MECHANISMS OF VIRAL MEMBRANE FUSION AND ITS INHIBITION

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■ **Abstract**   Viral envelope glycoproteins promote viral infection by mediating the fusion of the viral membrane with the host-cell membrane. Structural and biochemical studies of two viral glycoproteins, influenza hemagglutinin and HIV-1 envelope protein, have led to a common model for viral entry. The fusion mechanism involves a transient conformational species that can be targeted by therapeutic strategies. This mechanism of infectivity is likely utilized by a wide variety of enveloped viruses for which similar therapeutic interventions should be possible.

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INTRODUCTION

The fusion of lipid bilayers is central to a number of diverse biological processes, such as fertilization, vesicle trafficking, muscle development, and viral infection. To date, the most extensively studied of these events is the membrane fusion of enveloped viruses. When an enveloped virus infects a host cell, its membrane fuses with the host-cell membrane, allowing the contents of the virus to be transferred to the host. This fusion event is mediated by virally encoded surface glycoproteins.

Scope of this Review

In this review we focus on the fusion mechanisms mediated by two viral proteins, influenza hemagglutinin (HA) and human immunodeficiency virus type-1 (HIV-1) envelope protein (Env). We present these two viral proteins together, yielding a more complete picture of viral membrane fusion than can be accomplished by focusing on either individually. Biochemical and structural studies on the fusogenic proteins are emphasized. In addition, we discuss the current status of HIV-1 membrane fusion inhibitors.

Other Viral Membrane Fusion Reviews

Clearly, there are many areas of study in the field of viral membrane fusion. Although we try to at least touch on most subjects, many more thorough reviews on specific topics exist. Influenza HA has long been the paradigm for viral-mediated membrane fusion and has an extensive history in the literature. In 1987, an early, informative review was provided by Wiley & Skehel [1]. Since then, a number of comprehensive reviews have appeared [2–10]. The HA-mediated merging of lipid bilayers has been reviewed [11, 12], as has the role of fusion peptides in membrane fusion [13, 14]. Recently, HIV-1 Env was reviewed [9, 15–17]. The HIV-1 coreceptor has been thoroughly discussed [18, 19], and the roles of coiled coils in viral membrane fusion and vesicle membrane fusion have been compared [20, 21]. Thorough reviews of inhibiting HIV-1 viral entry have been provided [22–25], and finally, the fusion events of other viral families have been covered [7, 26].

ARCHITECTURE OF VIRAL MEMBRANE FUSION PROTEINS

Enveloped animal viruses encompass many viral families, each with a distinct virion morphology, genome structure, and life cycle (27). Despite extensive differences, these viruses also share common features. For example, most enveloped viruses bud from host-cell membranes, thereby acquiring a cell-derived lipid bilayer. Some viruses, such as paramyxoviruses, bud from the plasma membrane whereas other viruses, such as flaviviruses, bud from internal membranes like the endoplasmic reticulum. Enveloped viruses also share a common mechanism of infection. A glycoprotein on the surface of the virus mediates fusion between the viral membrane and the host-cell membrane, allowing release of the viral contents into the host cell.

The fusion glycoproteins of enveloped viruses, typically type-I integral membrane proteins, are encoded by the virus and synthesized by the infected cell. They are incorporated into the host-cell membrane and subsequently into the budding virus. Fusion glycoproteins comprise (*a*) a cleavable amino-terminal signal sequence directing it to the endoplasmic reticulum, (*b*) a large extraviral region (also referred to as the ectodomain), (*c*) a stop-transfer region that forms a transmembrane helix to anchor the protein in the viral membrane, and (*d*) a cytoplasmic tail. The cytoplasmic tail ranges from 20 amino acids for paramyxoviruses to ∼150 amino acids for some retroviruses. Typically, the envelope glycoproteins are synthesized as a precursor and then cleaved into two subunits that remain closely associated with each other. These proteins form higher-order oligomers and are glycosylated. Because of their location on the outer surface of the viral membrane, much of the host's immune response targets these glycoproteins. Also, these envelope glycoproteins contain a short region within their sequence called the "fusion peptide," which is required for mediating membrane fusion (for reviews, see 13, 14). The fusion-peptide region, rich in hydrophobic and glycine residues, interacts with the host-cell membrane at an early stage of the membrane-fusion process.

Viral entry is initiated when the surface glycoprotein binds to the appropriate cellular receptor(s) on the host-cell surface. Subsequent to binding, some enveloped viruses fuse with the cell-surface membrane at neutral pH, whereas others are endocytosed into clathrin-coated pits and fuse with the endosomal membrane when the pH is lowered. Viruses such as HIV-1 and human respiratory syncytial virus use the former method of viral entry, whereas viruses such as influenza and rabies utilize the latter.

Viral envelope fusion proteins proceed through a series of conformational changes in order to mediate fusion with the host cell. The initial cleavage of the glycoprotein precursor leaves the complex in a metastable state, primed for fusion, although the fusion peptide is not exposed. Conformational changes occur in response to binding to the host-cell receptors or exposure to low pH, exposing the

fusion peptide and allowing juxtaposition of the viral and host-cell membranes, which leads to membrane fusion.

MODEL STUDIES OF VIRAL MEMBRANE FUSION: Influenza HA and HIV-1 Env

Several decades of research illuminate a complex and elegant process by which influenza HA mediates viral membrane fusion, making it the prototypic enveloped viral membrane fusion glycoprotein (for reviews, see 1, 2, 7). More recently, there is increasing emphasis on biochemical and structural studies of the envelope glyco-protein of HIV-1. Although these two viruses are members of distinct viral families differing in genome organization, physical morphology, and replication strategies, at the molecular level they utilize a similar mechanism for gaining access to the interior of a host cell. Combining the experimental information available for these distinct virus glycoproteins creates a more complete picture of the viral entry process than when either virus is considered individually.

HA and Env are the sole viral proteins required for membrane fusion with the host cell by influenza and HIV-1, respectively. They are both synthesized as fusion-incompetent precursors, termed HA0 (for HA) and gp160 (for Env). They are then proteolytically cleaved into two subunits, a surface subunit and a transmembrane subunit, activating the fusion potential of the glycoproteins. The surface subunit of influenza, HA1, remains covalently associated to the transmembrane subunit, HA2, through a disulfide bond (Figure 1*a*). The interaction between the cleaved products of gp160 (a surface subunit, gp120, and a transmembrane subunit, gp41) is noncovalent. The surface subunits are responsible for recognizing and binding to specific receptors on the host cell. The transmembrane subunits contain the fusion-peptide region at their amino terminus and are anchored in the viral membrane via hydrophobic membrane-spanning helices. Despite numerous similarities between the two glycoproteins, the sites of entry of the two viruses differ. After HA1 binds to its receptor, sialic acid, the influenza virus is endocytosed and fusion is initiated with the endosomal membrane at low pH. In the case of HIV-1, gp120 binds to a cellular receptor, CD4, and subsequently a coreceptor, one of a family of seven transmembrane helix chemokine receptors (18), and the entry event is accomplished at the cellular surface at neutral pH.

Conformational Changes on Fusion Activation

The realization 20 years ago that exposure to low pH activates the membrane fusion potential of HA (28–30) led to intense scrutiny of the effect of low pH on HA structure. Consequently, prior to high-resolution structural information, it was discerned that exposure to low pH induces irreversible conformational changes in HA. Similarly, biochemical studies indicate HIV-1 Env changes conformation in response to CD4 binding—its fusion-activation signal.
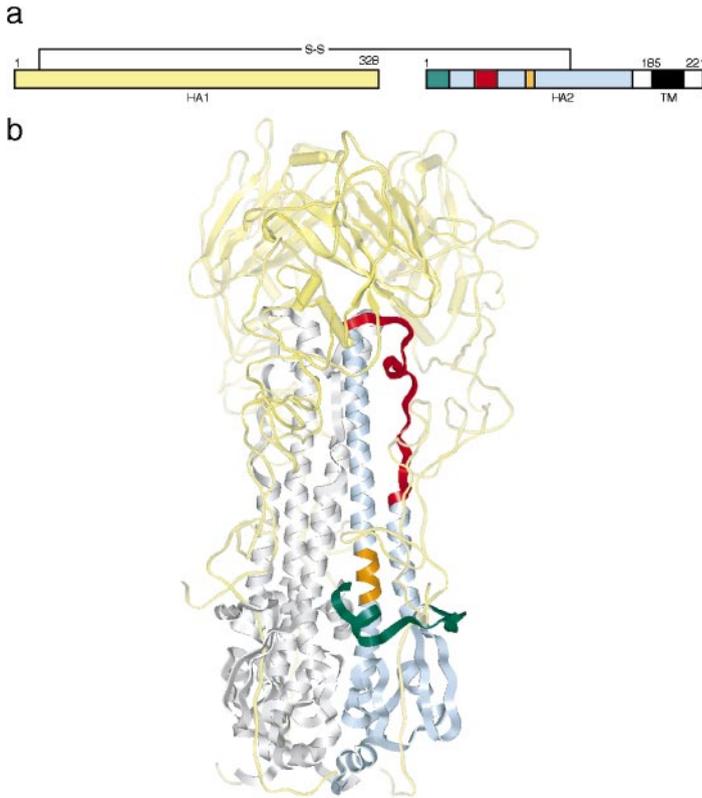
Figure 1   High-resolution structure of native influenza hemagglutinin (HA). (*a*) The primary structure of processed HA, including a disulfide bond between residue 14 of HA1 and residue 137 of HA2. (Color) The residues present in the bromelain-released form of processed HA (BHA) X-ray crystal structure in *b*. (*b*) The X-ray crystal structure of the trimeric BHA, including all of HA1 and the first 175 residues of HA2 (61). Three HA1 monomers (yellow) sit atop a stalk-region composed of three HA2 monomers (two gray, one mostly blue). The first 20 residues of the predicted fusion peptide (green) are indicated. Residues 55–76 (red), which are in a loop conformation, become helical during the HA-mediated fusion process (Figures 2 and 3). Residues 106–112 (orange), helical here, change to a loop conformation to form the trimer-of-hairpins during the fusion process (Figure 3). Figure drawn with Insight II 98.0 (Molecular Simulations, Inc, San Diego, CA).

First, on exposure to fusion-activating conditions (a temporary lowering of pH for HA and CD4 exposure for Env), both proteins become more hydrophobic: HA forms aggregates and can bind detergents and liposomes after exposure to low pH (31), whereas both proteins acquire the ability to bind bis-ANS, a hydrophobic fluorescent dye (32, 33). Second, HA and Env undergo changes in their proteolytic

susceptibility (34, 35). Third, there is evidence for alteration of the interaction between the surface and transmembrane subunits. Electron microscopy identifies a significant change in shape of HA, including a partial dissociation of globular head domains on the top of the molecule (31, 36). In laboratory-adapted strains of HIV-1, CD4 exposure can cause the dissociation of gp120 from gp41, a process known as shedding (37). Fourth, mutagenesis studies suggest the existence of distinct conformations. Specific point mutations in predicted coiled-coil domains of the glycoproteins do not alter the proteolytic processing or surface expression, yet they jeopardize infectivity and glycoprotein-mediated membrane fusion. Most likely, these mutations disrupt the fusion-active state of the protein, but not the native, nonfusogenic form (38–45). And fifth, the epitope accessibility of the proteins is altered.

In the case of HA, the changes in antigenicity with a change in pH indicate that structural rearrangements occur throughout the molecule, as the recognition of most epitopes is altered when HA is exposed to low pH (46–49). For example, monoclonal antibodies reactive with the tip of native HA are unable to immunoprecipitate HA after low-pH–induced conformational changes (50). Likewise, antibodies specific for epitopes exposed in the low-pH–activated HA, such as antibodies raised against the amino-terminal fusion peptide, are unable to immunoprecipitate native HA (50, 51).

In HIV-1, gp120 undergoes changes in antigenicity early in the fusion process. For example, the V3 loop on gp120 is recognized after receptor binding (52), and epitopes on gp41 become more accessible (53). Also, following binding of CD4, novel epitopes are revealed on the surface of gp120 that allow recognition by neutralizing antibodies (54). Neutralizing antibodies, such as 17b, block binding of gp120/CD4 complexes to the coreceptor (55, 56), which suggests that CD4 binding exposes the previously hidden coreceptor binding site (57). Consistent with this chain of events, after exposure to soluble CD4 (58), HIV-1 can fuse with cells expressing a coreceptor and lacking CD4 (59).

Structure/Function Studies

After decades of work, structure/function studies on the glycoproteins of both influenza and HIV-1 provide structural details of the predicted conformational changes. The conformational changes of HA are well defined, with high-resolution X-ray crystal structures available for three states: unprocessed precursor (HA0), and proteolytically processed HA in both the native and low-pH–activated conformations. There are also high-resolution data for gp120 and gp41. Some of these structures, as well as other important structure/function studies, are described below.

High-Resolution Structure of Influenza HA in the Native Conformation In 1981, Wilson et al (61) provided the first high-resolution view of a viral envelope glycoprotein. The native structure of influenza HA was solved by crystallization

of a large portion of the HA ectodomain that was cleaved from the viral surface by bromelain (BHA) (60, 61) (Figure 1*b*). BHA lacks the hydrophobic region that anchors HA to the viral membrane. It is a long trimer that extends 135 Å. At the top of the structure are three exposed globular head domains consisting strictly of HA1 and responsible for binding to sialic acid. The head domains sit atop a stalk, composed of the remainder of HA1 and HA2. This stalk contains a central trimeric coiled coil at its core, composed strictly of HA2. The amino terminus of HA2, which contains the fusion peptide, is buried in the native structure. The fusion peptide is located approximately 35 Å from the carboxy terminus of the structure and 100 Å from the distal tip.

Because BHA was prepared at neutral pH from virus that had not been exposed to low pH, it presumably represents the native conformation of the HA ectodomain (i.e. the conformation of HA on the surface of the virus following proteolytic processing yet preceding low-pH activation). Structural details are consistent with this interpretation. First, the distal end of the molecule (away from the presumed location of the viral-spanning region) is composed of HA1, and HA1 recognizes and binds to cell receptors. Second, the fusion peptide is sequestered in the interior of the structure, unavailable for mediating fusion. Earlier biochemical studies predicted the burial of the fusion peptide in the prefusogenic conformation.

The BHA structure was groundbreaking but left many questions unanswered. Most important, what conformational changes could expose the fusion peptide from its buried location in the folded structure, and how could the exposed fusion peptide interact with the cell membrane when they were over 100 Å apart?

The Spring-Loaded Model   Twelve years after the native HA structure was solved, synthetic peptide experiments led to a structural model of low-pH–induced conformational changes in HA (62). Computational methods identified a region of HA2 (residues 54–81) with a high propensity for forming a coiled coil. However, in the native X-ray crystal structure, most of this region maintains an extended loop conformation (residues 55–75) (Figure 1*b*). Biophysical studies on the synthetic peptide corresponding to this region confirmed that, in fact, it forms a coiled coil in solution. Therefore, it was proposed that in the transition from the prefusogenic, native structure to the fusogenic, low-pH–activated structure, the conformation of these residues changes from a loop to a coiled coil. This structural rearrangement would extend the central trimeric coiled coil and propel the fusion peptide to the opposite end of the molecule, allowing it to interact with the host-cell membrane (Figure 2). This proposal is called the spring-loaded mechanism, emphasizing the idea that the prefusogenic structure is primed for the conformational change that leads to fusion (62). One year later, the X-ray crystal structure of low-pH–activated HA was solved and confirmed the structural features of the spring-loaded mechanism [a more recent structure is depicted in Figure 3*a*] (63). In this fusogenic structure, residues 55–75 form an extension of the central coiled coil of the native structure. Although the fusion peptide is not present in the structure, this conformational change is expected to move the fusion peptide 100 Å from its position
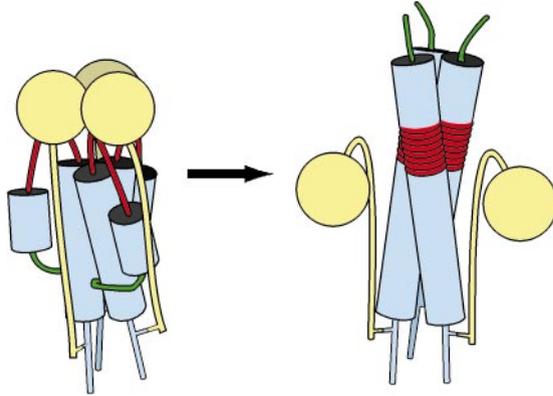
Figure 2   Spring-loaded mechanism for viral membrane fusion (62). In the native conformation of influenza hemagglutinin (HA) (*left*), the HA1 subunits (yellow balls) occupy the distal end of the structure, atop a trimeric coiled coil region of HA2 (blue). The fusion peptides (green) are buried in the core of the HA2 structure. On induction of low pH, fusion-activating conformational changes occur (*right*). The noncovalent interactions between HA1 and HA2 weaken and the loop regions of HA2 (red) "spring" into helical conformations, extending the central trimeric coiled coil and propelling the fusion peptides to the top of the structure to interact with the target membrane. (Adapted from Reference 62.)

in the native conformation. Mutations that reduce the helical propensity of the spring-loaded region reduce membrane fusion, indicating the importance of the loop-to-helix transition in the fusion event (39). With prolines at both positions 55 and 71, HA maintains surface expression and proteolytic maturation, yet no fusion activity occurs.

How is a shift in pH able to cause such major conformational changes? Evidence suggests the native prefusogenic conformation of HA is metastable, separated from the stable fusogenic state by a kinetic barrier (64). The pH change serves to destabilize the native state, making it easier to overcome the kinetic barrier. Indeed, low pH per se is not required for HA-mediated fusion. At neutral pH, high temperature or a chemical denaturant can activate the spring-loaded conformational change of HA and, therefore, HA-mediated membrane fusion (64).

High-Resolution Structures of Fusion-Active Conformations   In order to obtain structural information on HIV-1 gp41, a recombinantly expressed gp41 ectodomain lacking the amino-terminal fusion peptide was subjected to proteolysis (65). This technique, termed protein dissection, potentially reveals stable subdomains of a protein or protein complex. After gp41 was treated with proteases, a trimeric helical subdomain of gp41 remained. The subdomain is composed of two discontinuous peptides, N51 and C43, and is extremely stable, only unfolding at extreme temperatures. Generally, peptides from these two regions of gp41 are called N-peptides and C-peptides because they originate from the amino-terminal and
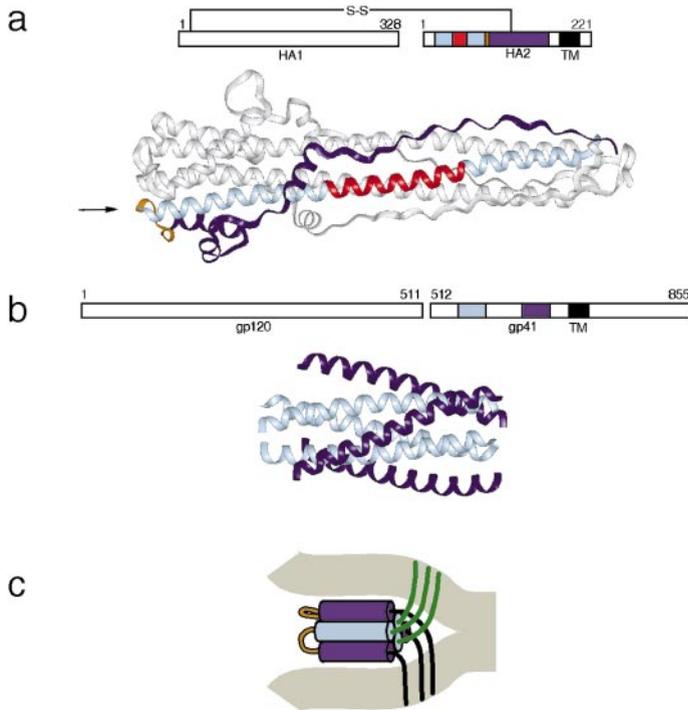
Figure 3   High-resolution views of fusion-active envelope glycoproteins and a model of the trimer-of-hairpins. (*a*) Primary structure and X-ray crystal structure of low-pH–activated influenza hemagglutinin (HA) (72; see also 63). The primary structure depicts processed HA, including a disulfide bond between residue 14 of HA1 and residue 137 of HA2. (*Color*) The residues present in the X-ray crystal structure. The X-ray crystal structure represents a stable trimeric domain of fusogenic HA2. One of the three helices (blue and red) that form the central coiled coil is surrounded by carboxy-terminal residues in an extended conformation (purple). Two monomers (gray) complete the trimer-of-hairpins. The amino- and carboxy-terminal ends of the protein are located at the same end of the structure. Residues 55–76 (red) participate in the loop-to-helix conformational change in the spring-loaded mechanism. Residues 106–111 (orange, arrow) change from helix-to-loop. (*b*) Primary structure and X-ray crystallography structure of fusogenic gp41 (67; see also 68, 69). The primary structure depicts processed gp120 and gp41. (Purple, blue) The residues present in the high-resolution structure. The X-ray structure is a trimer-of-hairpins, with three helical N-peptides in a coiled-coil conformation surrounded by three helical C-peptides. The amino- and carboxy-terminal ends of gp41 are located at the same end of the structure. (*c*) Model of the trimer-of-hairpins of HIV-1 that juxtaposes the viral and cellular membranes. (Blue) The N-peptides; (purple) the C-peptides. The fusion peptide (*green*) is inserted in the host cell membrane. The transmembrane domain of gp41 (*black*) is located in the viral membrane. High-resolution structures drawn with Insight II 98.0 (Molecular Simulations, Inc).

the carboxy-terminal regions of the gp41 ectodomain, respectively. Biophysical analysis led to the proposal that the N-peptides form a central trimeric coiled coil, with helical C-peptides bound to the outside of the coiled coil, packed antiparallel to the helices in the N-peptide coiled-coil core. The same protein dissection and biophysical analyses were performed on the ectodomain from simian immunodeficiency virus (SIV), a closely related retrovirus, and yielded the same results (66).

The structural model predicted from these biophysical studies was subsequently confirmed by X-ray crystallography for both HIV-1 (67–69) (Figure 3*b*) and SIV (70). The structure is described as a trimer-of-hairpins. In the trimer-of-hairpins, three helical C-peptides bind to the outside of the coiled-coil core of N-peptides in an antiparallel manner, with each C-peptide binding to a conserved hydrophobic groove formed by two N-peptides. Therefore, the intervening sequence of gp41 (not present in the structure) would be required to loop around from the base of the coiled coil to fold the C-peptide back to the same end of the molecule (Figure 3*c*). Indeed, a solution structure of SIV gp41 shows the loop between the two helical regions (71).

It is interesting that the low-pH–activated HA2 structure also contains a trimer-of-hairpins (Figure 3*a*) (63, 72). In addition to the loop-to-helix spring-loaded conformational change in influenza HA, there is a significant helix-to-loop change between native and low-pH–activated HA. Six carboxy-terminal residues near the base of the central trimeric coiled coil of the native structure become a loop in the low-pH–activated structure (residues 106–112) (see Figures 1 and 3*a*), reversing the direction of what was the carboxy-terminal end of the coiled coil. An additional low-pH–activated HA structure that was recently solved unequivocally demonstrates that the amino- and carboxy-terminal regions of the ectodomain come together at the same end of the folded structure, similar to HIV-1 (Figure 3) (72).

There are many reasons why the trimer-of-hairpins of HIV-1 gp41 and influenza HA are thought to represent the fusion-active conformation of the envelope glycoproteins. First, for HA, the molecules used for the structural investigations were prepared by exposure to low pH (63, 72), and low pH is required to activate the fusion potential of HA (28–30). Second, in the absence of the surface subunits, the transmembrane subunits are expected to fold to their fusogenic state. Indeed, in HIV-1, conformational changes induced by receptor binding and required for fusion activation are likely to ultimately lead to the shedding of gp120 (37). In influenza, biochemical studies predict a partial dissociation of HA1 from HA2 on fusion activation, and HA2 of influenza folds into its low-pH–activated form in the absence of HA1 (62, 73). Third, the final fusogenic structures of HA and HIV-1 described above are extremely stable and unlikely to unfold after they are formed (65, 72). In contrast, the native, metastable state of HA is easily altered by low pH, heat, or chemical denaturants (64). The native state of Env is also altered by increasing temperature (74). Fourth, the resulting conformational changes in the soluble ectodomain fragments of HA are consistent with biochemical and

morphological observations of the conformational changes that are required for
HA-mediated fusion (31, 35, 36, 46, 47, 49–51). As mentioned above, these obser-
vations predict an exposure of the fusion-peptide region. The low-pH–activated
structures display significant conformational differences from the native struc-
ture, including a spring-loaded change that would presumably displace the fusion-
peptide region from a buried region to an exposed location on the amino terminus
of the trimeric coiled-coil core (63, 72). Also, the high-resolution structures are
consistent with the shape of HA observed previously in electron micrographs of
low-pH–activated HA on the viral surface (36). Fifth, specific mutations that alter
fusion activity map to regions that change conformation between the native and
low-pH–activated structures in influenza or to the central trimeric coiled coil of
the HIV-1 gp41 structure (38–46, 63). These mutations do not affect processing
or cell-surface expression of the membrane-fusion proteins and, thus, presum-
ably do not substantially alter the native structure. Specifically, as mentioned
earlier, proline substitutions in the spring-loaded region of HA ablate fusion (39).
Finally, these structures are consistent with the biochemical data of inactivated
virus. When influenza is exposed to low pH in the absence of a target membrane,
it is inactivated, presumably by inserting its fusion peptide into its own or other
viral membranes (75–77). In the low-pH–activated structures, the amino and car-
boxy termini are located at the same end of the molecule, just as they would be in
the inactivated form. Indeed, a monoclonal antibody against residues 105–113 of
HA2 (see Figure 3*a*) binds to the tip of HA distal to the viral membrane of inac-
tivated virus (48). Therefore, the trimer-of-hairpins structure is thought to define
the fusion-active conformations of these viral envelope glycoproteins that occur
either concomitantly or just after membrane fusion. Hereafter, these structures are
referred to as fusogenic.

gp41-Derived Inhibitory Peptides   The conclusion that the trimer-of-hairpins is
indeed the fusogenic state is consistent with the inhibitory activity of gp41-derived
peptides. Several years before the structural studies on HIV-1 gp41, synthetic N-
and C-peptides were shown to inhibit HIV-1 infection (65, 78–81). The C-peptides,
effective at nanomolar concentrations, are much more potent than N-peptides,
which require micromolar concentrations for effectiveness. It was proposed that
C-peptides act by binding to or near the predicted helical region downstream from
the fusion peptide, which corresponds to the N-peptide region (65, 81, 82), and
therefore inhibits infection in a dominant-negative manner (65).

Several experimental observations support the dominant-negative inhibition hy-
pothesis. First, the inhibitory activity of the C-peptide is substantially decreased
in the presence of an equal amount of N-peptide (65). Second, mutant C-peptides
that destabilize formation of the trimer-of-hairpins structure also demonstrate
weakened antiviral potency (83, 84). Third, the hydrophobic binding surface on
the N-peptide coiled-coil core to which the C-peptides bind is highly conserved
between HIV-1 and SIV. Indeed, the comparable SIV C-peptide inhibits HIV-
1–mediated membrane fusion (70). Fourth, viruses that develop resistance to

C-peptides contain substitutions of several residues in the N-peptide region (85). And fifth, epitope-tagged C-peptide can immunoprecipitate gp41 (86). Thus, there is compelling evidence that C-peptides inhibit formation of the trimer-of-hairpins in a dominant-negative manner by binding to the N-peptide region of gp41.

A conundrum remains, however, with a simple dominant-negative model (16). How can the C-peptides—in an intermolecular interaction that occurs at nanomolar concentrations—prevent formation of an intramolecular interaction in which the N- and C-peptide regions of the same gp41 molecule interact to form a hairpin?

A Transient Fusion Intermediate   The conundrum is partially solved by the proposal of a transient intermediate in the fusion process—an intermediate formed after receptor binding but before formation of the trimer-of-hairpins (16, 69, 86) (Figure 4). In this intermediate, termed prehairpin, the N-peptide region is exposed, vulnerable to binding by synthetic C-peptides. To finish solving the conundrum, it is proposed that the C-peptide–inhibited gp41 ultimately becomes irreversibly inactivated.

Several experimental observations support the existence of the prehairpin intermediate. For example, C-peptides must be present during exposure to the host cell in order to be effective. If virus is preincubated with C-peptide, followed by removal of the C-peptide before addition of the target membrane, infection is not inhibited (86). Also, epitope-tagged C-peptide can immunoprecipitate gp41, but only after exposure to CD4 (and for some HIV-1 isolates, a coreceptor), demonstrating that a receptor-mediated conformational change is required to expose the N-peptide region (86).

The kinetics of C-peptide inhibition suggest that the transient intermediate experiences a lifetime of many minutes (16, 87). Conformational changes resulting from receptor binding begin in under 1 min (32), but C-peptides maintain potency even if added up to 15 min after receptor exposure (87).

A transient intermediate has also been proposed for influenza HA (2, 9), although it has yet to be verified experimentally. In influenza, it is likely that the conformational changes between the native structure and the fusogenic structure occur sequentially. First, the spring-loaded mechanism occurs, propelling the fusion peptide up to the target membrane; this step corresponds to the formation of the prehairpin in HIV-1. Next, the bottom of the trimeric coiled coil reverses direction to fold the molecule in half, bringing the two membranes into close contact, just as in the trimer-of-hairpins for HIV-1.

As mentioned above, N-peptides derived from HIV-1 gp41 exhibit anti-HIV activity, but with much lower potency than do the gp41 C-peptides (65, 79). The inhibition mechanism of N-peptides is unknown, however, because of their tendency to aggregate. It is possible N-peptides inhibit by targeting the C-peptide region of gp41 (65); alternatively, they may intercalate into the N-peptide region (44, 79). Recently, HIV-1 fusion inhibitors that specifically target the C-peptide region of gp41 were identified (88; DM Eckert & PS Kim, unpublished observations) (see also below), confirming the accessibility of the C-peptide region prior to
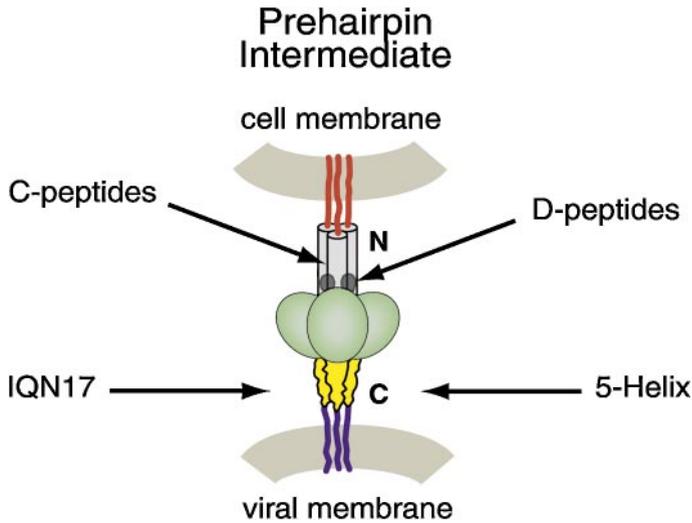
Figure 4   Model of the prehairpin intermediate and inhibitors. After the HIV-1 envelope protein (Env) binds CD4 and the coreceptor, a transient intermediate is formed in which gp41 spans both the viral and the cell membranes. The N-peptide region of gp41 (gray) is exposed and vulnerable to inhibitors. The N-peptide trimeric coiled coil contains three grooves that can be bound by C-peptides (65, 78, 80, 81). It also contains three prominent hydrophobic pockets at its base that can be targeted by potential small-molecule entry inhibitors, such as D-peptides (132). Binding of either C-peptides or D-peptides to the transient intermediate ultimately leads to irreversible inactivation of membrane fusion. Two additional types of inhibitors, 5-Helix (88) and IQN17-like molecules (DM Eckert & PS Kim, unpublished observations), bind the C-peptide region of gp41 (yellow), also inhibiting fusion. Although 5-Helix and IQN17 are depicted binding to the prehairpin intermediate, it is unknown whether these inhibitors target the prehairpin intermediate, the native state of gp41, or both.

formation of the trimer-of-hairpins. However, it is unknown whether the C-peptide is exposed prior to or during the formation of the prehairpin intermediate.

A Common Fusion Mechanism

With the high-resolution structures and other data described above, it is possible to describe a common model for both HA- and Env-mediated membrane fusion. The model described below is derived from several current hypotheses (2, 9, 12, 16) (Figure 5) and considers current experimental data.

In cells infected by either virus, the viral envelope glycoprotein is expressed as an unprocessed precursor, unable to fuse. Subsequently, the precursor is proteolytically processed, locking the protein into a metastable state. The structure of the HA0 unprocessed precursor, recently solved by X-ray crystallography (89), is almost identical to the native, processed form, differing primarily in the 18 residues surrounding the cleavage site. In the precursor, these residues are exposed
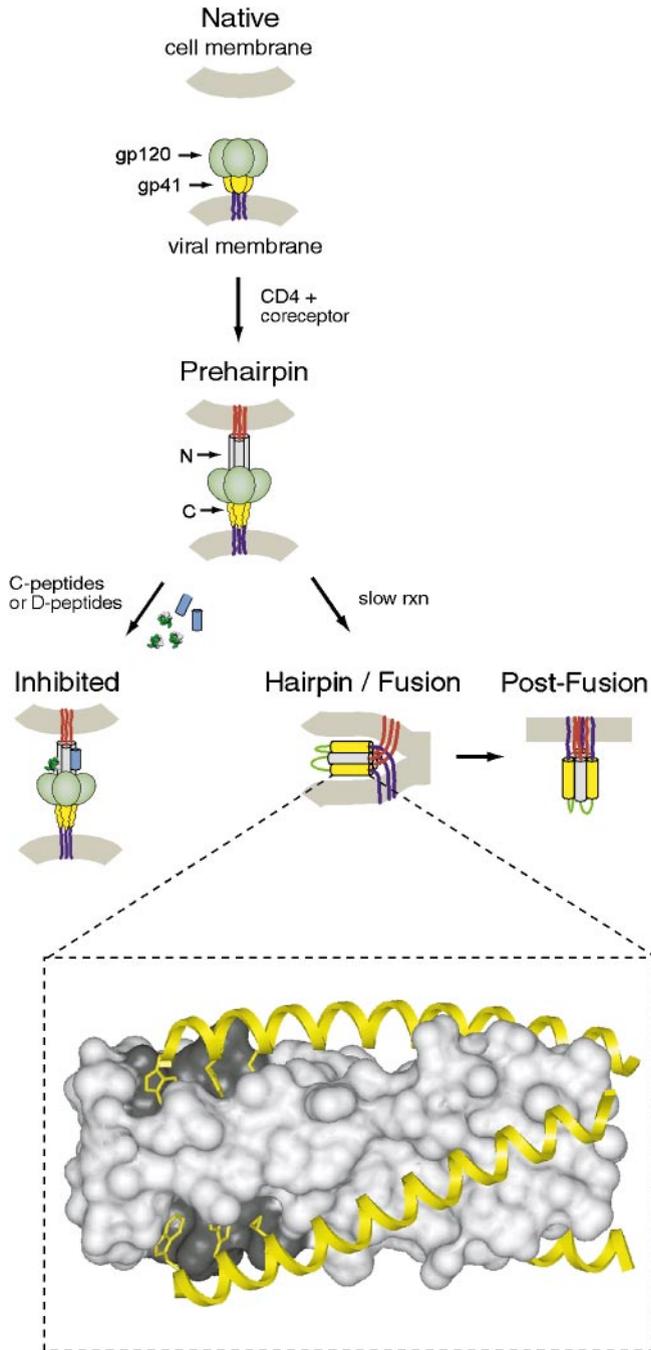
to solvent and folded as an extended, uncleaved loop. On cleavage, the newly created carboxy terminus of HA1 and amino terminus of HA2 separate. The fusion peptide is deeply buried in an interior region at the base of HA, primed for low-pH–activated structural rearrangement.

Data suggest the cleavage event locks the fusion glycoprotein into a metastable state, blocked from its most stable fold by a kinetic barrier. For example, the same HA conformational changes caused by exposure to low pH can also be activated at neutral pH by high temperature or urea denaturation (64). Mutants of HA that mediate fusion at a higher pH than wild type also require a lower temperature than wild type to fuse at neutral pH (90), consistent with metastability of the native state. In addition, when HA2 is expressed in the absence of HA1, it adopts the fusogenic structure (62, 73). Most likely, when HA0 folds as a single-chain precursor, it folds to its energetically most stable state (64). On proteolytic processing, that state becomes the metastable state for the cleaved molecule, essentially priming the virus for fusion.

When the processed protein is exposed to fusion-activating conditions, it overcomes the kinetic barrier of the native state and initiates the conformational changes necessary for fusion. In influenza, the HA1 domains lose their trimeric contacts and slightly dissociate from the compact glycoprotein structure. The fusion peptide is liberated from its buried position and propelled in a spring-loaded fashion to the amino terminus of the central trimeric coiled coil. Now exposed, the fusion peptide can interact with the host-cell membrane. In HIV-1, binding of

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Figure 5   Schematic representation of the current working model for viral membrane fusion. In the native state of the fusion protein, most of the exposed surface area is composed of the surface subunit. Much of the transmembrane subunit, including the fusion peptide, is not exposed. Following fusion-activating conditions (binding of CD4 and coreceptor for HIV-1 and low pH for influenza), conformational changes occur to free the fusion peptide from its unexposed location. For influenza hemagglutinin (HA), this occurs via a "spring-loaded" mechanism. In HIV-1, it is unknown whether fusion peptide release is spring loaded or whether the fusion peptide is simply uncovered by the movement of gp120. At least in HIV-1, the conformational changes result in the formation of the transient prehairpin intermediate. Such an intermediate is also likely to exist in HA-mediated fusion. The prehairpin intermediate spans two membranes, with its transmembrane region in the viral membrane and the fusion peptide deposited in the host-cell membrane. The N-peptide coiled coil, and likely the C-peptide region, is exposed, vulnerable to inhibitory molecules, at least for HIV-1. The prehairpin intermediate of HIV-1 constrains gp41 such that the N- and C-regions cannot interact. In the absence of inhibitors, the prehairpin intermediate resolves into the trimer-of-hairpins, and membrane fusion occurs. (*Inset*) The high-resolution crystal structure of HIV-1 gp41 (67). The N-peptide trimeric coiled-coil core is shown as a surface representation, whereas the C-peptides are depicted as helices (yellow). Three residues from each C-peptide bind to a small hydrophobic pocket (dark gray) that is the target for HIV-1 entry inhibitors (132). Inset drawn with Insight II 98.0 (Molecular Simulations, Inc).

gp120 to CD4 causes conformational changes in gp120, allowing attachment to the coreceptor, followed by further conformational changes in both gp120 and gp41 that greatly weaken their interaction. The transient prehairpin intermediate of gp41 is formed, freeing the previously buried fusion peptide to interact with the host-cell membrane and exposing the N-peptide region (and possibly the C-peptide region). It is unknown whether, as in influenza, there is a spring-loaded mechanism involved in forming the prehairpin intermediate in HIV-1, or whether the fusion peptide maintains the same position as in the prefusogenic state and is simply uncovered by the movement of gp120.

Analogous to HIV-1 Env, it seems likely that influenza HA also progresses through a transient intermediate state—after the spring-loaded mechanism but before the formation of the hairpin. When HA2 is exposed to low pH in the presence of target membranes that contain radioactively labeled, photoactivatable cross-linking reagents, only the fusion peptide becomes labeled (75), and evidence suggests that the labeling of HA2 precedes membrane fusion (91). In the absence of a target membrane, HA quickly becomes inactivated after acidification (76, 77). In the inactive state, the fusion peptide interacts with the viral membrane (75), probably forming the fusogenic structure anchored solely in the viral membrane.

In both viruses, after the fusion peptide of the glycoprotein inserts into the target membrane, the transmembrane subunit spans two membranes, those of the virus and those of the cell. In the fusogenic structure, both termini of the transmembrane subunit ectodomain are located at the same end of the folded molecule. This structure is extremely stable and presumably represents the most thermodynamically favorable form of the molecule. The progression from the transient intermediate to the energetically favorable fusogenic conformation brings the two membrane-proximal regions close together. It is likely that the energy gained by forming this highly stable structure overcomes the unfavorable process of pulling two phospholipid membranes into close proximity. To juxtapose the two membranes and maintain membrane contact on both ends, the fusion glycoprotein likely tilts over and lies parallel to both membranes. Flexibility in the membrane-proximal regions likely assists the tilting of the molecule. Ultimately, both transmembrane regions of the fusion glycoprotein (the fusion peptide and the original transmembrane helix) occupy the same membrane as the viral and cellular membranes become one.

The above description is a simplistic model of a complicated process. The molecular details that drive membrane juxtaposition to membrane fusion are only beginning to be discerned. A small sampling of those details is described here.

Electrophysiological experiments show that fusion between the influenza and host-cell membranes involves a flickering pore that expands over time to allow the release of the viral contents into the cell (92–94). Several pieces of evidence suggest that multiple HA molecules are required to gather at the fusion site to promote pore formation. First, there is a lag time between acidification and pore formation, and this lag time is shown to depend cooperatively on the density of HA on the cell (95). Second, when HA is present at low amounts on the cell,

hemifusion (the fusion of the outer monolayers of the membrane in the absence of inner monolayer fusion) occurs (96). Third, quick-freeze electron microscopy reveals an ordered array of HA trimers at the fusion site (97).

It has been proposed that pore formation occurs through a lipid intermediate in which the outer monolayers fuse first, likely forming a stalk between the two enveloped entities (98), followed by formation of the pore when the inner monolayers fuse (99–103). Indeed, the same lipid compositions that promote or inhibit stalk/pore formation likewise affect HA-mediated fusion (99, 101, 103).

OTHER MEMBRANE FUSION PROTEINS

Fusion Proteins of Other Enveloped Viruses

During the past several years, many X-ray crystal structures of enveloped viral fusion protein cores were published (67–70, 72, 104–109) (Figure 6). The four viral families with the most extensive structural information are orthomyxovirus, retrovirus, paramyxovirus, and filovirus. Most of the new structures depict a similar theme—the trimer-of-hairpins described above for influenza and HIV-1. In these structures there is a characteristic central trimeric coiled coil, presumably posed to present the fusion peptide at its tip. At the base of the coiled coil, the chain folds back, and supporting structures bind to the outside of the coiled coil; in most cases, at least part of the outer structure contains a helix. Because the structures for such a diverse array of fusion glycoproteins are so similar, it is likely these viruses utilize a similar mechanism of fusion, as described above.

Computational methods predict that many of the viral fusion glycoproteins not yet studied by high-resolution structural methods also form the trimer-of-hairpins. Heptad repeat regions were identified in many viral envelope proteins over a decade ago (110–113). However, an improved iterative method called LearnCoil-VMF (114), using a database of viral membrane fusion proteins with potential coiled-coil regions, provides a broader, more accurate prediction for many viruses within the retrovirus, paramyxovirus, and filovirus families. LearnCoil-VMF predicts N- and C-helical regions in the fusion glycoproteins of most paramyxoviruses and the lentivirus genus of retroviruses. Therefore, most of these proteins likely form the helical trimer-of-hairpins seen in HIV-1 (67–69) and SIV (70, 71) (Figure 6a). Indeed, even though paramyxovirus fusion glycoproteins contain more than 200 residues between the predicted helical regions, recent X-ray crystallography studies confirm formation of the trimer-of-hairpins structure (104, 115) (Figure 6b). In filoviruses and the remaining genera of retroviruses [mammalian C-type, avian C-type, D-type, and bovine leukemia virus—human T-cell leukemia virus (BLV-HTLV)], only the N-helical region is predicted by LearnCoil-VMF. This prediction is confirmed in the X-ray crystal structures of the glycoproteins of Moloney murine leukemia virus (a mammalian C-type retrovirus), human T-cell leukemia virus
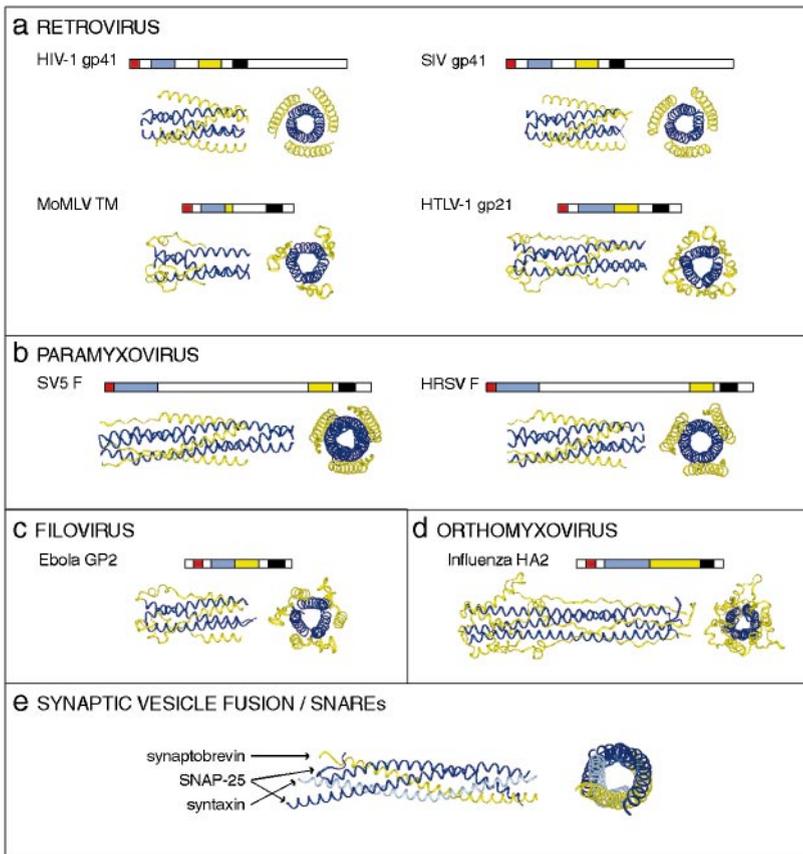
Figure 6   Trimer-of-hairpin structures of the transmembrane subunits of viral fusion glyco-
proteins and the coiled-coil bundle of the fusion proteins of synaptic vesicle fusion. (*a–d*)
The primary and high-resolution structures of eight enveloped virus fusion glycoprotein
transmembrane domains shown approximately to scale. In each primary structure, the fu-
sion peptide (red), N-peptide region (blue), C-peptide region (yellow), and transmembrane
domain (black) are depicted. In the trimer-of-hairpins side views, the amino- and carboxy-
terminal ends of the fusion proteins are located at the same end of the folded structure (right).
The top view is looking down from the amino terminus of the N-peptide coiled-coil core.
(*a*) The retrovirus family: HIV-1 gp41 (67; see also 68, 69), simian immunodeficiency virus
(SIV) gp41 (70; see also 71), Moloney murine leukemia virus transmembrane subunit (TM)
(105), and human T-cell leukemia virus type-1 gp21 (106). (*b*) The paramyxovirus family:
simian parainfluenza virus 5 F (104) and human respiratory syncytial virus F (115). (*c*) The
filovirus family: Ebola gp2 (107; see also 109). (*d*) The orthomyxovirus family: influenza
HA2 (72; see also 63). (*e*) High-resolution structure of the SNAREs involved in synap-
tic vesicle fusion (124) (side view and top view). (Blue) The plasma membrane SNAREs
(syntaxin and SNAP-25); (yellow) the vesicle SNARE (synaptobrevin). High-resolution
structures drawn with Insight II 98.0 (Molecular Simulations Inc).

(a BLV-HTLV), and the filovirus Ebola, in which the only substantial stretches of helix are composed of the N-peptides (Figure 6a,c). Nonetheless, these structures still contain the trimer-of-hairpins. The N-peptide region forms the familiar trimeric coiled-coil core, but the surrounding C-peptides pack around the N-peptide core in a more extended conformation with only short helical stretches, similar to HA (105–107, 109) (Figure 6d).

Enveloped viruses are known to cause many serious human diseases and disorders. For example, human respiratory syncytial virus, a paramyxovirus, is a major cause of bronchiolitis and pneumonia in infants and young children. Ebola, a filovirus, can cause a severe form of hemorrhagic fever. Because these and many other viruses likely have a similar fusion mechanism to that described in this review, they may be vulnerable to similar mechanisms of inhibition. It is interesting that the C-peptide regions of many of the paramyxoviruses, such as Sendai virus, measles, Newcastle disease virus, human parainfluenza virus, respiratory syncytial virus, and simian parainfluenza virus 5, can be utilized to inhibit virus infectivity (116–122). Additional methods for inhibiting HIV-1 entry, described below, may also apply to other enveloped viruses.

Vesicle Fusion Proteins

The coiled-coil helical bundle may be a global motif for promoting membrane fusion events. Recent evidence suggests that vesicle fusion is also mediated by formation of a coiled-coil structure that juxtaposes the fusing membranes (123, 124; for a review, see 125).

Vesicles move macromolecules from one membrane-bound cellular compartment to another through a series of membrane budding and fusion events. In exocytosis, vesicles fuse with the plasma membrane from within the cell to release macromolecules. For example, in synaptic vesicle exocytosis, the vesicle fuses with the plasma membrane of a neuron to release neurotransmitters into the synapse. A group of proteins called SNAREs, located on both the vesicle and target membranes, mediate fusion. In synaptic vesicle exocytosis, the vesicle contributes one SNARE (synaptobrevin), and the plasma membrane contributes two (syntaxin and SNAP-25).

X-ray crystallography studies suggest that the SNAREs of synaptic vesicle fusion mediate membrane fusion through the formation of a coiled-coil structure. Three SNARES form a highly stable bundle of four helices (124) (Figure 6e). (Each SNARE contributes one helix, except SNAP-25, which provides two.) The membrane-proximal regions are located at the same end of the helical bundle, just as in the trimer-of-hairpins of the viral protein. It is proposed that this bundle enables juxtaposition of the two membranes, leading to membrane fusion (123, 124).

Although vesicle exocytosis and viral membrane fusion are distinct biological processes that utilize diverse proteins for fusion, the underlying mechanism appears similar—the use of a coiled-coil structure to juxtapose the membranes. Quite

possibly, coiled-coil domains that participate in additional membrane fusion events will be identified.

INHIBITING HIV-1 ENTRY

Since the discovery of HIV two decades ago, over 20 million deaths have been attributed to acquired immune deficiency syndrome (AIDS). Currently, over 36 million people worldwide are infected with the virus, corresponding to one HIV-positive person for every 200 people in the world (25a). At the end of 2000, the 16 anti-HIV-1 drugs approved by the Food and Drug Administration target only two viral proteins. The two current viral targets are reverse transcriptase, which is responsible for transcribing the HIV-1 RNA genome to DNA, and protease, which processes the HIV-1 Gag/Pol polyprotein and the subsequent Gag protein (126). Because of the high rate of viral turnover (127) and the error-prone nature of reverse transcriptase, viruses resistant to these small-molecule drugs often emerge. Currently in the United States, combination therapy, in which three or more drugs are administered concomitantly, is the routine treatment. Although combination therapy is often successful at lowering viral load, there are significant problems associated with it. Some patients develop immediate adverse effects and are therefore intolerant of available drugs. Patients who are more tolerant face expensive, arduous treatment. Among these patients, some harbor viral strains resistant to several drugs (128), and long-term adverse effects of treatment can develop (126, 129, 130). Also, because of increasing viral resistance, the threat of an outbreak of a virus immune to all available drugs is rising (128). Therefore, drugs that target an additional step of the viral life cycle, such as viral entry, would be useful, especially if they are less toxic and less susceptible to viral resistance than current therapies.

The increased understanding of viral entry opens the door to the design and discovery of HIV-1 entry inhibitors (16, 22, 23, 25). As there are multiple protein molecules involved in the entry process, both on the virus and on the host cell, there are multiple potential targets for intervention. As mentioned, the inhibitory activity of the C-peptides demonstrates the feasibility of targeting the transient gp41 structure that emerges during viral infection. Indeed, several additional inhibitors were discovered that target gp41 prior to the formation of the trimer-of-hairpins (88, 131–133) (see below). Also, the recent identification of the HIV-1 coreceptors (for a review, see 18), as well as the completion of a high-resolution structure of gp120 core bound to CD4 and an antibody mimicking the coreceptor (134) (see below), made it possible to discover molecules that inhibit HIV-1 from binding to the cell.

Anti–HIV-1 molecules that inhibit entry stop the virus before it infects the host cell, unlike currently used drugs that act only after infection has occurred. Identified drugs that stop the virus from invading cells may be useful as prophylactic agents, creating a barrier to the initial infection event. The knowledge utilized for

the design of drugs to inhibit HIV-1 entry could also provide useful leads for effective HIV-1 vaccines.

Targeting the Transient Fusion Intermediate

The gp41 transient intermediate of viral entry is a promising target for inhibition (16). C-peptides, which bind to this intermediate, have shown reasonable success in human clinical trials as injected therapeutics (135). Participants who received 100 mg of the C-peptide T20 twice daily experienced viral reduction levels similar to patients treated with one reverse transcriptase or protease inhibitor.

There are disadvantages, however, to the therapeutic use of C-peptides. First, because of their size, C-peptides are not amenable to oral routes of entry and must be injected. Second, producing the lengthy C-peptide requires expensive chemical synthesis, and large amounts of the peptide are required to observe an antiviral effect in humans. It would be preferable to identify an orally bioavailable small molecule that mimics the function of the C-peptide as an alternative therapy.

Recent progress has been made toward identifying such molecules (for a review, see 24). In the gp41 X-ray crystal structure there is a small pocket in the conserved hydrophobic groove of the N-peptide trimeric coiled coil (Figure 5). Three hydrophobic residues from the C-peptide, two tryptophans and an isoleucine, bind this pocket. The pocket was proposed to be an attractive target for drug discovery for many reasons (67, 83). First, the pocket is small (400 Å^3) and provides a structurally defined binding surface, ideal for binding by a small molecule of 500–600 Da. Second, many of the residues lining the pocket are critical for membrane fusion (38, 40, 42–45). Third, C-peptide inhibitory activity depends on its ability to bind the pocket (83). Fourth, drugs that target this pocket may elude the emergence of resistant virus because (*a*) the residues that constitute the pocket are highly conserved among all known HIV-1 isolates as well as among SIV isolates, and (*b*) the mRNA encoding this region is an integral part of the structured Rev-response element (136, 137), which suggests there is selective pressure not to mutate at both the protein and RNA levels. Finally, although there are effective C-peptides that do not contain pocket-binding residues (e.g. T20), such C-peptides are more vulnerable to the emergence of resistant viruses than are those containing the pocket-binding residues (e.g. T649) (85).

Three methods to identify pocket binders have been reported (131–133). In one attempt, utilizing the X-ray crystal structure of the gp41 core and molecular docking techniques, a database of 20,000 small organic molecules was screened for potential fitting into the hydrophobic pocket. Of the 16 compounds with the best fit, two were reported to inhibit formation of the trimer-of-hairpins, with one inhibiting HIV-1 infection at micromolar concentrations (131). Further work is required to show that this compound is indeed binding to the pocket region of the N-peptide. A second method used a combinatorial chemistry approach. C-peptides with a combinatorial library of three organic chemical moieties replacing the pocket-binding region were screened for improved binding to N-peptides

(133). One member of the library improved the inhibitory activity of a 30-residue C-peptide but was not able to inhibit HIV-1 infection on its own. A third method utilized mirror-image phage display (138) to identify small peptides composed of D-amino acids that bind to the pocket (132).

The target for the mirror-image phage display was designed to overcome a potential hurdle in identifying pocket binders. gp41 N-peptides aggregate in the absence of C-peptides (65, 139) and therefore are not good targets for identifying pocket-binding ligands. A hybrid coiled-coil molecule, IQN17, was designed to avoid aggregation and properly present the gp41 pocket. In IQN17, a soluble trimeric coiled coil derived from GCN4-pI$_Q$I (140) is fused to the pocket-forming residues of gp41. IQN17 is helical, soluble, and trimeric, as shown by biophysical studies. Peptides were identified that bind to IQN17 but that do not bind to a control molecule with a point mutation that occludes the pocket. A 1.5 Å cocrystal structure and a simple nuclear magnetic resonance assay demonstrate that the D-peptides bind specifically to the gp41 pocket region of IQN17. These D-peptides inhibit HIV-1 infectivity at micromolar concentrations. Because of low potency, the D-peptides themselves may not prove useful for therapy, although their identification has validated the concept that targeting the gp41 coiled-coil pocket, and only the pocket, is a viable therapeutic option. In addition, the D-peptides in combination with the IQN17 target that accurately represents the gp41 pocket provide key tools for identifying other potentially useful pocket-binding compounds.

Targeting the C-Peptide Region of gp41

As mentioned, N-peptides derived from gp41 exhibit anti–HIV-1 characteristics at micromolar concentrations (65, 79), but the mechanism of N-peptide inhibition is not known. N-peptides have a strong tendency to aggregate (65, 139) and may either target the C-peptide region of gp41 (65) or intercalate into the gp41 amino-terminal coiled coil (44, 79).

To determine whether the C-peptide region is a useful target for inhibiting the formation of the trimer-of-hairpins structure, a new inhibitor, denoted 5-Helix, was recently designed (88). 5-Helix is a 25-kDa protein consisting of five of the six helices of the gp41 trimer-of-hairpins joined by short peptide linkers. The design harnesses the C-peptide binding ability of N-peptides while reducing their tendency to aggregate. 5-Helix, which lacks a third C-peptide, binds to C-peptide with high affinity and inhibits a variety of HIV-1 strains at nanomolar concentrations. A control molecule, 6-Helix, consists of the entire trimer-of-hairpins structure and does not bind C-peptide or inhibit HIV-1 infection. With its ability to specifically target the C-peptide region of gp41, 5-Helix demonstrates a new avenue for inhibiting HIV-1 entry (Figure 4).

In a similar approach, hybrid coiled-coil peptides that decrease the aggregation of N-peptides dramatically increase the anti–HIV-1 inhibitory activity of the N-peptides (DM Eckert & PS Kim, unpublished data). A panel of hybrid coiled-coil peptides was synthesized in which one of two stable, soluble, trimeric coiled

coils (GCN4-pI$_Q$I or IZ) was fused to the amino terminus of N-peptides of varying lengths. For example, IZN17 is composed of a designed trimeric isoleucine zipper (IZ) fused to 17 residues of the N-peptide. The hybrid coiled coils increase the inhibitory activity of the comparable N-peptide by two to three orders of magnitude, with the best inhibitor working at low nanomolar concentrations. Like 5-Helix, these peptides likely inhibit by binding to the C-peptide region of gp41 (Figure 4).

High-Resolution View of gp120

Recently, the high-resolution structure of gp120 bound to CD4 and a molecule mimicking the coreceptor was described (134; for review, see 141, 142) (Figure 7). The structure provides atomic detail on potential binding sites for entry inhibitors as well as suggestions as to why targeting these sites may prove difficult.

The primary sequence of gp120 is composed of five regions of high sequence variability among known HIV-1 isolates (termed V1–V5) interspersed with five conserved regions. The X-ray crystal structure shows a mostly deglycosylated gp120 conserved core (missing V1, V2, V3, and the amino and carboxy termini) bound to two domains of CD4 and to the antigen-binding fragment (Fab) of the monoclonal antibody 17b that binds to the coreceptor recognition site. gp120 contains two structural domains, an inner domain, presumed to contain the gp41 contacts, and an outer domain. These regions are joined by a small "bridging" domain (Figure 7).

The CD4 binding site is large and located at the interface of all three gp120 domains. The binding surface contains both conserved residues required for CD4 binding as well as variable residues. CD4 interacts with the variable residues through hydrogen bonding to main-chain atoms. Also, cavities exist within the binding surface that are not contacted by CD4. Immune recognition of this CD4 binding site is probably difficult for three reasons: (*a*) There is a mix of conserved and variable regions in the binding site, (*b*) the V1 and V2 regions likely mask the CD4 binding site prior to binding (143), and (*c*) the CD4-bound state may represent an otherwise energetically unfavorable conformation of gp120 (134, 142). For these same reasons, it may also be difficult to target this area with therapeutic molecules.

The Fab fragment binds to the side of the bridging domain composed of part of the fourth conserved domain and the base of the V1 and V2 stem. This region is presumed to be the coreceptor-binding site. Although the V3 loop is absent in the X-ray crystal structure, it seems likely that this region is shielded by the V3 loop before CD4 binding. First, in the high-resolution structure, there is a large gap between the core gp120 and the 17b antibody light chains that would most likely be filled by the V3 loop (143). Second, antibodies that recognize the V3 loop can inhibit coreceptor binding (55). Third, V3-recognizing antibodies compete for gp120 binding with the neutralizing antibodies, such as 17b, that recognize the coreceptor binding site (144).
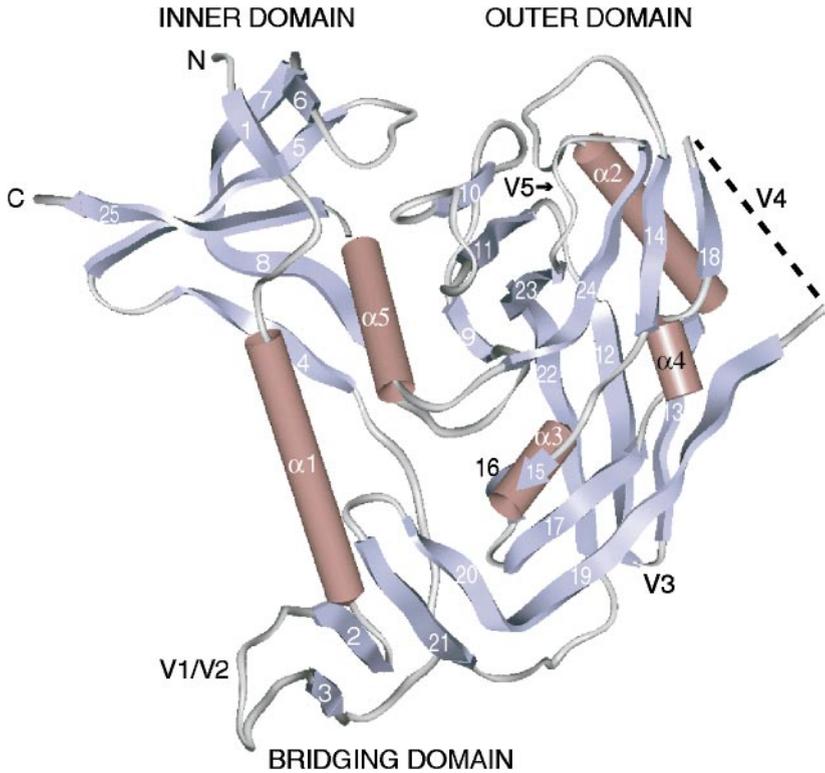
Figure 7 High-resolution X-ray crystal structure of core gp120. In this view, the CD4 binding site is facing the viewer, and the coreceptor-binding site is at the bottom of the structure. (Pink) α-Helices; (purple) β-sheets. The inner domain, which presumably contacts gp41, is composed of β-sheets 1, 4–8, and 25 as well as α-helices 1 and 5. The outer domain is composed of β-sheets 9–19 and 22–24 and α-helices 2–4. The bridging domain is composed of α-helices 2, 3, 20, and 21. The V1/V2 and V3 variable regions were both replaced with Gly-Ala-Gly tripeptide sequences. The positions of the V1/V2 and V3 substitutions are indicated. V4 was disordered in the structure (dashed line). The amino and carboxy termini of core gp120 are indicated. Figure drawn with Insight II 98.0 (Molecular Simulations, Inc). (Adapted from Reference 134.)

Inhibiting the gp120/CD4 Interaction

Early efforts to inhibit entry of HIV-1 focused on inhibiting binding of the envelope protein to CD4 on the surface of cells by competition with a soluble version of CD4. Soluble CD4 (sCD4) is composed of the ectodomain of CD4 and inhibits laboratory-adapted strains of HIV-1 (58, 145). When used against primary isolates, however, sCD4 was much less successful and actually increased the infectivity of some isolates (for a review, see 146). In laboratory-adapted strains, sCD4 induces shedding of gp120 from gp41 (37), thereby ablating the virus's potential to bind to

and fuse with host cells. The same extent of shedding is not observed in primary isolates that have a lower affinity for sCD4 (147). In addition, the initial interaction of gp120 with CD4 induces changes in gp120 that allow it to bind the coreceptor with higher affinity (59, 148, 149; for a review, see 18). Indeed, as mentioned above, after exposure to sCD4, HIV-1 Env can mediate fusion with cells expressing the coreceptor but lacking CD4 (59). Thus, in primary isolates, when gp120 shedding does not occur, sCD4 likely promotes fusion by facilitating binding to the coreceptor.

Recent designs of sCD4-like molecules, however, demonstrate promising results. Pro542 is a tetrameric version of sCD4 in which the gp120-binding region of CD4 is fused to the constant region of the human immunoglobulin, IgG2 (150). It has an increased affinity for gp120 and has shown some success in decreasing viral loads of HIV-1–infected patients in phase I clinical trials (151). Lower-molecular-weight, sCD4-like molecules were made by grafting critical gp120-binding motifs from CD4 onto toxins (peptides of ∼30 residues) of similar structure (152, 153). The toxin chimeras are capable of mimicking CD4-induced conformational changes in gp120 (152) and inhibiting CD4 binding and viral infectivity (153).

There are several other molecules undergoing preclinical or clinical evaluation that interfere with the gp120/CD4 interaction (for reviews, see 22, 23, 25). FP213999, Zintevir, and cyanovirin-N are anti–HIV-1 molecules identified for their ability to inhibit viral infection in vitro (154–156). Subsequently, each was proposed to inhibit the gp120/CD4 interaction (157–159). PRO 2000, a 5-kDa napthalene sulfonate polymer, was shown to inhibit gp120/CD4 interaction in an ELISA assay (160). Despite their ability to inhibit the gp120/CD4 interaction in vitro, the antiviral activities of cyanovirin-N and PRO 2000 are nonspecific (157, 161). Ultimately, any of these molecules that do not demonstrate ideal behavior as therapeutics in clinical studies may be useful as topical preventive medications.

Chemokine Receptors

Chemokine receptors provide an additional potential therapeutic target. Most HIV-1 variants use one or both of two specific chemokine receptors, CXCR4 and CCR5 (for a review, see 18, 19, 162), for cell entry. CXCR4 is expressed on T-cells and is therefore utilized by T-cell–tropic HIV-1 (now called X4 isolates). CCR5 is expressed on macrophages and is consequently the coreceptor of choice for macrophage-tropic HIV-1 (or R5 isolates). Both receptors are expressed on primary T-cells. These cells are vulnerable to infection by R5, X4, and dual-tropic (R5X4) viruses. Although CXCR4 and CCR5 are the two most common coreceptors, at least 11 additional coreceptors have been reported. It is not known whether any of the additional coreceptors have significance in vivo.

The chemokine receptors are attractive targets for several reasons. First, the chemokine receptors are static targets not prone to mutation, in contrast to viral targets. Second, rare individuals with homozygous deletions in the CCR5 gene

are resistant to R5 virus infection (163, 164). Third, each coreceptor has specific chemokine ligands, and these ligands are effective in blocking entry of HIV-1 isolates that utilize that specific coreceptor. For example, the chemokine ligands of CCR5 (RANTES, MIP-1α, and MIP-1β) are effective inhibitors of R5 HIV-1 strains (165–168), whereas SDF-1α inhibits CXCR4-mediated entry (169, 170).

Treatment with chemokines themselves may cause unfavorable side effects. Chemokines bind chemokine receptors to guide the movement of white blood cells during an immune response to injury or infection. Therefore, the use of chemokines as therapeutic molecules may cause unwanted activation or interference with normal signaling of the inflammatory pathway. In response to this concern, chemokine derivatives that uncouple viral inhibition activity from natural receptor function were created (171–175). For example, a RANTES derivative lacking eight amino-terminal amino acids no longer activates chemotaxis, yet is only slightly less effective at inhibiting CCR5-mediated HIV-1 entry (171). Also, in a step toward potent, small-molecule drugs, several low-molecular-weight compounds were identified that bind to either CXCR4 or CCR5 and inhibit HIV-1 infection (176–178). A bicyclam, AMD3100, is the most advanced in therapeutic trials (178).

It is unknown whether the targeting of chemokine receptors will be a successful therapeutic approach. Although individuals with homozygous deletions in CCR5 do not exhibit any negative side effects (163, 164), CXCR4 knockout mice exhibit an embryonic-lethal phenotype (179, 180). In addition, it is possible that blocking specific coreceptors will lead to a possibly detrimental selection for viruses with altering tropism. In a SCID-hu mouse model sensitive to HIV-1 infection, a rapid switch in coreceptor usage was seen after a short treatment with an anti-CCR5 agent (174), and in an HIV-1 infection, a switch to an X4 viral population usually coincides with the onset of AIDS.

HIV-1 Vaccine Strategies Targeting gp120 and/or gp41

A safe vaccine that could be widely administered and could either prevent or decrease the rate of infection would be the most useful method for combating new infections in the world. Current vaccine efforts are twofold: (*a*) to elicit cell-mediated responses that target HIV-1 infected cells, and (*b*) to raise a neutralizing antibody response that effectively targets viruses. Significant progress has been made in generating a cell-mediated HIV-1–specific immune response (181, 182). Rodents and nonhuman primates generate HIV-1–specific cytotoxic T-lymphocytes in response to a variety of immunogens, including live recombinant vectors, HIV-1 peptides, and plasmid DNA–encoding HIV-1 proteins. Raising an effective neutralizing antibody response has been much more elusive.

Only three antibodies that potently neutralize a wide range of HIV-1 isolates in vitro have been isolated from HIV-1–infected patients—two specific for gp120 epitopes (b12 and 2G12) and one that recognizes a gp41 epitope (2F5) (183–187).

Passive transfer of a mixture of these antibodies can successfully protect Rhesus macaques against challenge by a SHIV containing either a laboratory-adapted or a primary isolate HIV-1 Env (188, 189). However, neutralizing antibodies were administered at extremely high levels (ranging from 30–400 mg/kg) to observe this effect.

Although the HIV-1 Env protein is extremely immunogenic, attempts to raise potent neutralizing antibodies in the laboratory against a broad range of HIV-1 viruses with viral and protein immunogens have been largely unsuccessful (for reviews, see 190–192). This is likely due to a variety of reasons. First, much of the sequence of Env is highly variable between viral strains, and therefore neutralizing antibodies are often strain-specific. Second, the high mutation rate of the virus likely allows quick escape from potentially neutralizing antibodies. Finally, most protein immunogens used thus far have probably not properly represented the trimeric conformation of Env found on the surface of the virus.

Recent efforts attempt to reproduce the native structure of Env. For example, Binley et al (193) created a stable gp120/gp41 complex, SOS gp140, with an engineered disulfide bond to keep the two subunits covalently associated. The antigenic profile of SOS gp41 is similar to that of native Env. The same disulfide-bonded construct, with deleted variable loops on gp120, may expose hidden conserved epitopes on gp120 (194). In addition, Yang et al (194a) have created soluble stabilized Env trimers that elicit neutralizing antibodies more efficiently.

In a different approach, it was suggested that eliciting an antibody response against transiently exposed conserved conformations of proteins involved in HIV-1 may be successful at neutralizing a broad range of viral strains (132, 195). The gp41 prehairpin intermediate, or other Env conformations, such as the coreceptor-binding site, exposed transiently during the fusion process, may provide useful targets. These regions are likely too transient for HIV-1–infected patients to develop an immune response to them. However, potential immunogens could present these structures in stable, exposed conformations. For example, a covalent gp120/CD4 protein antigen could stably expose the conserved coreceptor-binding site (196). Also, gp120 molecules lacking glycosylation or some of the variable regions may increase the exposure of neutralizing epitopes (197). Additionally, molecules such as 5-Helix (88) and IQN17 (132), which accurately represent the potential transient conformations of gp41, may be able to raise a neutralizing response.

CONCLUSION

Extensive biochemical and structural studies on viruses from different families provide a general mechanism for viral envelope glycoprotein-mediated membrane fusion. Viruses synthesize their fusion glycoproteins in an inactive form. In this state, the fusion glycoprotein adopts a thermodynamically stable conformation.

Subsequently, the fusion glycoprotein is proteolytically processed into two subunits, a surface subunit and a transmembrane subunit. No longer free to sample all conformational space, the processed protein is trapped in the same conformation as the precursor, primed for fusion. The protein waits in a metastable state for the appropriate activation signal, whether an induction of low pH or receptor binding. After the signal arrives, the glycoprotein unleashes its fusion potential. No additional energy, such as ATP hydrolysis, is required. Through a spring-loaded mechanism, at least in the case of influenza HA, the fusion peptide is propelled out of the interior of the protein and inserted into the target membrane. The protein now spans both membranes, and in HIV-1 Env, this prehairpin intermediate is vulnerable to inhibition for many minutes. Subsequently, the protein then adopts its most stable fold, the trimer-of-hairpins. The energy harnessed through acquisition of the stable state likely promotes fusion of the two membranes. Recent evidence on SNARE-mediated synaptic vesicle fusion suggests that coiled-coil helical bundles may be a global motif for promoting membrane fusion events.

The dissection of the viral membrane fusion process has led to a new strategy in HIV-1 therapy development—targeting viral entry. Current efforts attempt to inhibit HIV-1 binding to CD4 and the coreceptor, as well as gp41-mediated membrane fusion. Because many enveloped viruses likely use the same mechanism of entry, similar therapeutic strategies may be effective against a wide range of viral diseases.

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