yellow arrows, respectively. (B) The mature HA after cleavage but before low-pH triggering. The only change from the structure in (A) is insertion of the fusion peptide (the N terminus of HA$_2$, in yellow) into a crevice along the three-fold axis (dark yellow asterisk). A purple arrow points to the loop between the shorter, N-proximal helix and the longer, central helix. A purple asterisk indicates the position of residues that will move to the top of the molecule during the low pH-induced transition. (C) HA$_2$ after exposure to low pH. The same structure can be obtained by refolding HA$_2$ expressed in *E. coli*. It is the minimal free-energy state of HA$_2$ unconstrained by covalent association with HA$_1$. The long loop in the prefusion structure (purple arrow in [B]) has now become helical, elevating the N terminus of the protein (the fusion peptide itself is not included in this structure) to the top of the molecule (purple asterisk). A break and reversal of direction in the central α-helix of the prefusion trimer likewise projects the C terminus of the protein to the top. The figure is aligned with respect to (B) so this break is roughly at the same height in both panels. In the actual transition, the chain reversal is likely to occur by melting and rezipping of the C-terminal helical segment, as shown in Fig. 3C. For detailed references, see Skehel and Wiley (2000).

a cooperative conformational rearrangement ensues (Daniels *et al.*, 1985).

The conformational change triggered by proton binding (Fig. 2B,C, and Fig. 3) has the following characteristics (Skehel and Wiley, 2000). (1) The HA$_1$ receptor-binding domains separate from each other. Because HA$_1$ is linked to HA$_2$ by a disulfide bond, it cannot dissociate completely, but the final conformation of HA$_2$ requires that HA$_1$
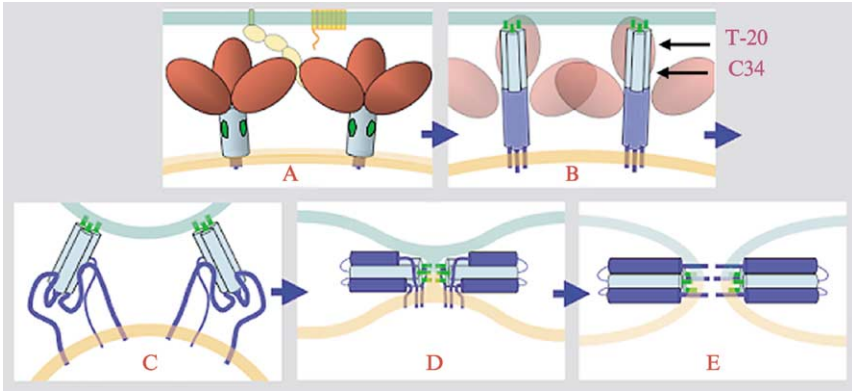
F<small>IG</small> 3. Diagram of membrane fusion mediated by class I viral fusion proteins. (A) Receptor binding (shown here for HIV or SIV Env, where the schematic receptor and coreceptor symbolize CD4 and CXCR4 or CCR5). (B) Dissociation of the receptor-binding domain (in the case of HA, a disulfide bond prevents complete dissociation, but the structural rearrangement requires that HA_1 move away from the threefold axis) and projection of the fusion peptide toward the target cell membrane. This state is known as the "prefusion intermediate" (or, sometimes, the "prehairpin intermediate"). The arrows show the positions along the core helical bundle at which, on HIV-1 gp41, the inhibitor peptides T-20 and C34 are expected to bind. (C) Folding back of the C-terminal part of the molecule. This zipping up of a segment of the fusion protein sometimes designated "helical region 2" (HR2); helical region 1 (HR1) forms the inner core of the postfusion trimer) draws the two membranes together as the N-terminal fusion peptide, inserted into the target cell membrane, and the C-terminal transmembrane segment, which is anchored in the viral membrane, are forced by the refolding to approach each other. (D) Hemifusion stalk formation. Provided that they insert only into the outer leaflet of the bilayer, the fusion peptides can migrate into the stalk, as proposed here, and stabilize it. (E) Fusion pore formation. Hemifusion structures flicker transiently into unstable pores, which reseal. The pore can be trapped by the final refolding step, in which the three transmembrane segments snap into place around the inserted fusion peptides. In the case of HA, this step may be driven by formation of a set of interactions that cap the inner core helices.

move away from the threefold axis to allow HA_2 to form new threefold contacts. (2) HA_2 straightens by a loop-to-helix transition of a segment previously tucked beneath HA_1 (Bullough *et al*., 1994; Carr and Kim, 1993). This rearrangement thrusts the N-terminal fusion peptide up (using the coordinates of Fig. 2 as a reference for "up" and "down"), where it can encounter the membrane of the target cell. The extended intermediate, with fusion peptides inserted into the target cell membrane, may have a lifetime of seconds to minutes. (3) The long helix of HA_2 breaks and reverses direction, and the distal part of the HA_2 chain

zips up along the stem formed by long central helix, which now runs from the fusion peptide to the breakpoint. In its final conformation, the HA_2 ectodomain has its N terminus, which bears the fusion peptide, adjacent to its C terminus, which leads to the transmembrane anchor (Chen *et al.*, 1999). In other words, the two membrane-interacting segments on each chain are next to each other. Thus, assuming that all three fusion peptides insert into the target membrane and that the C-terminal transmembrane segments remain anchored in the viral membrane, the zipping up of the distal part of HA_2 will drag the two membranes together (Fig. 3).

Only the inital (Fig. 2B) and final (Fig. 2C) conformations of HA_2 can be studied easily by structural methods. Evidence for the pathway described previously and shown diagrammatically in Fig. 3 comes from biophysical studies of HA-mediated fusion and from the effects of various mutations on the fusion process (reviewed extensively elsewhere and outlined only in summary here).

1. *The low-pH trigger*. The threshold pH for fusion can be affected by a variety of amino acid substitutions, and some that increase the threshold pH can be selected by isolating virus resistant to amantadine (which raises the pH of endosomes). These mutations map in a variety of locations, which have in common that they appear to contribute to interactions that hold together the HA trimer (Daniels *et al.*, 1985). Thus, there appears not to be a single, localized trigger, but rather a cumulative effect of various groups that bind protons at pH 5 and above.

2. *Fusion peptide emergence and insertion*. The amino acid sequence of the loop in HA_2 has a particularly strong helical coiled-coil signature, and peptides with this sequence form stable coiled coils in solution (Carr and Kim, 1993). Release of the constraints imposed on the conformation of this peptide by HA_1 is therefore likely to lead to coiled-coil formation and translocation of the fusion peptide away from the base of the trimer. The phrase "spring-loaded" has been used to describe this mechanism (Carr and Kim, 1993). Experiments with target membranes containing photoactivatable cross-linking reagents demonstrate insertion of the fusion peptide into the target cell membrane, probably before fusion (Tsurudome *et al.*, 1992). A peptide having the amino acid sequence of the first 20 residues of HA_2 forms a gently kinked, amphipathic helix in the presence of detergent micelles (Han *et al.*, 2001). On a membrane, this structure would lie partially embedded in the outer monolayer, with the two ends dipping somewhat more deeply into the bilayer outer leaflet than the apex of

the kink. That is, the fusion peptide probably inserts only into the proximal leaflet of a membrane lipid bilayer. The effects of mutations in the fusion peptide vary with position (Qiao *et al*., 1999; Steinhauer *et al*., 1995). They do not correlate in any simple way with the structure just described, but it may be difficult to deconvolute the requirements for its conformation in the mature HA_1–HA_2 trimer from those for productive membrane insertion.

3. *Fold-back*. The best evidence for an extended "prefusion intermediate," with the fusion peptides inserted into the target cell membrane but with the C-terminal parts of the fusion protein still at the opposite end of the molecule, comes from work on HIV-1 gp41 (Furuta *et al*., 1998; Rimsky *et al*., 1998) (see later). In the mature HA trimer at neutral pH, the long axial helices diverge from each other at about the position at which the folding back occurs (Fig. 2B,C), and it is plausible that in the extended intermediate, the "lower" part of the molecule is unstable and locally unfolded. There has been some confusion in the literature about the folding back process. Although the expressions "fold back" and "jack-knifing" have been used to describe the conformational change, the only way it can plausibly occur in practice is by melting at least some of the C-terminal segments of the HA_2 ectodomain and zipping up these segments along the central core (Fig. 3). If the links between the fusion peptides and the central core are flexible, then the zipping process will necessarily displace the position of the chain reversal (the fold-back point) laterally, away from the constriction. During the transition from the extended intermediate to the folded-back conformation, threefold symmetry is broken; it is restored only in the final, postfusion structure.

The free energy required to deform the two membranes, squeeze them together, and initiate fusion is probably more than can be obtained from refolding of a single trimer, and it is likely that several HA trimers cooperate to form a fusion pore. Evidence for this statement includes the observation that the lag time between acidification and fusion of cells expressing HA on their plasma membranes depends on the HA surface density (Danieli *et al*., 1996). One picture for the process would invoke formation of a structurally defined ring of extended intermediates, surrounding the position at which fusion will occur. An alternative, more stochastic model, is also possible, as described in more detail later, in Section IV.A.

4. *Hemifusion*. The fold-back step brings the two membranes close together. Are there further structural features that accelerate hemifusion? Distortion of one or both membranes by introduction of positive curvature would be one way to do so; stabilization of a hemifusion stalk

once formed, thereby preventing reversal, would be another. We need to know more about the conformation and properties of an inserted fusion peptide before we can assess these possibilities. The discovery, that class II fusion peptides insert only into the outer part of the target membrane, suggests a possible mechanism for hemifusion stalk stabilization (see later).

5. *Fusion pore formation*. A noteworthy feature of the rearranged structure of HA_2 ectodomain is a tight "cap," formed by the way the C-terminal part of the polypeptide chain associates with the N-terminal end of the central, three-helix bundle (Fig. 2C) (Chen *et al*., 1999). This cap is likely to snap the folded-back conformation into place. In the model for the fusion reaction diagrammed in Fig. 3, the final step involves stabilization of the fusion pore by interposition of the trans-membrane anchors of HA_2. Formation of the HA_2 cap may be a critical feature of this step. When HA is linked to a glycophosphatidylinositol (GPI) anchor or to a truncated transmembrane anchor that does not cross the bilayer, it catalyzes hemifusion quite readily, but it promotes fusion poorly, if at all (Kemble *et al*., 1994; Melikyan *et al*., 1995). Catalyzing fusion does not require a specific structure or amino acid sequence on the inside of the membrane; the transmembrane anchor must simply traverse the bilayer completely (Armstrong *et al*., 2000). Truncation of the SV5 fusion protein produces a similar result (Dutch and Lamb, 2001). Snapping the HA_2 cap into place and dragging the cytosolic end of the transmembrane anchor into a newly opened pore will prevent the pore from resealing (which it will otherwise tend to do) and force the fusion reaction to proceed to completion.

B. Influenza C HEF

The fusion protein of influenza C, known as HEF (for hemagglutinin, esterase, fusion protein) contains, in addition to the receptor-binding (hemagglutinating) and fusion activities, an esterase domain, which hydrolyzes the 9-*O*-acetyl-sialic acid receptor, to promote viral escape from the cell surface. With the exception of the added esterase module, the protein is recognizably similar to the HA of influenza A (Rosenthal *et al*., 1998) (Fig. 4). The esterase domain resembles in structure other acetyl hydrolases. In the amino acid sequence, it falls in two parts— one at the N-terminal end of the sialic acid-binding domain and the other at the C-terminal end. That is, the receptor-binding domain is an insert into the esterase, which in turn is an insert between the N- and C-terminal segments of HEF_1. One can thus imagine that
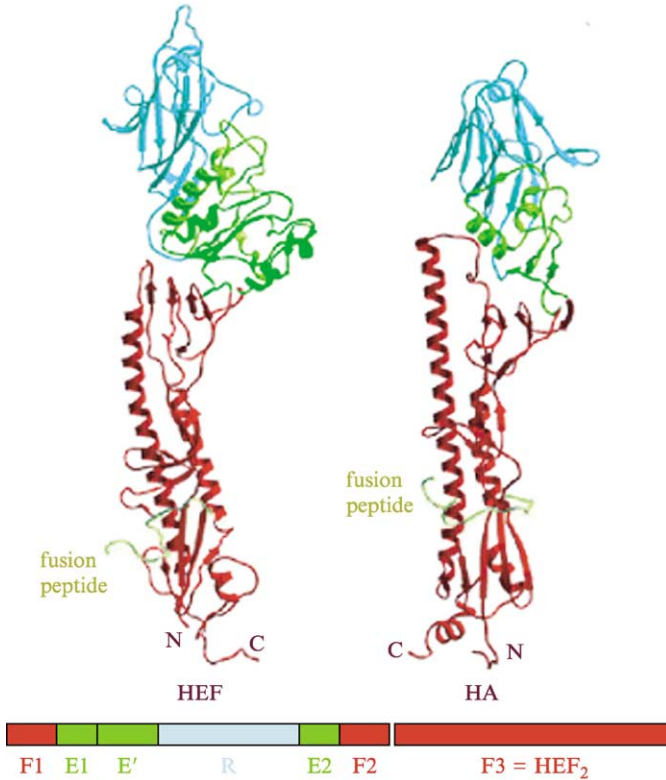
FIG 4. *Top*: The ectodomain of influenza virus C HEF and its structural and apparent evolutionary relationship to influenza A HA. *Bottom*: A key to the color scheme for the HEF polypeptide chain. The N- and C- terminal segments of HEF_1 and all of HEF_2 (except for the fusion peptide) are in red. The acetylase enzymatic domain ($E1 + E'' + E2$) is in green; the receptor-binding domain (R) is in blue. Think of the red fragments as an elementary fusion protein and the green and blue fragments as insertions. In HA, most of the acetylase domain has been deleted, except for the E′ fragment, which becomes an adaptor to connect the elementary fusion protein with the receptor-binding domain. Adapted from Rosenthal *et al*. (1998).

the N- and C-terminal segments of HEF_1 (or HA_1) and the entirety of HEF_2 (or HA_2) constitute an elementary fusion module, into which other functions have been inserted in the course of evolution (Rosenthal *et al*., 1998; Skehel and Wiley, 2000).

C. HIV and SIV Env

The HIV and SIV envelope glycoproteins have two essential functions in viral entry. They attach the virus to target cells and they catalyze membrane fusion. The single-chain envelope glycoprotein precursor, gp160, is cleaved after trimerization by a furin-like protease in a late compartment of the export pathway (Allan *et al*., 1985; Robey *et al*., 1985; Veronese *et al*., 1985). The two fragments, gp120 and gp41, remain associated noncovalently, but the contact is relatively weak, and gp120 tends to dissociate ("shed") from mature virions (Moore *et al*., 1990).

Fusion occurs at the cell surface and does not require a change in pH. Rather, infection of suitable cell types is determined by a requirement for both a primary receptor, CD4 (Dalgleish *et al*., 1984), and a coreceptor, one of several members of the chemokine receptor family (Feng *et al*., 1996). Binding of CD4 induces a conformational change in gp120/gp41, detected by altered antigenic properties, enhanced proteolytic sensitivity of the gp120 moiety, and enhanced shedding (Sattentau and Moore, 1991; Sattentau *et al*., 1993). The change increases affinity of gp120/gp41 for the coreceptor, probably by organizing the site for coreceptor binding (Trkola *et al*., 1996; Wu *et al*., 1996). Coreceptor attachment leads to fusion, probably because the coreceptor induces gp120 dissociation, fusion peptide exposure, and gp41 refolding (Chan *et al*., 1997; Weissenhorn *et al*., 1997). The diagram in Fig. 5 summarizes current thinking.

Direct data are available for only two of the postulated structures in the complete envelope protein transformation. These show gp120 complexed with CD4 and the Fab from monoclonal antibody (mAb) 17b (Kwong *et al*., 1998) and the rearranged form of the gp41 ectodomain (Chan *et al*., 1997; Weissenhorn *et al*., 1997). Both structures correspond to postfusion states (or at least to posttriggering states). Both structures are also of molecules truncated in important ways to facilitate structural studies. Thus, the gp120 "core" that has been crystallized (in complex with CD4 and the Fab from mAb 17b) is simply the receptor- and coreceptor-binding element, stripped of nonessential variable loops and lacking N- and C-terminal segments. These terminal segments of gp120 can be thought of as part of the basic fusion module, by analogy with influenza A HA and influenza C HEF (Pollard *et al*., 1992) (Fig. 6). The soluble form of the gp41 ectodomain (Blacklow *et al*., 1995; Lu *et al*., 1995), studied in several crystal forms (Chan *et al*., 1997; Tan *et al*., 1997; Weissenhorn *et al*., 1997) and also by nuclear magnetic resonance (NMR) (Caffrey *et al*., 1998), lacks the

$$(gp160)_3 \xrightarrow{\text{(furin)}} (gp120/gp41)_3 \xrightarrow{\text{CD4}} (gp120^\dagger/gp41)_3{:}CD4 \xrightarrow{\text{chR}} (gp120^\ddagger/gp41)_3{:}CD4{:}chR \longrightarrow 3\ gp120^*$$

$$(gp41)_3 \longrightarrow (gp41^\dagger)_3 \longrightarrow (gp41^*)_3$$
$$\text{"prefusion intermediate"}$$

$$\text{Overall: } (gp160)_3 \longrightarrow 3\ gp120^* + (gp41^*)_3$$
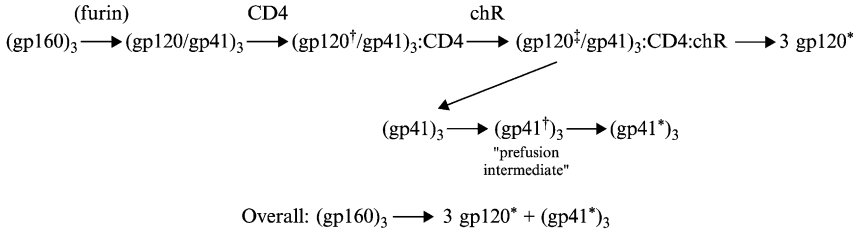
FIG 5. States of the HIV-1 envelope protein, as detected by biochemical, immunological, physicochemical, and structural analyses. The trimeric gp160 precursor is cleaved by a furin-like protease to gp120 and gp41, which retain their threefold association. Binding of CD4 (one per trimer may be sufficient, but the degree of cooperativity is not determined) induces a conformational change in gp120 ($gp120^\dagger$); binding of a suitable chemokine receptor (chR) may induce or lock in a further change ($gp120^\ddagger$). Ultimately gp120 is probably shed from the gp41 stem ($gp120^*$). Once liberated, the gp41 trimer undergoes a two-stage, fusion-inducing conformational change: a transition to an extended, prefusion intermediate, $(gp41^\dagger)_3$, with the fusion peptide inserted into the target cell membrane, followed by a folding back to form the final, trimer-of-hairpins structure $(gp41^*)_3$, generating membrane fusion in the process.

first 30 residues of gp41, that is (the fusion peptide plus some residues that follow it) and (in most cases) a loop in the middle.

The structure of the gp120 core bound with CD4 and the Fab of mAb 17b has been determined for two different HIV-1 isolates (Kwong *et al.*, 1998, 2000) (Fig. 7). The affinity of gp120 for mAb 17b is enhanced by CD4 binding, and the antibody footprint is thought to mark the co-receptor site (Rizzuto *et al.*, 1998). Thus, the crystal structure probably shows the released form of gp120 (Fig. 5).

In what is generally agreed to be its final, postfusion conformation, the ectodomain of gp41 is a trimer of α-helical hairpins, as shown in Fig. 8. The crystal structures do not contain the residues immediately proximal to either membrane (or to the single, fused membrane at the end of the fusion process), including a number of residues critical for inhibition by the peptide drug DP178/T-20/enfuvirtide (Wild *et al.*, 1994). The prefusion conformation of gp41 and the unliganded structure of gp120 remains undetermined, and we therefore know rather little about how they might fit together into the gp160 trimer.

From the available data, what can we deduce about the conformational changes that lead to fusion? The rearrangements in gp120 that initiate the process are probably quite extensive (Myszka *et al.*, 2000). The receptor-binding core folds into two domains, termed "inner" and "outer" (Fig. 7). One hairpin loop from each of these
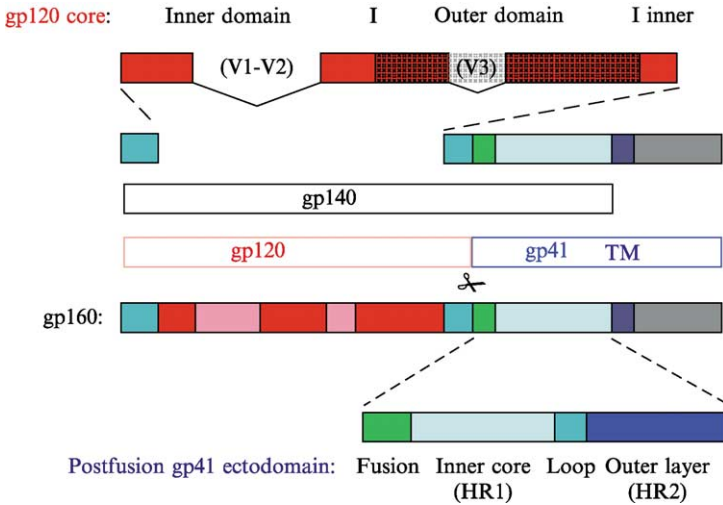
F<small>IG</small> 6. Primary structure of the HIV and SIV envelope proteins. The bar labeled "gp160" represents, schematically, the regions of the envelope precursor. The parts of gp160 corresponding to gp120, gp41, and gp140 (the gp160 ectodomain) are shown as open bars. The scissors symbol shows the furin cleavage point. The gp120 core can be considered a receptor-binding insertion into an elementary fusion protein, as diagrammed in the top two bars. Compare with Fig. 4—the gp120 core is the analog of the R and E′ regions of influenza HA. The principal elements of the gp41 ectodomain are diagrammed in the bottom bar.

domains contributes to the "bridging sheet," a β sheet that locks the relative positions and orientations of the two domains and that creates the site for coreceptor interaction. It has been suggested that the bridging sheet is not present before encounter with CD4 and that receptor binding locks the sheet into place (Kwong *et al.*, 1998). The inner and outer domains could thus have quite different relative orientations in the precursor conformation. The inner domain contains both N and C termini of the gp120 core. They are essentially adjacent to each other in the structure, consistent with the notion that the receptor-binding element is an insert into an elementary fusion module (compare with Fig. 4).

The conformational changes induced by CD4 binding weaken interactions with the rest of the trimer and lead to gp120 dissocation. This event in turn liberates gp41 to rearrange. The likely rearrangement, leading to the final trimer of hairpins, follows the mechanism already outlined for influenza HA and shown in Fig. 3. The N-terminal helices
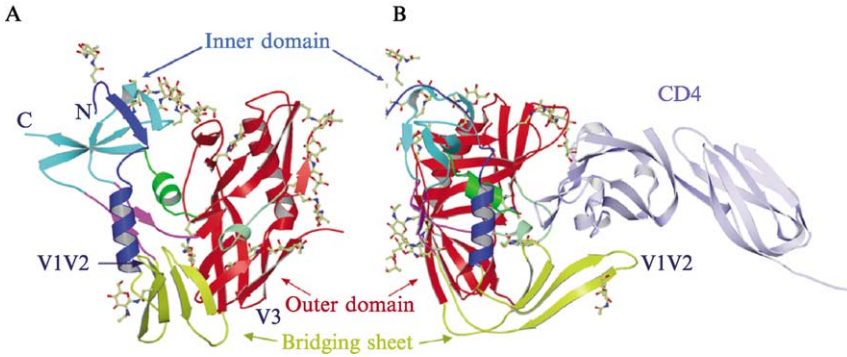
FIG 7. Structure of the gp120 core in the CD4-bound state (Kwong *et al*., 1998). (A) Ribbon representation, showing the inner and outer domains, linked by a bridging sheet. The locations of the V1–V2 and V3 loops, deleted from the core construct (compare with Fig. 6), are shown. The locations of carbohydrate chains are shown by molecular ball-and-stick representations of those sugars found to be well-ordered in the crystal structure. The view is into the CD4-binding pocket. N and C termini of the core are labeled. (B) Side view of the same structure, with the first two immunoglobulin-like domains of CD4 also shown.

of each hairpin cluster into a three-helix coiled coil, forming the inner core of the rearranged gp41. The fusion peptides project from the N-terminal end of this inner core. The C-terminal helix of each hairpin runs along the outside of the core, so that the transmembrane anchor at which it terminates lies adjacent to a fusion peptide. Although missing from the crystal structures, the loop between inner core helix and outer layer helix probably has a structure that resembles the conformation seen in fragments of the fusion proteins from Moloney murine leukemia virus (Fass *et al*., 1996) and Ebola virus (Weissenhorn *et al*., 1998), both of which have a conserved disulfide bond in this region, in common with HIV and SIV Env.

Peptides derived from the outer layer of rearranged gp41 inhibit fusion and viral infectivity by targeting the intermediate shown in Fig. 3B (Rimsky *et al*., 1998). Evidence for the properties of this intermediate comes from kinetic studies, in which binding of outer layer-derived peptides has been analyzed by detecting their effects on fusion (Furuta *et al*., 1998; Jones *et al*., 1998; Munoz-Barroso *et al*., 1999). The intermediate has a measurable lifetime. For example, in cell–cell fusion assays, conformational changes in gp120/gp41 begin within 1 min of mixing donor and target cells, but a peptide corresponding to residues in the outer layer helix inhibits fusion even if added 15 min later (Munoz-Barroso *et al*., 1999). Two classes of outer layer peptide

FIG 8. The inner core (HR1) and outer layer (HR2) of the gp41 ectodomain in the postfusion state of the gp41 trimer (Weissenhorn *et al.*, 1997). The structure as determined crystallographically contains six peptides—three inner and three outer. The dashed lines show covalent connectivities that would be present in the intact gp41 trimer. The residue numbers (for gp41, counting from the N terminus of the fusion peptide) for the beginning and end of the inner and outer layer helices are shown for the red subunit.

have been studied. One includes residues in the stretch just preceding the transmembrane segment (e.g., DP178/T-20/enfuvirtide, now a licensed antiretroviral drug [Kilby and Eron, 2003; Wild *et al.*, 1994]). The other includes residues toward the N terminus of the outer layer helix (e.g., a peptide called C34; Chan *et al.*, 1998). These two classes may block fusion at slightly different stages. There is some evidence that C34-like peptides block both hemifusion (lipid mixing) and full fusion (content mixing), whereas T-20-like peptides block only the

latter (Kliger *et al.*, 2001). Figure 3 is consistent with this difference. According to Fig. 3, early stages in zipping up the outer layer, blocked by C34, would be required to bring together the two membranes and hence for the initial hemifusion step; later stages would probably be required primarily to complete the process and to lock the open fusion pore in place.

Peptides from the inner core of rearranged gp41 also inhibit fusion and viral infectivity, probably by trimerizing and capturing outer layer segments before the zipping-up step (Jiang *et al.*, 1993; Lu *et al.*, 1995; Wild *et al.*, 1992). The inner core is quite insoluble, and a more efficient way to study this mode of inhibition is to cover two of its three grooves with outer layer peptides, allowing one groove free to capture an outer layer segment from gp41 as it refolds. A single-chain version of such a structure (in which three inner layer segments, interspersed with two outer layer segments, have been concatenated with suitable short linkers) is indeed an effective inhibitor of gp41-mediated fusion and of HIV-1 infectivity (Root *et al.*, 2001).

Inspection of the trimer of inner core helices shows that there is a particularly striking hydrophobic pocket on each of the three symmetric surfaces of the three-helix bundle, near the end distal to the fusion peptide (Chan *et al.*, 1997). This pocket is occupied in the final, post-fusion structure by Trp-117 and adjacent residues from an outer layer helix. A 16-residue cyclic peptide synthesized from D-amino acids can bind tightly at this site and in so doing inhibit fusion and HIV infectivity (Eckert *et al.*, 1999). This pocket is also the target of a small molecule selected from a biased combinatorial library, synthesized onto the N terminus of a partial outer layer peptide (Ferrer *et al.*, 1999; Zhou *et al.*, 2000).

III. Class II Viral Fusion Proteins

The fusion machinery of flaviviruses and alphaviruses resembles that of the class I fusion apparatus in certain broad respects: it requires activation by proteolytic cleavage and it uses the reduced pH of an endosome as a trigger for conformational change. The cleavage does not, however, modify the fusion protein itself, but rather a second, "protector" protein, which in its uncleaved state blocks the conformational rearrangement. Moreover, the fusion peptide is a loop within the folded polypeptide chain, and we refer to it here as the "fusion loop."

Until structures were determined (Lescar *et al.*, 2001; Rey *et al.*, 1995), the similarity of alphavirus and flavivirus surface proteins was

not evident. During alphavirus infection, proteins pE2 (precursor of E2) and E1 are synthesized, and pE2 is cleaved to E2 plus E3 during transport of a heterodimer to the cell surface. Budding occurs at the plasma membrane. Some alphaviruses retain E3; others shed it. During flavivirus infection, proteins prM (presursor of M) and E are incorporated into immature virions, which bud into the endoplasmic reticulum. Processing of prM to M (the C-terminal fragment dissociates) occurs late in the export pathway. The structures of E1 and E are very similar; those of pE2 and prM are not yet known. E1 and E2 assemble into a T = 4 icosahedral lattice on the surface of an alphavirus particle; E also forms an icosahedral array, with 180 subunits (90 dimers), but not one with a quasi-equivalent design (Kuhn *et al*., 2002).

A. *Flaviviruses*

The envelope protein, E, does not project from the viral membrane like the "spikes" of many viruses; instead, it forms a relatively thin, tightly packed layer on the virion surface (Fig. 9A; note that sequence similarities among all flavivirus envelope proteins allow us to apply structural results from one virus to analysis of another) (Kuhn *et al*., 2002; Rey *et al*., 1995). A central, β-sandwich domain (domain I) organizes its folded structure (Fig. 9B) (Modis *et al*., 2003; Rey *et al*., 1995). Two long and elaborate loops, stabilized by disulfide bridges, emanate from this central domain, forming a distinct subdomain termed domain II. At the tip of domain II is a hydrophobic sequence, identified from its conservation among all flaviviruses as the fusion loop (Allison *et al*., 2001). A third domain (domain III) follows the others in the polypeptide-chain sequence; it has an immunoglobulin-like fold, and various lines of evidence indicate that it binds receptors (at least in the case of several, well-studied flaviviruses). Between domain III and the transmembrane anchor are about 50 amino acid residues—a segment that has been called the "stem" (Allison *et al*., 1999). Although not a part of the protein that crystallizes, its conformation on the virion has been deduced from cryo-EM image reconstructions. It is a pair of α helices with an intervening loop, sandwiched between the main part of the E protein and the outer surface of the lipid bilayer (W. Zhang *et al*., 2003). The transmembrane anchor is also a helical hairpin, as the virus relies on signal protease (in the endoplasmic reticulum [ER] lumen) to cleave E from a precursor polyprotein.

E is a dimer on the surface of a mature virion (Heinz *et al*., 1991; Kuhn *et al*., 2002). Its stable (stemless) ectodomain ("soluble E" or sE, comprising domains I, II, and III—approximately 395 of the 450
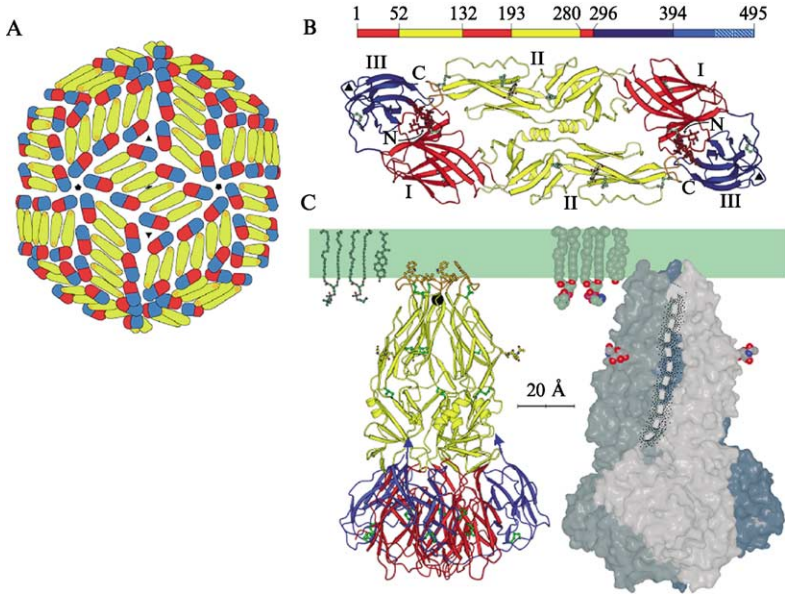
FIG 9. The flavivirus fusion protein E. (A) Organization of E dimers in the virion surface. Each subunit is shown in three colors: domain I in red, domain II in yellow, and domain III in blue—based on the structure described by Kuhn *et al.*, (2002). (B) The soluble ectodomain, sE of dengue virus type 2, in the dimeric prefusion conformation found on mature virions (Modis *et al.*, 2003). The domain colors are as in (A). The bar above the ribbon diagram shows the relationship of domains to primary structure. The "stem" segment between residue 394 and the transmembrane anchor is not included in the three-dimensional structure. (C) The sE trimer (Modis *et al.*, 2004). The proteins are shown in relation to a schematic lipid bilayer to illustrate the likely degree of penetration of the fusion loops (*top*) into the membrane. The ribbon diagram (*left*) is colored as in (A) and (B). Arrows at the C terminus of the polypeptide chain suggest its presumed continuing direction. The surface rendering (*right*) includes a dashed arrow to show the proposed course of the stem peptide, which would lead to the transmembrane anchor.

residues that lie outside the viral membrane) is also a dimer in solution at reasonable concentrations (Heinz *et al.*, 1991). The fusion loop at the tip of domain II lies at the dimer interface, sequestered from potential membrane insertions until lowered pH triggers rearrangement (Rey *et al.*, 1995). On the immature virion, E and prM form heterodimers, which in turn form somewhat asymmetric trimer clusters, and prM (rather than another E) appears to protect the fusion loop from exposure (Y. Zhang *et al.*, 2003). Cleavage of prM releases all but 40 ectodomain residues, leading to dimerization of E and formation of the tightly packed surface array (Heinz *et al.*, 1994).

When exposed to lowered pH, E rearranges yet again into trimers (Allison *et al*., 1995). The rearranged, presumably postfusion, structure of sE has been determined [for dengue virus type 2 (Modis *et al*., 2004) and for TBE virus (Bressanelli *et al*., 2004)] by taking advantage of the observation that sE dissociates reversibly to monomers in solution at low pH, but trimerizes irreversibly if liposomes are present (Stiasny *et al*., 2002). The conformational change involves reorientations of the three domains with respect to each other and probably an extension of the stem (not present in the sE trimer that crystallizes; Fig. 9C). The clustered domains II present their fusion loops at one end of the trimer. The loops themselves have essentially the same conformation as they do in the prefusion dimer, and the extent of their joint hydrophobic surface leads to the conclusion that they probably interact only with the outer leaflet of the target membrane bilayer and that they can dip by no more than 5 to 10-Å into its hydrophobic region. The reorientation of domain II with respect to domain I is a rotation of about 20°. The reorientation of domain III is substantially greater; it flips over in such a way that its C terminus now projects back toward the fusion loops. The structure leads directly to the proposal that in the fusion rearrangement of intact E, the three stems extend along the lateral faces of the clustered domains II, forcing the C-terminal transmembrane segments to approach the fusion loops. This feature of the conformational transition accomplishes essentially the same result as the fold-back of class I viral fusion proteins: it brings together the two membrane-associated parts of the protein and thus can cause the target cell membrane, into which the fusion loops are presumably inserted, to approach the viral membrane, into which the transmembrane anchor penetrates (Fig. 10).

Structural analysis of the rearrangements that E undergoes during fusion suggests at least two distinct strategies for discovering fusion inhibitors. One follows from the analogy with class I viral fusion and the approach embodied in the development of T-20/enfuvirtide: use of a peptide containing sequences derived from the stem. Provided that the stem interacts as strongly with the threefold clustered domains II as the outer layer helices of gp41 interact with the trimeric core, then stem-derived peptides would be expected to frustrate the zipping-up step (Fig. 10D), just as T-20 or C34 frustrate the zipping up of gp41. A second strategy follows from the observation that a detergent molecule can occupy the hydrophobic pocket at the domain I–domain II interface (Modis *et al*., 2003). This pocket disappears during the fusion rearrangement, so small molecules that lodge there would probably retard refolding.
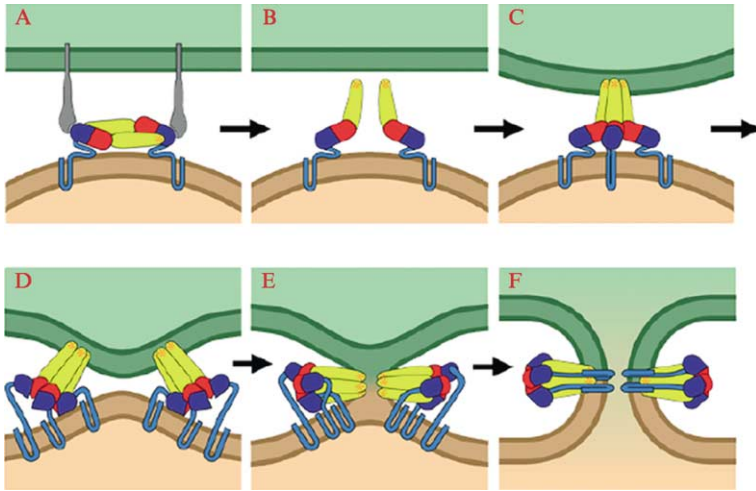
FIG 10. Diagram of membrane fusion by class II viral fusion proteins. (A) Receptor binding through domain III of E (flaviviruses). (B) Lowered pH in an endosome leads to dissociation of the dimer interactions. On release of dimer constraints, monomers can flex outward, presenting their fusion loops to the target cell membrane. (C) Insertion of the fusion loops into the target cell membrane and initial formation of trimer contacts among the projecting domains II. (D) Domain III flips over and the stem zips up along the outside of the trimer. (E) Hemifusion stalk. The diagram shows a proposed role for the inserted fusion loops—stabilization of the hemifusion stalk. (F) Formation of a fusion pore. Completing the zipping up of the stem drives fusion forward, because the cytosolic tails enter the pore and commit it to dilation.

B. Alphaviruses

The structures of two representative alphaviruses—Sindbis virus and Semliki Forest virus (SFV)—have been examined in considerable detail by electron cryomicroscopy (Lescar *et al.*, 2001; Pletnev *et al.*, 2001; Zhang *et al.*, 2002), and crystal structures of the E1 protein from SFV have been obtained, both for the pre- and postfusion forms (Gibbons *et al.*, 2004b; Lescar *et al.*, 2001). E1 has the same architecture as flavivirus E. Each of the domains in E1 resembles closely its E counterpart, including the position and conformation of the fusion loop (Fig. 11). The stem segment of E1 is substantially shorter (30 residues instead of 55). The E1 domain III does not bind receptor; this function resides instead on E2. The soluble ectodomain fragment, E1*, which lacks all but about 10 residues of the stem, is a monomer.

E1 and E2 are a closely associated heterodimer on the surface of the virion (Lescar *et al.*, 2001; Pletnev *et al.*, 2001; Zhang *et al.*, 2002). Their
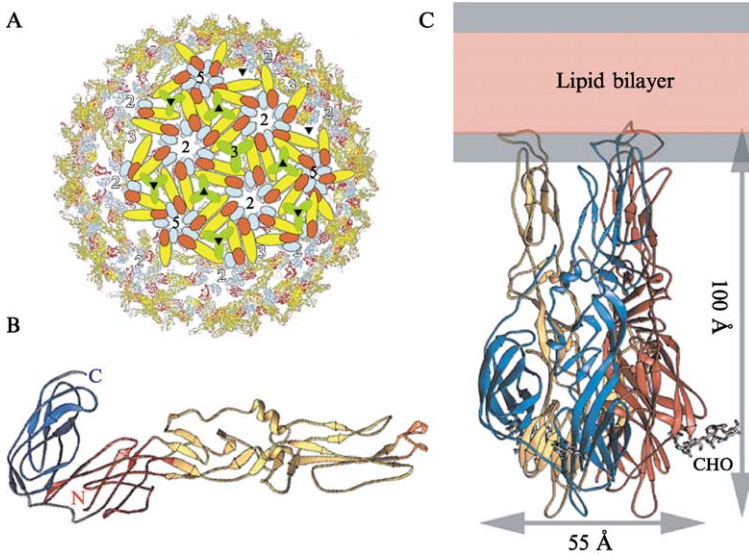
FIG 11. The alphavirus fusion protein E1. (A) Organization of E1 and E2 on the surface of virions. Simplified representations of subunits have been superimposed on a model of the fit of the SFV E1 crystal structure into an image reconstruction of the virion from electron cryomicroscopy (Lescar *et al.*, 2001). E1 is red (domain I), yellow (domain II), and blue (domain III). The E2 trimer (for which only a 9-Å structure is currently known, from electron microscopy) is represented by a green trefoil. It projects outward, capping the fusion loop of E1. Numbers (5, 3, and 2) show the positions of fivefold, threefold, and twofold icosahedral symmetry axes; triangles show the positions of local threefold axes in the T = 4 icosahedral surface lattice. (B) The soluble ectodomain, E1*, of Semliki Forest virus. Domain colors as in (A). (C) The E1* postfusion trimer. Each subunit is a single color. The stem of E1, shorter than the stem of flavivirus E proteins (compare with Fig. 9), would link the C terminus of E1* to the transmembrane anchor. (B) and (C) are adapted from Gibbons *et al.* (2004b).

transmembrane anchors are in contact as they pass through the membrane. E2, for which no high-resolution structure is yet available, projects outward and over E1, covering the E1 fusion loops. Three E2 subunits cluster around the threefold and quasi-threefold axes of the T = 4 icosahedral surface lattice, creating a spike like feature in images from electron microscopy; the three associated E1 subunits form a triangular "skirt" around the base of the E2 spike (Fig. 11A).

When alphavirus particles are exposed to lowered pH, protein packing in the icosahedral surface lattice changes substantially (Wahlberg *et al.*, 1989, 1992). The E1–E2 heterodimers dissociate, and E1 trimerizes. Thus, the E2 subunits move away from the threefold axis,

permitting E1 to cluster there. As outlined later, the analogy to what happens in influenza HA rearrangement is direct: the receptor-binding fragment, which covers the fusion fragment, loses its threefold interactions and allows the latter to associate along the threefold axis, initially as an extended prefusion intermediate and subsequently as a folded-back, postfusion trimer.

Trimerization and membrane insertion of the soluble E1* from SFV can be induced by lowering the pH in the presence of liposomes (Ahn *et al*., 2002; Gibbons *et al*., 2004a; Klimjack *et al*., 1994). The lipid requirements are the same as those for fusion of intact virions: the target membrane must contain cholesterol and sphingolipid. Electron microscopy of liposomes with inserted E1 or E1* gives similar pictures: arrays of uniform trimers (Gibbons *et al*., 2003). Thus, the presence of the C-terminal transmembrane anchor does not affect the way the fusion loops insert into the membrane. In the absence of a liposome bilayer into which to insert, E1* remains monomeric at lowered pH, like flavivirus sE. In both cases, the bilayer catalyzes the trimeric association, presumably by concentrating the monomers and organizing them in essentially parallel orientations. The irreversibility of the monomer-to-trimer transition probably resides in both cases in local β-sheet reorganizations at the two interdomain boundaries.

The crystal structure of the SFV E1* trimer, extracted from liposomes after the procedure just described, shows that the low pH-induced conformational change is essentially just like the one outlined for flavivirus E (Figs. 9–11). The clustering of domain II is less pronounced at the fusion loop tip, so that the three loops are not in contact, as they are in the trimer of dengue sE, and the hydrophobic insertion surface is therefore somewhat different (Fig. 11C). Nonetheless, one can infer the same restricted extent of insertion into the target bilayer, both from an analysis of hydrophobicity at the tip of domain II and from a comparison with the images of membrane-inserted trimers mentioned previously. Why cholesterol should be necessary for membrane insertion of the SFV fusion loop is not immediately evident from the structure. A mutation in E1 that partially alleviates the cholesterol requirement (Vashishtha *et al*., 1998) lies close to the lipid headgroup region in the model for a membrane-interacting trimer, but the segment of the protein that bears it (see Fig. 11B,C) may still be too far from the membrane to interact with headgroups directly.

In the SFV E1* trimer crystals, there is a lateral interaction between fusion loops of subunits in adjacent trimers. It has been suggested that this contact might represent the way in which a ring of trimers could

cooperate in creating a fusion pore (Bressanelli *et al*., 2004; Gibbons *et al*., 2004b) (see Section IV.A).

C. Class II Fusion Mechanism

Fusion by class II proteins may proceed with somewhat different kinetics than fusion by HA and other class I proteins. In the case of fusion by SFV E1, once a pore opens, it proceeds to dilate; flickering is infrequent (Samsonov *et al*., 2002). Thus, folding over of domain III and zipping up of the stem may be strongly concerted processes, so that once a pore has opened, the transmembrane anchors snap firmly into place. It is possible that this property reflects intertrimer cooperativity, as discussed later (Section IV.A).

We can outline five steps in the class II fusion process that parallel the five steps described previously for class I fusion by influenza virus HA.

1. *The low-pH trigger*. Mutations that alter the threshold pH for flavivirus fusion cluster around a hydrophobic pocket at the hinge between domains I and II (Modis *et al*., 2003). A nonionic detergent molecule inserts into this pocket when crystals of DEN-2 sE are grown in its presence. Several of these mutations change the size of hydrophobic side chains, suggesting that their effect is on the free energy cost of altering the domain I–domain II hinge position, rather than on the pK of a titrating residue. Candidates for proton-binding residues are two conserved histidines at the domain I–domain III interface in the prefusion dimer (Bressanelli *et al*., 2004). One might expect protonation of these histidines to destabilize that interface, inducing dissociation of the dimer as domain III swings away both from its own domain I and from the fusion loop region of the other subunit.

2. *Fusion loop emergence and insertion*. Unlike class I fusion peptides, which when exposed cause the fusion protein to aggregate (e.g., into "rosettes"), class II fusion loops appear to be relatively stable when exposed to an aqueous environment. Flavivirus sE fragments dissociate reversibly at low pH in the absence of liposomes, and the SFV E1 ectodomain monomer crystallizes without any special protection for its fusion loop. Even three clustered fusion loops, as in the DEN-2 sE trimer, do not seem to bind a detergent micelle (Modis *et al*., 2004). Nonetheless, they clearly insert stably into bilayers, as seen directly by electron microscopy for SFV E1 ectodomain, DEN-2 sE, and tick-borne encephalitis (TBE) sE (Gibbons *et al*., 2003; Modis *et al*., 2004; Stiasny *et al*., 2004). Although the insertion has not been visualized at high resolution, we can infer by comparing the

high-resolution structures with images from negative-stain electron microscopy that the fusion loops penetrate only a short distance into the outer bilayer leaflet. Conserved tryptophans in the loops may lodge at the polar group–hydrocarbon interface, as frequently found in integral membrane proteins.

3. *Fold-back*. Class I fusion proteins are generally trimers both before and after the fusion transition, but trimerization is actually part of the transition in the class II proteins. The structures suggest that the finger-like domains II might initiate the trimer cluster, which could then lock in place as each monomer reverses on itself. The stem segments are likely to be quite flexible, and the description we gave of class I folding back as first a melting out (of the C-terminal part of HA_2) and then a zipping up probably applies to class II proteins as well. In this case, the melted-out part would be the stem. Even if the two α-helical segments of the flavivirus stem retained their secondary structure during the refolding, the stem could still extend to nearly 100 Å in length, giving more than enough slack for the process diagrammed in Fig. 10D,E.

4. *Hemifusion*. As stated earlier, fusion proteins could catalyze the hemifusion step by promoting appropriate membrane curvature or by stabilizing the hemifusion intermediate (or both). Insertion of the fusion loops only into the outer bilayer leaflet will indeed produce some degree of positive curvature in the target membrane. A simple area calculation suggests, however, that it would take a relatively large number of trimers—more than are likely to contribute to one fusion event—to produce the degree of curvature pictured in some theoretical models of hemifusion (see, e.g., Kuzmin *et al*., 2001). A more important consequence of the limited insertion may be the possibility for the fusion loops to migrate into the hemifusion stalk itself, thereby stabilizing the stalk and preventing its reversal (fission). This notion is attractive for two reasons. First, it depends on a general property of the fusion segment (limited insertion), and it could apply to class I fusion mechanisms as well. Second, it explains the observation of stable hemifusion (so far, for class I fusion only) when proceeding to full fusion is substantially retarded. This proposed incorporation of fusion loops into the hemifusion stalk is included in the the diagram in Fig. 10.

5. *Fusion pore formation*. Finishing the refolding steps, by bringing the transmembrane segments back onto the threefold axis of the protein trimer, can also lock a fusion pore into place, provided that the fusion loops have not migrated through the hemifusion stalk and into the viral membrane. At its waist, the curvature of the stalk (as seen

from outside the cell by the fusion protein) is positive, and the fusion loops would be expected to stabilize it and perhaps to influence its geometry. Conversely, migration of fusion loops into a hemifusion stalk would be energetically favorable, holding them there until the opening of a pore. These proposals all require testing: the structural data available so far show only the initial and final states of the fusion process.

IV. Some Questions

A. How Many Trimers Are Needed for Fusion?

Diagrams such as those in Figs. 3 and 10 represent a schematic cross-section through a (presumably) axially symmetric fusion structure. But how many fusion proteins actually surround the hemifusion stalk? Is the number precise, determined by cooperative, lateral interactions among the proteins, or is it variable? What is the minimal number of fusion proteins required to generate a productive fusion event?

The minimal number of fusion proteins required for a productive encounter is set in part by the free energy barrier to fusion and the free energy recovered from the overall refolding reaction. The latter value will obviously vary from case to case. The former has been estimated to be roughly 30–50 kcal/mol (Kuzmin et al., 2001). In principle, this value could be recovered from refolding of one or a few trimers.

Evidence for cooperativity among trimers in HA-mediated fusion supports the notion that more than one trimer participates (Danieli et al, 1996). A possible mechanistic basis for cooperativity in class II fusion has been described in connection with analysis of the SFV E1* trimer structure (Gibbons et al., 2004). Lateral contacts between the outer rims of fusion loops from adjacent trimers in the crystals of the low pH-induced form of SFV E1* suggest that these interactions may create a ring of five trimers, angled at roughly 45° to the viral membrane, with their fusion loops (15 in all) forming a hydrophobic "crater" at the tip of a fusion "volcano" (Bressanelli et al., 2004; Gibbons et al., 2004b). These 15 loops might indeed be sufficient to stabilize a nipple-like outpouching of the target cell membrane, a first step toward formation of a hemifusion stalk, and their coordinated participation could promote rapid transit through the hemifusion stage to an open fusion pore, as observed (Samsonov et al., 2002).

Another possible source of cooperativity, even without specific inter-trimer interactions at any step in the fusion process, comes from

common anchoring of several participating trimers in both viral and target cell membranes. The prefusion intermediate (Figs. 3B, 10C) presumably flickers toward the zipped-up conformation but proceeds along that direction only when it meets a low enough barrier. If squeezing the two membranes together requires more than one trimer—that is, if the resistance of the membranes to deformation counteracts the tendency of a single extended intermediate to collapse by zipping up into the folded-back structure—then only when several trimers proceed toward the zipped-up conformation in concert will the refolding proceed. In this view, cooperativity is achieved not by defined protein–protein interactions (ring formation), but rather by coupling through the elastic properties of the membranes undergoing fusion. A hemifusion stalk might be generated by as few as one or two trimers or by as many as can fit around the stalk (probably five or six), depending on circumstances, concentrations, and of course the actual free energy recovery from a single trimer. One advantage of this picture is that it does not require additional protein–protein contacts between necessarily flickering and partly disordered intermediate structures.

B. What Is the Structure of the Hemifusion Intermediate?

The detailed structure of the hemifusion state for two bilayers has not yet been determined. Several possible geometries are still consistent with available data. Instead of opening up as a fusion pore, the stalk shown in Fig. 1 could resolve as a hemifusion diaphragm. This state, which can spread laterally, is more likely to be a nonproductive dead end, as rupture of the diaphragm would be required to achieve content mixing and as the fusion protein would probably be relegated to the periphery.

The proposal, embodied in Figs. 3 and 10, that the fusion peptide or fusion loop can migrate into the hemifusion stalk, derives from the conclusion that these segments interact solely with the proximal leaflet of a lipid bilayer and that this migration is feasible. Because hemifusion states can be trapped by suitable mutations in the fusion protein, it will in principle be possible to test this proposal directly.

C. Are There Additional Structural Classes of Viral Fusion Proteins?

The distinction between class I and class II viral fusion proteins is essentially an architectural rather than a mechanistic one. Class I proteins assemble as trimers, are primed by proteolytic cleavage, and

rearrange to a postfusion structure based on a trimeric coiled coil. Class II proteins assemble as heterodimers with a protector protein. Cleavage of the latter is the priming event, and the fusion-inducing rearrangement includes trimer formation. The class II proteins studied so far are clearly homologs. The same is not so evident for the class I proteins. Those of the lentiviruses, oncoretroviruses, and filoviruses have some apparent evolutionary kinship, but how they relate to influenza HA (for example) is less obvious. In any case, it is unlikely that the categories just defined exhaust the possible structural solutions to the problem of promoting bilayer fusion. It is probable, however, that fusion proteins of classes yet to be discovered will resemble the ones we know already in how they actually fuse membranes. That is, we can expect them to work through the sequence of fusion peptide exposure, fusion peptide insertion, and overall folding back that we have already analyzed in detail.

D. Can Small Molecules Inhibit the Fusion Transition?

Peptides can inhibit the HIV gp41 fold-back step. How general is this strategy, and can a more drug-like small molecule do the same? An outer layer peptide from Ebola virus fusion protein GP2 blocks entry of VSV pseudotyped with Ebola virus glycoprotein GP1–GP2 (Watanabe *et al*., 2000). Thus, HIV gp41 is not unique in having an outer layer that binds tightly enough to work as an inhbitor *in trans*. The cyclic peptide that targets a pocket on the inner core of the HIV gp41 trimer has a footprint not much larger than that of a more conventional drug, so even the zipping-up phase of the refolding reaction might be susceptible to small molecule inhibitors. Because HIV fuses at the cell surface, the gp41 fusion transition is more likely to be accessible to a peptide than that of a protein that refolds in an endosome. Small molecules that can penetrate to low-pH compartments may therefore be necessary if the principle demonstrated by the successful use of T-20/enfuvirtide is to be applied to the entry of many other viral pathogens.

REFERENCES

Ahn, A., Gibbons, D. L., and Kielian, M. (2002). The fusion peptide of Semliki Forest virus associates with sterol-rich membrane domains. *J. Virol.* **76:**3267–3275.
Allan, J. S., Coligan, J. E., Barin, F., McLane, M. F., Sodroski, J. G., Rosen, C. A., Haseltine, W. A., Lee, T. H., and Essex, M. (1985). Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. *Science* **228:**1091–1094.

Allison, S. L., Schalich, J., Stiasny, K., Mandl, C. W., and Heinz, F. X. (2001). Mutational evidence for an internal fusion peptide in flavivirus envelope protein E. *J. Virol.* **75:**4268–4275.

Allison, S. L., Schalich, J., Stiasny, K., Mandl, C. W., Kunz, C., and Heinz, F. X. (1995). Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. *J. Virol.* **69:**695–700.

Allison, S. L., Stiasny, K., Stadler, K., Mandl, C. W., and Heinz, F. X. (1999). Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E. *J. Virol.* **73:**5605–5612.

Armstrong, R. T., Kushnir, A. S., and White, J. M. (2000). The transmembrane domain of influenza hemagglutinin exhibits a stringent length requirement to support the hemifusion to fusion transition. *J. Cell Biol.* **151:**425–437.

Blacklow, S. C., Lu, M., and Kim, P. S. (1995). A trimeric subdomain of the simian immunodeficiency virus envelope glycoprotein. *Biochemistry* **34:**14955–14962.

Bressanelli, S., Stiasny, K., Allison, S. L., Stura, E. A., Duquerroy, S., Lescar, J., Heinz, F. X., and Rey, F. A. (2004). Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. *EMBO J.* **23:**728–738.

Bullough, P. A., Hughson, F. M., Skehel, J. J., and Wiley, D. C. (1994). Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* **371:**37–43.

Caffrey, M., Cai, M., Kaufman, J., Stahl, S. J., Wingfield, P. T., Covell, D. G., Gronenborn, A. M., and Clore, G. M. (1998). Three-dimensional solution structure of the 44 kDa ectodomain of SIV gp41. *EMBO J.* **17:**4572–4584.

Carr, C. M., and Kim, P. S. (1993). A spring-loaded mechanism for the conformational change of influenza hemagglutinin. *Cell* **73:**823–832.

Chan, D. C., Chutkowski, C. T., and Kim, P. S. (1998). Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target. *Proc. Natl. Acad. Sci. USA* **95:**15613–15617.

Chan, D. C., Fass, D., Berger, J. M., and Kim, P. S. (1997). Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89:**263–273.

Chen, J., Skehel, J. J., and Wiley, D. C. (1999). N- and C-terminal residues combine in the fusion-pH influenza hemagglutinin HA_2 subunit to form an N cap that terminates the triple-stranded coiled coil. *Proc. Natl. Acad. Sci. USA* **96:**8967–8972.

Cohen, F. S., Markosyan, R. M., and Melikyan, G. B. (2002). The process of membrane fusion: Nipples, hemifusion, pores, and pore growth. *Curr. Top. Membr.* **52:**501–529.

Dalgleish, A. G., Beverley, P. C., Clapham, P. R., Crawford, D. H., Greaves, M. F., and Weiss, R. A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312:**763–767.

Danieli, T., Pelletier, S. L., Henis, Y. I., and White, J. M. (1996). Membrane fusion mediated by the influenza virus hemagglutinin requires the concerted action of at least three hemagglutinin trimers. *J. Cell Biol.* **133:**559–569.

Daniels, R. S., Downie, J. C., Hay, A. J., Knossow, M., Skehel, J. J., Wang, M. L., and Wiley, D. C. (1985). Fusion mutants of the influenza virus hemagglutinin glycoprotein. *Cell* **40:**431–439.

Dutch, R. E., and Lamb, R. A. (2001). Deletion of the cytoplasmic tail of the fusion protein of the paramyxovirus simian virus 5 affects fusion pore enlargement. *J. Virol.* **75:**5363–5369.

Eckert, D. M., Malashkevich, V. N., Hong, L. H., Carr, P. A., and Kim, P. S. (1999). Inhibiting HIV-1 entry: Discovery of D-peptide inhibitors that target the gp41 coiled-coil pocket. *Cell* **99:**103–115.

Fass, D., Harrison, S. C., and Kim, P. S. (1996). Retrovirus envelope domain at 1.7 Å resolution. *Nat. Struct. Biol.* **3:**465–469.

Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996). HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272:**872–877.

Ferrer, M., Kapoor, T. M., Strassmaier, T., Weissenhorn, W., Skehel, J. J., Oprian, D., Schreiber, S. L., Wiley, D. C., and Harrison, S. C. (1999). Selection of gp41-mediated HIV-1 cell entry inhibitors from biased combinatorial libraries of non-natural binding elements. *Nat. Struct. Biol.* **6:**953–960.

Furuta, R. A., Wild, C. T., Weng, Y., and Weiss, C. D. (1998). Capture of an early fusion-active conformation of HIV-1 gp41. *Nat. Struct. Biol.* **5:**276–279.

Gibbons, D. L., Ahn, A., Liao, M., Hammar, L., Cheng, R. H., and Kielian, M. (2004a). Multistep regulation of membrane insertion of the fusion peptide of Semliki Forest virus. *J. Virol.* **78:**3312–3318.

Gibbons, D. L., Erk, I., Reilly, B., Navaza, J., Kielian, M., Rey, F. A., and Lepault, J. (2003). Visualization of the target-membrane-inserted fusion protein of Semliki Forest virus by combined electron microscopy and crystallography. *Cell* **114:**573–583.

Gibbons, D. L., Vaney, M. C., Roussel, A., Vigouroux, A., Reilly, B., Lepault, J., Kielian, M., and Rey, F. A. (2004b). Conformational change and protein–protein interactions of the fusion protein of Semliki Forest virus. *Nature* **427:**320–325.

Han, X., Bushweller, J. H., Cafiso, D. S., and Tamm, L. K. (2001). Membrane structure and fusion-triggering conformational change of the fusion domain from influenza hemagglutinin. *Nat. Struct. Biol.* **8:**715–720.

Heinz, F. X., Mandl, C. W., Holzmann, H., Kunz, C., Harris, B. A., Rey, F., and Harrison, S. C. (1991). The flavivirus envelope protein E: Isolation of a soluble form from tick-borne encephalitis virus and its crystallization. *J. Virol.* **65:**5579–5583.

Heinz, F. X., Stiasny, K., Puschner-Auer, G., Holzmann, H., Allison, S. L., Mandl, C. W., and Kunz, C. (1994). Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM. *Virology* **198:**109–117.

Jahn, R., Lang, T., and Sudhof, T. C. (2003). Membrane fusion. *Cell* **112:**519–533.

Jiang, S., Lin, K., Strick, N., and Neurath, A. R. (1993). HIV-1 inhibition by a peptide. *Nature* **365:**113.

Jones, P. L., Korte, T., and Blumenthal, R. (1998). Conformational changes in cell surface HIV-1 envelope glycoproteins are triggered by cooperation between cell surface CD4 and co-receptors. *J. Biol. Chem.* **273:**404–409.

Kemble, G. W., Danieli, T., and White, J. M. (1994). Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell* **76:**383–391.

Kilby, J. M., and Eron, J. J. (2003). Novel therapies based on mechanisms of HIV-1 cell entry. *N. Engl. J. Med.* **348:**2228–2238.

Kliger, Y., Gallo, S. A., Peisajovich, S. G., Munoz-Barroso, I., Avkin, S., Blumenthal, R., and Shai, Y. (2001). Mode of action of an antiviral peptide from HIV-1: Inhibition at a post-lipid mixing stage. *J. Biol. Chem.* **276:**1391–1397.

Klimjack, M. R., Jeffrey, S., and Kielian, M. (1994). Membrane and protein interactions of a soluble form of the Semliki Forest virus fusion protein. *J. Virol.* **68:**6940–6946.

Kuhn, R. J., Zhang, W., Rossmann, M. G., Pletnev, S. V., Corver, J., Lenches, E., Jones, C. T., Mukhopadhyay, S., Chipman, P. R., Strauss, E. G., Baker, T. S., and Strauss, J. H. (2002). Structure of dengue virus: Implications for flavivirus organization, maturation, and fusion. *Cell* **108:**717–725.

Kuzmin, P. I., Zimmerberg, J., Chizmadzhev, Y. A., and Cohen, F. S. (2001). A quantitative model for membrane fusion based on low-energy intermediates. *Proc. Natl. Acad. Sci. USA* **98:**7235–7240.

Kwong, P. D., Wyatt, R., Majeed, S., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A. (2000). Structures of HIV-1 gp120 envelope glycoproteins from laboratory-adapted and primary isolates. *Struct. Fold. Des.* **8:**1329–1339.

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393:**648–659.

Lescar, J., Roussel, A., Wien, M. W., Navaza, J., Fuller, S. D., Wengler, G., and Rey, F. A. (2001). The fusion glycoprotein shell of Semliki Forest virus: An icosahedral assembly primed for fusogenic activation at endosomal pH. *Cell* **105:**137–148.

Lu, M., Blacklow, S. C., and Kim, P. S. (1995). A trimeric structural domain of the HIV-1 transmembrane glycoprotein. *Nat. Struct. Biol.* **2:**1075–1082.

Markin, V. S., Kozlov, M. M., and Borovjagin, V. L. (1984). On the theory of membrane fusion: The stalk mechanism. *Gen. Physiol. Biophys.* **5:**361–377.

Melikyan, G. B., White, J. M., and Cohen, F. S. (1995). GPI-anchored influenza hemagglutinin induces hemifusion to both red blood cell and planar bilayer membranes. *J. Cell Biol.* **131:**679–691.

Modis, Y., Ogata, S., Clements, D., and Harrison, S. C. (2003). A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc. Natl. Acad. Sci. USA* **100:**6986–6991.

Modis, Y., Ogata, S., Clements, D., and Harrison, S. C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature* **427:**313–319.

Moore, J. P., McKeating, J. A., Weiss, R. A., and Sattentau, Q. J. (1990). Dissociation of gp120 from HIV-1 virions induced by soluble CD4. *Science* **250:**1139–1142.

Munoz-Barroso, I., Salzwedel, K., Hunter, E., and Blumenthal, R. (1999). Role of the membrane-proximal domain in the initial stages of human immunodeficiency virus type 1 envelope glycoprotein-mediated membrane fusion. *J. Virol.* **73:**6089–6092.

Myszka, D. G., Sweet, R. W., Hensley, P., Brigham-Burke, M., Kwong, P. D., Hendrickson, W. A., Wyatt, R., Sodroski, J., and Doyle, M. L. (2000). Energetics of the HIV gp120–CD4 binding reaction. *Proc. Natl. Acad. Sci. USA* **97:**9026–9031.

Pletnev, S. V., Zhang, W., Mukhopadhyay, S., Fisher, B. R., Hernandez, R., Brown, D. T., Baker, T. S., Rossmann, M. G., and Kuhn, R. J. (2001). Locations of carbohydrate sites on alphavirus glycoproteins show that E1 forms an icosahedral scaffold. *Cell* **105:**127–136.

Pollard, S. R., Rosa, M. D., Rosa, J. J., and Wiley, D. C. (1992). Truncated variants of gp120 bind CD4 with high affinity and suggest a minimum CD4 binding region. *EMBO J.* **11:**585–591.

Qiao, H., Armstrong, R. T., Melikyan, G. B., Cohen, F. S., and White, J. M. (1999). A specific point mutant at position 1 of the influenza hemagglutinin fusion peptide displays a hemifusion phenotype. *Mol. Biol. Cell* **10:**2759–2769.

Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C., and Harrison, S. C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* **375:**291–298.

Rimsky, L. T., Shugars, D. C., and Matthews, T. J. (1998). Determinants of human immunodeficiency virus type-I resistance to gp41-derived inhibitory peptides. *J. Virol.* **72:**986–993.

Rizzuto, C. D., Wyatt, R., Hernandez-Ramos, N., Sun, Y., Kwong, P. D., Hendrickson, W. A., and Sodroski, J. (1998). A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* **280:**1949–1953.

Robey, W. G., Safai, B., Oroszlan, S., Arthur, L. O., Gonda, M. A., Gallo, R. C., and Fischinger, P. J. (1985). Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients. *Science* **228:**593–595.

Root, M. J., Kay, M. S., and Kim, P. S. (2001). Protein design of an HIV-1 entry inhibitor. *Science* **291:**884–888.

Rosenthal, P. B., Zhang, X., Formanowski, F., Fitz, W., Wong, C. H., Meier-Ewert, H., Skehel, J. J., and Wiley, D. C. (1998). Structure of the haemagglutinin–esterase-fusion glycoprotein of influenza C virus. *Nature* **396:**92–96.

Samsonov, A. V., Chatterjee, P. K., Razinkob, V. I., Eng, C. H., Kielian, M., and Cohen, F. S. (2002). Effects of membrane potential and sphingolipid structures on fusion of Semliki Forest virus. *J. Virol.* **76:**12691–12702.

Sattentau, Q. J., and Moore, J. P. (1991). Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. *J. Exp. Med.* **174:**407–415.

Sattentau, Q. J., Moore, J. P., Vignaux, F., Traincard, F., and Poignard, P. (1993). Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding. *J. Virol.* **67:**7383–7393.

Siegel, D. P. (1993). Energetics of intermediates in membrane fusion: Comparison of stalk and inverted micellar intermediate mechanisms. *Biophys. J.* **65:**2124–2140.

Skehel, J. J., and Wiley, D. C. (1998). Coiled coils in both intracellular vesicle and viral membrane fusion. *Cell* **95:**871–874.

Skehel, J. J., and Wiley, D. C. (2000). Receptor binding and membrane fusion in virus entry: The influenza hemagglutinin. *Annu. Rev. Biochem.* **69:**531–569.

Steinhauer, D. A., Wharton, S. A., Skehel, J. J., and Wiley, D. C. (1995). Studies of the membrane fusion activities of fusion peptide mutants of influenza virus hemagglutinin. *J. Virol.* **69:**6643–6651.

Stiasny, K., Allison, S. L., Schalich, J., and Heinz, F. X. (2002). Membrane interactions of the tick-borne encephalitis virus fusion protein E at low pH. *J. Virol.* **76:**3784–3790.

Stiasny, K., Bressanelli, S., Lepault, J., Rey, F. A., and Heinz, F. X. (2004). Characterization of a membrane-associated trimeric low-pH induced form of the class II viral fusion protein E from tick-borne encephalitis virus and its crystallization. *J. Virol.* **78:**3178–3183.

Tan, K., Liu, J., Wang, J., Shen, S., and Lu, M. (1997). Atomic structure of a thermostable subdomain of HIV-1 gp41. *Proc. Natl. Acad. Sci. USA* **94:**12303–12308.

Trkola, A., Dragic, T., Arthos, J., Binley, J. M., Olson, W. C., Allaway, G. P., Cheng-Mayer, C., Robinson, J., Maddon, P. J., and Moore, J. P. (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature* **384:**184–187.

Tsurudome, M., Gluck, R., Graf, R., Falchetto, R., Schaller, U., and Brunner, J. (1992). Lipid interactions of the hemagglutinin HA$_2$ NH$_2$-terminal segment during influenza virus-induced membrane fusion. *J. Biol. Chem.* **267:**20225–20232.

Vashishtha, M., Phalen, T., Marquardt, M. T., Ryu, J. S., Ng, A. C., and Kielian, M. (1998). A single point mutation controls the cholesterol dependence of Semliki Forest virus entry and exit. *J. Cell Biol.* **140:**91–99.

Veronese, F. D., DeVico, A. L., Copeland, T. D., Oroszlan, S., Gallo, R. C., and Sarngadharan, M. G. (1985). Characterization of gp41 as the transmembrane protein coded by the HTLV-III/LAV envelope gene. *Science* **229:**1402–1405.

Wahlberg, J. M., Boere, W. A., and Garoff, H. (1989). The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to low pH during virus maturation. *J. Virol.* **63:**4991–4997.

Wahlberg, J. M., Bron, R., Wilschut, J., and Garoff, H. (1992). Membrane fusion of Semliki Forest virus involves homotrimers of the fusion protein. *J. Virol.* **66:**7309–7318.

Watanabe, S., Takada, A., Watanabe, T., Ito, H., Kida, H., and Kawaoka, Y. (2000). Functional importance of the coiled-coil of the Ebola virus glycoprotein. *J. Virol.* **74:**10194–10201.

Weissenhorn, W., Carfi, A., Lee, K. H., Skehel, J. J., and Wiley, D. C. (1998). Crystal structure of the Ebola virus membrane fusion subunit, GP2, from the envelope glyco-protein ectodomain. *Mol. Cell* **2:**605–616.

Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J., and Wiley, D. C. (1997). Atomic structure of the ectodomain from HIV-1 gp41. *Nature* **387:**426–430.

Wild, C., Oas, T., McDanal, C., Bolognesi, D., and Matthews, T. (1992). A synthetic peptide inhibitor of human immunodeficiency virus replication: Correlation between solution structure and viral inhibition. *Proc. Natl. Acad. Sci. USA* **89:**10537–10541.

Wild, C. T., Shugars, D. C., Greenwell, T. K., McDanal, C. B., and Matthews, T. J. (1994). Peptides corresponding to a predictive α-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection. *Proc. Natl. Acad. Sci. USA* **91:**9770–9774.

Wu, L., Gerard, N. P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardoso, A. A., Desjardin, E., Newman, W., Gerard, C., and Sodroski, J. (1996). CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* **384:**179–183.

Zhang, W., Chipman, P. R., Corver, J., Johnson, P. R., Zhang, Y., Mukhopadhyay, S., Baker, T. S., Strauss, J. H., Rossmann, M. G., and Kuhn, R. J. (2003). Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. *Nat. Struct. Biol.* **10:**907–912.

Zhang, Y., Corver, J., Chipman, P. R., Zhang, W., Pletnev, S. V., Sedlak, D., Baker, T. S., Strauss, J. H., Kuhn, R. J., and Rossmann, M. G. (2003). Structures of immature flavivirus particles. *EMBO J.* **22:**2604–2613.

Zhou, G., Ferrer, M., Chopra, R., Kapoor, T. M., Strassmaier, T., Weissenhorn, W., Skehel, J. J., Oprian, D., Schreiber, S. L., Harrison, S. C., and Wiley, D. C. (2000). The structure of an HIV-1 specific cell entry inhibitor in complex with the HIV-1 gp41 trimeric core. *Bioorg. Med. Chem.* **8:**2219–2227.

Zhang, W., Mukhopadhyay, S., Pletnev, S. V., Baker, T. S., Kuhn, R. J., and Rossmann, M. G. (2002). Placement of the structural proteins in Sindbis virus. *J. Virol.* **76:**11645–11658.