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Review

The HIV Env-mediated fusion reaction

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Abstract

The current general model of HIV viral entry involves the binding of the trimeric viral envelope glycoprotein gp120/gp41 to cell surface receptor CD4 and chemokine co-receptor CXCR4 or CCR5, which triggers conformational changes in the envelope proteins. Gp120 then dissociates from gp41, allowing for the fusion peptide to be inserted into the target membrane and the pre-hairpin configuration of the ectodomain to form. The C-terminal heptad repeat region and the leucine/isoleucine zipper region then form the thermostable six-helix coiled-coil, which drives the membrane merger and eventual fusion. This model needs updating, as there has been a wealth of data produced in the last few years concerning HIV entry, including target cell dependencies, fusion kinetic data, and conformational intermediates. A more complete model must include the involvement of membrane microdomains, actin polymerization, glycosphingolipids, and possibly CD4 and chemokine signaling in entry. In addition, kinetic experiments involving the addition of fusion inhibitors have revealed some of the rate-limiting steps in this process, adding a temporal component to the model. A review of these data that may require an updated version of the original model is presented here.

Keywords: HIV; Fusion; Env; SIV; Virus; CD4; gp120; gp41; CXCR4; CCR5; Six-helix bundle

1. Introduction

HIV/SIV deliver their genetic material into the cell by direct fusion of the viral membrane with the plasma membrane of the host cells [1]. The fusion is mediated by Env glycoproteins [2–5], which are organized into oligomeric, probably trimeric spikes [6], and anchored in the viral membrane by the gp41 transmembrane protein. The Env glycoprotein, gp120, forms surface trimeric spikes, which are associated by noncovalent interactions with each subunit of the trimeric, normally hidden, gp41 [7]. The similarity between structural motifs of gp120–gp41 and influenza hemagglutinin (HA) leads to the notion that the native conformation of gp41 is metastable and it is stabilized by gp120 [8,9]. The triggering mechanisms that activate Env are quite complex involving target cell CD4 [10], co-receptors [11–16], and perhaps other cell surface components [17–19]. These interactions trigger a barrage of conformational changes in Env that drive the membrane fusion process [20–25] (see Fig. 1). The final outcome of the reaction is the formation of a six-helix bundled gp41 ectodomain (Fig. 2) core structure consisting of three N helices paired with three anti-parallel C helices [26–29]. The six-helix bundle structure is (Fig. 2) similar to the proposed fusogenic structures of envelope fusion proteins from influenza, Moloney murine leukemia virus, simian parainfluenza virus 5, Ebola virus, and simian immunodeficiency virus, as well as to the snarepin fusion machinery involved in intracellular fusion events [30]. The formation of the six-helix bundle presumably occurs concomitantly with membrane fusion [31]. HIV fusion can be inhibited by peptides that mimic the sequences of the N- and C-terminal helices by binding to the N terminal heptad repeat triple helices, or to the C-terminal regions of Env, thereby preventing six-helix bundle formation [32–34].

The model shown in Fig. 1 is based on structural information of the gp41 core and on studies with fusion inhibitors. It has served as a guide to direct our thinking about the way the envelope glycoprotein mediates membrane fusion. However, some crucial pieces of the puzzle are missing. For instance, although we know that the first step

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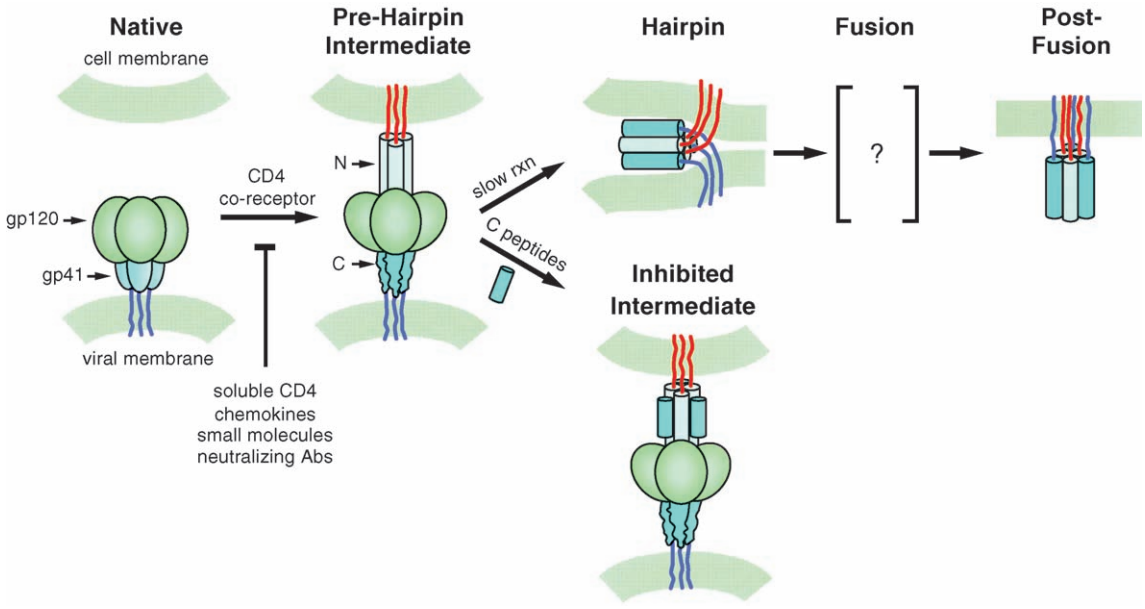

Fig. 1. Model of HIV-envelope-mediated membrane fusion according to Chan and Kim [4]. Upon binding to CD4 and co-receptor, gp120 undergoes conformational changes that allow gp41 fusion peptide insertion into the target membrane and the formation of a pre-hairpin structure. As the gp120 dissociates from gp41, the latter undergoes a slow reaction, which transforms the pre-hairpin into the six-helix bundle. It is during this process that the envelope-induced fusion is sensitive to inhibition by C-terminal peptides. (Current data show that Env–CD4 interactions trigger the pre-hairpin state that becomes sensitive to C-terminal inhibitors and that co-receptor engagement leads to rapid six-helix bundle formation [48]). The formation of the six-helix bundle promotes complete fusion, in which the fusion peptide and the transmembrane segment of gp41 lie parallel on a contiguous bilayer. Reprinted from Ref. [4], with permission from Elsevier.

involves exposure of a pre-hairpin that is sensitive to entry inhibitors, it has not been shown that the fusion peptide inserts into the target membrane under these conditions. Other steps that occur before or after six-helix bundle formation have not been resolved. These include further conformational changes in Env, aggregation of viral proteins, lipid-fusion peptide interactions, hemi-fusion, fusion pore formation, and pore widening.

In order to get a handle on these intermediate processes, we need to know the rate at which the reaction proceeds. Therefore, kinetic assays and methodologies have been developed to dissect intermediate steps in the fusion reaction. In Section 2, we will describe these kinetic approaches. The role of the target membrane in assembling the receptors that trigger Env and participate in the formation of fusion complexes will be discussed in Section 3. Section 4 will describe various approaches to reveal the temporal sequence of conformational changes in Env as the fusion reaction proceeds. The final steps that couple the protein conformational changes to the lipid rearrangements required for fusion will be discussed in Section 5.

2. Kinetics of the HIV fusion reaction

Fluorescent probes have been extensively used in the study of viral membrane fusion (for a review, see Ref. [35]). Studies of fusion of intact virus with cells have utilized fluorescent dequenching assays, which are based on the redistribution of a lipid dye incorporated into the viral membrane to the target membrane as a result of fusion. However, because of the difficulty in purifying HIV virus into highly active homogenous stock, noninfectious particles and general debris can lead to nonspecific transfer and, therefore, a dequenching artifact, which occurs on roughly the same time scale as HIV fusion. However, a photosensitized labeling methodology provided a reliable time course of fusion of HIV and SIV with biological membranes [36]. The most striking result is that at 37 °C SIV reaches maximum fusion with a $t_{1/2}$ of 19 min. This indicates that the CD4 and co-receptor-induced triggering events leading to HIV/SIV Env-mediated fusion are stochastic, leading to a much slower fusion kinetics as compared to the low pH fusion induced by influenza HA. The lack of synchrony in the activation of HIV/SIV Envs therefore provides an opportunity for the C terminal peptide inhibitors to bind to the pre-hairpin grooves, which become transiently exposed following CD4-induced triggering of HIV-1 Env.

Kinetics of HIV/SIV Env-mediated membrane fusion have extensively been studied using envelope glycoproteins expressed in cells interacting with target cells bearing CD4 and appropriate co-receptor [37]. Although there are subtle differences between fusion of intact virus with cells and Env-mediated cell–cell fusion, the basic mechanisms that underpin both phenomena are the same. Although syncytia counting [38] and gene reporter assays [39] of cell–cell fusion have been used to monitor HIV Env-mediated fusion,

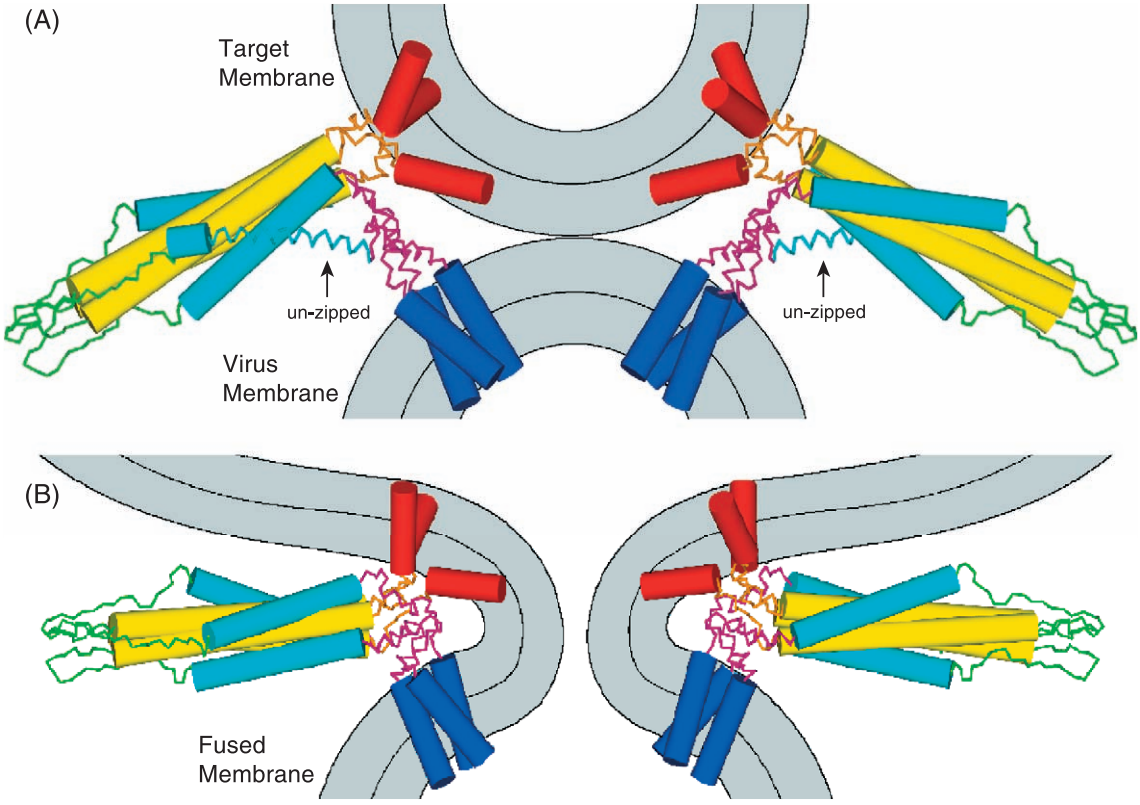

Fig. 2. Cartoon representations of atomic-scale models of HIV gp41 catalyzing virus/target cell membrane fusion. Two opposing members are shown to illustrate the likely larger group of proteins radially aligned to form a fusion pore. Helical segments of the proteins are shown as cylinders, and the intervening segments are shown as alpha-carbon traces. The fusion peptides are colored red, the N- and C-terminal helices of the six-helix bundle are colored yellow and cyan, respectively, and the anchors in the virus membrane are colored blue. The intervening segments between the fusion peptides and the N-terminal helices are in orange, between the N- and C-terminal helices in green, and between the C-terminal helices and the viral anchors in magenta. The coordinates for the residues of the six-helix bundle were obtained from Chan et al. [26] (1AIK.pdb), and for the extension of the N-terminal helices and the loops to the C-terminal helices were derived from the structure of SIV from Caffrey et al. [29] (2EZO.pdb). (A) Pre-fusion state: Partial incorporation of two of the three C-terminal helices into the core bundle of three N-terminal helices is presumed to provide the energy for bringing the membrane-embedded fusion peptides and viral anchors into proximity, thus placing the two membranes into apposition. Having the third C-terminal helix "unzipped" allows for the binding of mimetic peptides to the bundle, which are known to inhibit the fusion mechanism beyond this stage. The oblique angle of the fusion peptides is presumed to destabilize the outer, apposed layer of the target cell membrane. (B) Small fusion pore: Incorporation of the final C-terminal helix into the bundle provides the energy to bring the fusion peptides and viral anchors closer together, thus initiating merging of the outer leaflets and then fusion of the membranes. See text for further explanation.

a more precise measurement of fusion kinetics is obtained using dye transfer assays [40–44].

Initial fusion events have been detected after a time lag of 10–15 min following co-culture at 37 °C, which is believed to be correlated to the binding of gp120 to CD4 receptor [41,45,46]. The observation that the CD4-independent mutant 8× [47] displays a reduced time lag as compared to that of wild-type fusion supports this hypothesis [48]. This lag time can be dramatically shortened for HIV-1 through pre-incubation at 25 °C, which allows CD4 docking to take place but prevent co-receptor binding [31]. Similar decreases have been found in some systems with pre-incubation at 31 °C [38]. This binding and engagement of CD4 represent the first rate-limiting step. This lag is followed by a relatively quick rise in fusion yield that saturates at some level (usually after about 50–100 min), creating a curve easily fit to a sigmoidal function [48].

Although it is reasonable to assume that the relative densities of envelope and receptors will modulate these curves, it has been observed that envelope densities affect only the saturation fusion levels and not the rapidity of the kinetics [44]. The lack of pre-warming cells before fusion, though, may have altered those observed lag times. It has also been shown that the rapidity of fusion is greatly increased by increased co-receptor levels or by an enhanced envelope affinity for co-receptor [49]. The rapidity of these kinetics have been shown to correlate well with sensitivity to fusion inhibitors such as DP178, also known as T20 [48,49], and may account for drug resistance found in patients receiving this treatment [50]. Thus, in the cascade of conformational changes that gp120/gp41 goes through, conformational-dependent epitopes of the proteins are exposed, allowing for windows of opportunity for inhibitors that bind to these transient epitopes, to adhere and inhibit. Further examinations of time-dependent changes in gp120/

gp41 structure that govern the kinetics are examined in Sections 4.1 and 4.2.

3. The role of the target membrane in HIV fusion

Although in Fig. 1 only one trimer of Env is depicted as being triggered to produce a fusion event, it appears that for this process to take place efficiently, several HIV Env trimers must interact with host cell receptors, CD4, and co-receptors, such as CXCR4 or CCR5, that determine the tropism of different HIV-1 isolates. This process appears to be highly cooperative and is affected by receptor density as well as by Env-receptor affinity [51]. It has been estimated that four to six CCR5 receptors [52] and multiple CD4 receptors [53] are required to form a fusion pore. In primary CD4+ T cells that typically express fewer than 10,000 CCR5 or CXCR4 molecules and about 65,000 CD4 molecules per cell [54], the cooperativity required for HIV-1 Env-mediated fusion will unlikely be satisfied through random collisions between the receptors in a fluid mosaic membrane [55]. The idea that co-receptor clusters large enough to cooperatively trigger a productive HIV-1 Env-mediated fusion event are assembled in domains of the plasma membrane therefore emerged.

In the last decade, the notion of heterogeneity of phases in the plasma membrane and in particular the presence of specialized membrane microdomains, or lipid rafts, has been highlighted [56,57]. Their formation could be driven by a special interaction between different lipids, such as cholesterol, sphingomyelin, and glycosphingolipids, which have a propensity to form, in dynamic equilibrium, a more ordered domain separate from the more liquid plasma membrane. The special environment they provide drives an uneven distribution of proteins on the plasma membrane depending on their affinity for rafts. The main means of observation of those domains has so far been isolation by flotation on a sucrose gradient. Their more ordered organization provides them with a resistance to solubilization with some nonionic detergents under certain temperatures. Solubilization of the cells under those conditions allows then their recovery and the study of their composition. They have been shown to be enriched in GPI anchored proteins, receptors (EGF, PDGF, CD4, TCR), and signaling molecules (src, Gproteins, MAPK kinase, shc, GRB, SOS). Due to the very small size of the rafts beyond the resolution of fluorescence microscopy, labeling of those proteins reveals an even distribution. However, cross-linking of those proteins has been shown to result in co-patching of the small domains to form bigger aggregates that can be readily observed in the form of a punctuate fluorescence. Beyond the advantage of visualization of the rafts, it provides insights into the dynamic of those domains and their ability to reorganize in response to a stimulus.

Rafts were shown to serve as entry and exit sites for microbial pathogens and toxins, such as influenza virus [58], measles virus [59], and cholera toxin [60]. They also appear to play a role at various stages of HIV-1 replication cycle such as virion packaging [61], assembly and budding from infected cells [62–64], and viral entry [18,65]. A way to test the involvement of rafts is to alter their formation. Methyl beta cyclodextrin (MβCD) has a high affinity for cholesterol, a main constituent of rafts. Treatment of cells with this drug in appropriate conditions removed enough cholesterol from the plasma membrane to disrupt the rafts. Such a treatment did inhibit the infectivity of HIV-1 leaving the infectivity of other viruses such as VSV unaffected [66]. In addition, replenishment of depleted cells with cholesterol did restore the HIV-1 Env-mediated entry, indicating that the cells were not permanently damaged by their treatment with MβCD. Furthermore, it has been recently shown [67] that MβCD treatment did not alter the HIV-1 permissivity of cells expressing high level of receptors but selectively inhibited fusion of cells expressing lower levels of receptors. This indicates that the cells depleted of cholesterol are not drastically damaged and that cholesterol per se is not necessary for the fusion to proceed but that the raft organization seems crucial for HIV-1 entry in cells expressing low levels of receptors, probably through their concentration in distinct domains.

If CD4 is considerably enriched in rafts [68,69], CCR5 is enriched to a lesser extent [65,66] and CXCR4 is poorly enriched [66,67] if at all [70]. Along with this notion, immuno-precipitation experiments [71–73] showed constitutive association of CD4 with CCR5 but very weak association with CXCR4. The rafts do not seem therefore to naturally contain sufficient clustering of co-receptor and CD4. Indeed, using fluorescence microscopy, a number of groups have found CD4 and co-receptors to be randomly distributed on the surface in an unstimulated state [74]. Rafts have, however, the faculty to reorganize upon stimulation. Following association of virus or gp120, co-localization of these molecules has been detected by fluorescence microscopy [74] and an increase of the presence of CXCR4 in raft seen in sucrose density gradient. According to other reports [70], this phenomenon is not observed, which raises interest in the dynamics and the time frame of this reorganization. The nature of this reorganization that would allow the formation of clusters large enough to support the cooperativity of HIV-1 Env-mediated entry is not well understood. It seems, however, that the formation of the fusion complex, at least in cells expressing low levels of receptors [67], requires cholesterol [65] and a functional actin [74]. Interestingly, some reports also showed that tyrosine kinase inhibitors could inhibit the syncytia formation induced by different X4 tropic strains in various cell lines [75].

The reorganization of the rafts can be mediated by the binding of a ‘‘raft’’ protein [76] or by a protein that would change its affinity for rafts upon binding [77]. As mentioned earlier, rafts are particularly enriched in signaling molecules, most notably members of the Src-tyrosine kinase family. The raft aggregation can therefore provide a favorable environment for signal transduction to proceed. Raft clus-

tering leads indeed to the accumulation of local tyrosine phosphorylation and actin in those domains [76]. It seems therefore that actin and signaling (notably tyrosine kinase) may participate in the formation and/or the stabilization of a bigger complex [76,78]. CD4 being the receptor of HIV and present in rafts is of particular interest. Taking CD4 out of rafts, either by depleting the cells of cholesterol or more elegantly by mutating it so that it would still bind to gp120 but not partition in the rafts [79], inhibited the co localization of CD4 and co-receptors upon treatment with gp120 and, therefore, HIV-1 fusion. The localization of CD4 in rafts seems to be important for the fusion process although this idea is still controversial [79a]. In lymphocytes, CD4 cross-linking has been shown to induce tyrosine kinase activation and simultaneous association of p56Lck with cytoskeleton [80]. Interestingly, it was also shown that expression of p56Lck retards the internalization of CD4 and increases its ability to form syncytia [75]. P56 anchors CD4 to microvilli [81], which have been shown to be the site of localization of clusters of CD4 and co-receptors [82]. Those membrane protrusions rich in actin and cholesterol-based domains may provide a possible site for fusion [67].

Where and how large clusters could be formed, allowing HIV-1 Env-mediated fusion to proceed, is still poorly understood. The requirement of rafts, intact actin, and signaling in the formation of syncytia suggests that the process would involve the clustering of domains promoted by actin and signaling processes. CD4 is localized in rafts and could facilitate this clustering upon gp120 binding, but how this relates to the mechanism of co-receptor recruitment and what role co-receptor engagement and signaling might play, even though co-receptors are not regularly localized in rafts, is currently poorly understood. Gp120 has notably been shown to have signaling capabilities on both CD4 and co-receptors separately [83,84]. Some experiments have shown that mutations made in CD4 and/or CCR5 affecting their signaling capacities do not have an effect on HIV-1 entry [85]. However, Pertussis toxin has been shown to deactivate CCR5 and, in some cases, to block the entry of R5 utilizing strains [86]. Clearly, CD4 signaling alone cannot support fusion, as supported by experiments in which CD4 independent strains efficiently recruit co-receptors to allow their entry [87]. The active participation of CD4, co-receptors, and other possible participants such as glycosphingolipids, possibly through signaling and actin rearrangement of rafts to form clusters large enough to support HIV entry, needs to be further characterized.

4. Conformational intermediates

4.1. CD4 and co-receptor-induced conformational changes in HIV Env revealed by Mabs

In order to study the initial interaction between HIV and target cells, a soluble form of CD4 (sCD4) has been employed as a receptor mimic in the analysis of the gp120–CD4 fusion intermediate [88]. CD4 binds in a recessed cavity on gp120 and this high affinity interaction forms a gp120–CD4 complex for which structural information is available [89]. As this is the sole conformation of gp120 for which the X-ray crystal structure is known, our understanding of receptor-induced conformational changes remains incomplete. Co-receptor engagement is generally thought to induce further conformational changes in the envelope complex facilitating gp41 fusogenic activity. Although the Env conformational changes leading to fusion are depicted in Fig. 1 as resulting from concerted Env-CD4–co-receptor interactions, it appears that these changes are due to sequential contacts as discussed in detail below.

4.1.1. Conformational changes in gp120 due to CD4 engagement

In the absence of complete structural information, conformational changes in gp120 during receptor engagement have been inferred from immunochemical, biochemical, and mutagenic analysis. Upon engagement, sCD4 induces well-documented conformational changes in the envelope protein, the most dramatic of which results in the dissociation of gp120 from gp41 under specific assay conditions [88]. Such dissociation occurs more readily for X4 tropic viruses than for R5 tropic, probably reflecting subtle alterations in the oligomeric envelope structures [90,91]. This event has been proposed to trigger the fusogenic potential of gp41 [9], presumably by releasing steric constraints and allowing gp41 to transition from a metastable conformation into a highly stable "six-helix bundle" [26–29].

Analysis of sCD4-induced conformational changes in gp120 rapidly identified structural changes that permit co-receptor interactions and subsequent viral entry. The gp120 V3 loop was initially identified as the principal determinant of co-receptor specificity [92]. Structural changes in the V3 loop occur following CD4 binding and are detected due to altered proteolytic sensitivity and antigenic profile [93]. Shortly thereafter, the contribution of other conserved gp120 structures was realized. One of the most well-studied conformational changes following sCD4 engagement of gp120 is the exposure of conserved, discontinuous epitopes, which are recognized by the human antibodies, 17b, 48D, and the murine monoclonal antibody CG10 [94–96]. These epitopes are located near or within the bridging sheet and the V3 loop on gp120 [89]. CD4 binding has been implicated in moving the V2 loop [95] and promoting the exposure and/or stabilization of the bridging sheet [89], thus facilitating the binding of antibodies to these epitopes. Although sCD4 binding to soluble gp120 [94] or HIV-infected cells [95] readily induces conformational changes permitting reactivity with the co-receptor binding site antibodies, analyses in viral–cell and cell–cell fusion systems [97–99] indicate that the co-receptor-binding domain remains largely occluded during viral entry. In agreement, no enhanced reactivity is observed with co-receptor binding

site antibodies during the fusion of Env and target cells, either in the presence or absence of co-receptor expression [97]. Collectively, these studies suggest that the close physical proximity of fusing membranes, rather than co-receptor interactions impedes detection of this conformational change.

Although co-receptor binding site antibodies fail to neutralize fusion, these antibodies can potently block sCD4-activated fusion with target cells expressing co-receptor alone [98]. These findings confirm that membrane-anchored envelope is primed by CD4 to promote interactions with co-receptor, similar to conclusions that have been reached based on analysis with soluble molecules [92,100]. In contrast to that shown in Fig. 1, these studies support a two-step model for receptor engagement that involves initial interactions between gp120 and CD4, followed by conformational changes that permit interaction of the gp120–CD4 complex with co-receptor.

Additional regions of gp120 also undergo conformational changes due to CD4 binding. These epitopes are located in the C1–C4/C5 region of gp120, which is implicated in gp41 interactions [101]. When soluble gp120 is incubated with sCD4, these conformational changes are readily detected. However, following the engagement of envelope expressing cells with sCD4, these interface epitopes remain occluded due to gp41 interactions. Likewise, during cell–cell fusion, these epitopes do not become exposed following CD4 binding. Instead, they become accessible to antibody binding following dissociation of gp120 from gp41, which is dependent on co-receptor interaction [97].

4.1.2. Conformational changes in gp41 due to CD4 engagement

In addition to triggering conformational changes in gp120, CD4 binding also promotes conformational changes in gp41. As for gp120, investigating the antigenicity of gp41 before and after sCD4 engagement has provided important structural information. Two regions of gp41 have been found to be particularly immunogenic in vivo. One of these regions spans amino acid residues 598–604 and includes a nonhelical hydrophilic region that forms a disulfide loop in the six-helix bundle. The other region includes residues 644–663 and comprises a portion of the C terminal α-helix. In the absence of sCD4 binding, antibodies to these epitope clusters react minimally with infected cells. Following sCD4 binding, a substantial increase in immuno-reactivity is observed probably reflecting conformational changes in gp41 [102]. Alternatively, such immuno-reactivity could be due in part to conformational changes in gp120 resulting in unmasking gp41 epitopes or additionally due to dissociation of gp120 from gp41. However, increased reactivity has been observed in the absence of gp120 dissociation [102], indicating that subunit dissociation is not absolutely required for these CD4 induced conformational changes.

A monoclonal antibody designated NC1, generated against the core of fusion active gp41, also reacts with HIV-infected cells following sCD4 engagement [103]. Reactivity with NC1 shows less than a two-fold enhancement following sCD4 binding and due to the assay conditions may in part reflect gp120 shedding [104]. Whether gp41 undergoes CD4-induced conformational changes permitting reactivity with NC1 in the absence of gp120 dissociation remains speculative. Due to the specificity of NC1, these conformational changes would likely reflect formation of the six-helix bundle conformation. Recently, enhanced NC1 reactivity has been observed following interactions between HIV-1 Env-expressing cells and susceptible target cells (A. Dimitrov et al., in preparation).

Contrasting results are observed with a disparate gp41 monoclonal antibody, 2F5, which reacts with a continuous epitope (a.a. 656–671) proximal to the transmembrane domain of gp41. The 2F5 epitope is strongly exposed on the surface of infected cells [102,103,105], and interestingly, sCD4 binding promotes a decrease in immuno-reactivity [102]. This decrease may reflect the resolution of gp41 into a stable six-helix bundle following gp120 dissociation, as the 2F5 epitope is occluded once N and C helices interact to form six-helix bundles. However, a slight decrease in 2F5 signal (1.3-fold) is seen following sCD4 incubation with HIV-infected cells under conditions where shedding would not be appreciable [102]. As for NC1, such altered immuno-reactivity may reflect six-helix bundle formation, but as yet it remains unclear whether CD4 engagement can promote such conformational changes.

In agreement with observations from sCD4 experiments, conformational changes in gp41 have been observed using membrane-bound CD4 [102]. Early analysis employed an immunochemical approach to investigate the exposure of immuno-dominant gp41 epitopes at the interface of HIV infected and target cells. Staining with gp41 antibodies reveals that previously cryptic gp41 epitopes become exposed at the fusion interface. Recent analysis of temporal gp41 conformational changes also demonstrates selective gp41 reactivity at the interface of attached Env and target cells [105]. Gp41 conformational changes, as detected by antibody binding, occurs rapidly upon interaction of Env and target cells and remains localized to the cell–cell interface until the initiation of fusion. The kinetics of epitope exposure, combined with the localized immuno-reactive pattern and the sensitivity of short-term co-cultures to T20, lead to the hypothesis that the immuno-reactive gp41 antigen is reflecting exposure of the gp41 pre-hairpin fusion intermediate. In this study, it appears that CD4 engagement is sufficient to induce the exposure of this intermediate as similar gp41 immuno-reactivity is observed in the absence of co-receptor.

Numerous studies have noted the accessibility of the 2F5 epitope on envelope expressing cells [102,103,105]. Upon sCD4-envelope engagement, 2F5 demonstrates decreased immuno-reactivity [102], but during the co-culture of Env and target cells, reactivity with 2F5 remains unaltered. This discrepancy may reflect differences in assay sensitivity or

alternatively may reflect concentration or structural differences of soluble versus cell-surface CD4.

4.1.3. Co-receptor-induced conformational changes

For the last two decades, it has been appreciated that conformational changes elicited upon CD4 engagement are not sufficient to permit fusion. This has led to the hypothesis that co-receptor engagement induces further conformational changes in the envelope–CD4 complex, culminating in activation of gp41. If the CD4-induced conformational changes in gp41 reflect exposure of the pre-hairpin intermediate as has been hypothesized, then what is the role of co-receptor in inducing further conformational changes? To address this question, the effect of co-receptor engagement on envelope conformation has been investigated by probing for altered immuno-reactivity. During cell–cell fusion, the exposure of epitopes that are located at the gp120–gp41 interface occurs immediately preceding fusion. Reactivity with antibodies to these epitopes is observed as a discrete signal at the interface of Env and target cells and is not observed in the absence of co-receptor. Hence, co-receptor engagement is implicated in facilitating dissociation of gp120 from gp41 and permitting antibody reactivity [97].

Conformational changes in gp41 have also been investigated using a gp41 peptide inhibitor called DP178, also known as T20. T20 was fortuitously discovered to inhibit HIV entry and most likely targets a gp41 ‘‘pre-hairpin intermediate’’ [106] that is characterized as having exposed N and C helices. Furuta et al. [34] probed conformational changes in gp41 by determining the ability of epitope-tagged T20 to immuno-precipitate intermediate gp41 structures under a variety of assay conditions. It appears that for some strains of HIV, sCD4 or membrane-anchored CD4 is sufficient for exposing the pre-hairpin intermediate while for other HIV isolates, co-receptor engagement is clearly a prerequisite. Presumably, in the latter case, both co-receptor and CD4 are required to drive pre-hairpin formation. Other studies using gp41-derived peptides suggest that the pre-hairpin structure appears at some point after the gp120–CD4 complex forms, and persists during a fusion lag phase that precedes lipid mixing [31,48,105] and then disappears concomitant with co-receptor engagement (see below). Because both CD4 and co-receptor are necessary for six-helix bundle formation, it would be interesting to see the time course of gp41 capture by T20 following interactions between Env and CD4 and/or co-receptors. Collectively, these studies highlight the advances in our understanding of receptor-induced conformational changes, for both gp120 and gp41, and illustrate the complexities in understanding receptor-induced conformational changes for HIV.

4.2. Kinetic studies with entry inhibitors

Studies using inhibitors to arrest fusion intermediates provide important insights into the sequential nature of receptor induced conformational changes. The inhibitory effects of anti-CD4 antibodies (e.g. Leu3A and Q4120) that block gp120-CD4 binding, chemokine receptor antagonists (e.g. AMD3100, TAK779) that block gp120-co-receptor binding, peptide inhibitors of six-helix bundle formation (e.g. T20, C34), and anti-six-helix bundle antibodies can be studied as a function of their time of addition [38,42,48]. HIV-1 Env-mediated fusion is completely inhibited when Leu3A, C34, or AMD3100 is added to the fusion reaction mixture during the first 10–15 min of co-culture. Leu3A added at later time points has a lowered inhibitory effect that rapidly is reduced to zero, while inhibition by AMD and C34 is still at about 50% of control. In the case of HIV-1 Env-mediated fusion, a considerable length of time was found between the fall of Leu3A inhibition and AMD3100 inhibition, suggesting a time scale of tens of minutes between CD4 binding and CXCR4 engagement [48]. In the case of HIV-2 Env-mediated fusion, this window of time is considerably reduced (S.A. Gallo and R. Blumenthal, in preparation). This delay is also found in the formation of the six-helix bundle, through the use of C34 and T20 [42]. Fusion levels as a function of time of addition of C34 and AMD3100 have been found to be equivalent, indicating that engagement of gp120 by CXCR4 and formation of six-helix bundles follow similar kinetics and that once CD4 binding has occurred, the engagement of co-receptor is the rate-limiting step. The model shown in Fig. 1 needs to be re-assessed in regard to these data showing that Env-CD4 interactions trigger the pre-hairpin state that becomes sensitive to C-terminal inhibitors and that co-receptor engagement leads to rapid six-helix bundle formation.

The evolution of six-helix bundle formation has been measured in a similar fashion by adding a rabbit anti-six-helix bundle antibody at different times after co-culture after first priming cells with a 2 h incubation at 31 °C [38]. It was found that selected populations of cells which have already bound to CD4 need about 30 min to form six-helix bundles, which is consistent with the above finding, assuming six-helix bundle and co-receptor engagement happen concomitantly. This assumption has been shown to be valid not only through kinetic measurements (as above), but through the sensitivity of the reversibly temperature arrested fusion state (TAS) to peptide inhibition. Target and effector cells incubated at 25 °C experience arrested fusion at the point of CD4 binding, but before co-receptor engagement, as evidenced by the inability of sCD4 (added at the end of TAS) to inhibit fusion while CXCR4 binding peptide, T22, was still able to inhibit. Release from this state through elevated temperatures to 37 °C promotes fusion with a marked increase in kinetics above that of fusion without TAS pre-incubation. Addition of T20 during TAS allows for inhibition of fusion while T20 addition at the time of release from this arrested state yields ineffectual inhibition, indicating that six-helix bundle formation occurs quickly after co-receptor binding [31].

The addition of steroyl lyso-phosphatidyl choline (Lyso-PC) can also reversibly arrest fusion, but at the point of

membrane merger by inhibiting negative membrane curvature [107] (see below). Thus, Lyso-PC arrested state (LAS) represents a stage in the fusion cascade that is further evolved than that of TAS. If T20 is added at LAS with subsequent washing, fusion is inhibited, indicating that the six-helix bundle has not yet formed [31]. This supports the hypothesis that the transition to bundle formation occurs concomitantly with fusion, inducing pore formation and expansion. An alternative hypothesis is that the six-helix bundle forms before membrane merger and the higher-order clustering of these bundles provides the final driving force for fusion. This is supported by the positive staining of effector/target cell contacts arrested at 32 °C by anti-six-helix bundle antibodies [38]. It is also possible that T20 can interfere with fusion at an incomplete stage of six-helix bundle formation (see Fig. 2).

All of this kinetic information is important in dissecting HIV entry and especially in determining which steps are rate limiting. For instance, it seems a great deal of time is spent between CD4 binding and co-receptor binding. This may be due to a requirement of multiple co-receptors acting in concert. HIV fusion is likely to be a slow, stochastic process, and its rate is clearly dependent on the density of the involved molecules. Thus, there may be a need for the spatial recruitment of several CXCR4 molecules to be in close proximity to each other and to CD4. This is in agreement with the raft dependence of fusion as described in Section 3. This co-localization of receptors and subsequent interaction with envelope maybe a bottleneck in the fusion process and may represent a new area for drug development.

Along these lines, it may be quite important to determine the windows of opportunity with which fusion is sensitive to different inhibitors. T20 inhibition is greatly enhanced when added during TAS, as well as when it is pre-incubated with sCD4, indicating that fusion becomes sensitive to T20 as early as CD4 engagement. It has also been clearly shown that the rapidity of fusion greatly affects the inhibitory ability of anti-viral peptides and chemokine agonists [48]. Fusion kinetics can be modulated by changing target cell co-receptor levels or by using envelope glycoproteins with different co-receptor affinities [49]. Increased receptor density or enhanced envelope affinity for co-receptor increases the chance of triggering the CD4 engaged envelope and therefore increases the rate of transformation of the gp41 ectodomain from the pre-coiled-coil state to six-helix bundle, decreasing the window of sensitivity to T20 [49]. Thus, kinetic data may have great relevance to disease management, as well as to the elucidation of the mechanism of viral entry.

4.3. Fusion peptide insertion

According to the model shown in Fig. 1, the gp41 fusion peptide inserts into the target membrane in the pre-hairpin state. However, no evidence is available for this state in the case of HIV/SIV Env-mediated fusion. The model is based on analogies with influenza HA-mediated fusion. In the case of HA-mediated fusion, two lines of evidence suggest fusion peptide insertion prior to fusion: (1) By lowering the temperature, a long-lived, low-pH HA fusion intermediate has been identified that is committed to fusion [108]. Following a temperature jump to 37 °C at neutral pH, this intermediate proceeds to fusion at a similar rate and extent as that seen for low-pH fusion. The committed state is insensitive to treatments with trypsin or DTT, which release HA1, but is reversed by treatment with proteinase K and thermolysin, which affect HA2. The committed state thus represents interactions between HA2, presumably including the fusion peptide, and the target membrane. (2) Photolabeling studies of the interactions between intact influenza virus with liposomes demonstrated insertion of HA2 into the target membrane as a kinetically distinct step prior to the actual fusion of the viral and target membranes [109–111]. Using energy transfer from a membrane-incorporated fluorophore to activate [^{125}I]iodonaphthylazide label, photolabeling of HA was also demonstrated using biological membranes as targets [112]. Durrer et al. [113] confirmed that it is only the N-terminal fusion peptide part of HA2 (residues 1 to >22) in those systems that become labeled. However, under conditions of HA inactivation, the fusion peptides were found to insert back into the viral membranes (which also contain the C-terminal, transmembrane anchors) [114,115].

Attempts have been made to determine whether HIV/SIV Env inserts into target membranes in the pre-fusion state by photosensitized labeling [36]. Although incubation of HIV Env-expressing cells with target cells at 23 °C does not lead to fusion, the gp41 in this "temperature-arrested state" is sensitive to the C terminal peptides, indicating that the N-terminal triple-stranded coiled coil is accessible under these conditions [31,48]. By measuring incorporation of [^{125}I]INA into both HLA DR and HIV/SIV Env (both present in membranes of Env expressing cells but not in target cells), upon interaction with fluorescently labeled target cells, it is possible to examine whether gp41/gp32 insertion into the target membrane takes place prior to actual fusion. If this was the case, preferential labeling of gp32/gp41 (i.e. its fusion peptide) over HLA DR, whose transmembrane anchor will only be labeled under conditions of actual fusion, will occur. No preferential labeling of gp32 was seen at 23 °C [36], indicating that no fusion peptide insertion takes place under these conditions. However, the assay might not be sufficiently sensitive to measure insertion prior to fusion.

Insertion of the HIV fusion peptide into viral membranes is suggested by experiments showing perturbation of these membranes when CD4 and co-receptor-associated beads were added to cells expressing the HIV-1 envelope glycoprotein [116]. The CD4 and co-receptor-induced permeabilization of Env-expressing cells occurred with the same specificity with respect to co-receptor usage as cell fusion.

Moreover, natural ligands for the co-receptors and C-terminal gp41 peptide inhibitors of HIV-1 fusion blocked this effect. A similar membrane destabilization of Env-expressing cells was seen when incubated with target cells at a ratio of target cell/Env-expressing cell $\geq$5:1. However, no destabilization of target cell membranes was observed when incubated at an Env-expressing cell/target cell ratio $\geq$5:1. Incubation at a target cell/Env-expressing cell ratio $\approx$ 1:1 resulted in cell–cell fusion without leakage. Therefore, it appears that relocation of the fusion peptide following the CD4 and co-receptor-induced conformational changes in Env initiates destabilization of the Env-expressing cell membrane rather than the target membrane bilayer.

Experiments with mutant HIV-1 Envs indicate that the fusion peptide is indeed responsible for this effect. Substitution of Val residue at position 2 of gp41 fusion peptide with Glu [117] resulted in no leakage. By contrast, Envs with an unmodified fusion peptide but with a deletion of the sequence Δ665–682 in the membrane-proximal domain [118] (see below) were perfectly leakage-competent (A.S. Dimitrov and R. Blumenthal, in preparation). Both mutant Envs did not mediate cell fusion. The observation that the Δ665–683 Env self-inserts its fusion peptide but does not cause fusion suggests that self-insertion of the fusion peptide is not sufficient for HIV-1 Env-mediated fusion. Self-insertion at high activation of HIV-1 Env may represent inactivation as was shown for influenza HA [114,115].

There is some evidence, on the other hand, supporting the insertion of the fusion peptide into the target membrane. Target cells expressing membrane-anchored T20 are not susceptible to infection by HIV-1 virions [119], suggesting that the fusion peptide must be inserted into the target membrane in order for anti-parallel binding of the T20 to the N-terminal grooves to occur (Fig. 2). If fusion could occur solely via self-insertion, which presumably would create a pre-hairpin structure to form perpendicular to the membrane, only parallel binding of T20 would be allowed and therefore, fusion would be rendered insensitive to inhibition, which is clearly not the case. Similar data on SIV Env-mediated fusion were obtained using fatty acyl linked SIV gp41-derived T20 [120]. Therefore, the evidence favors "pull" models that couple the energy of coil–coil formation to the movement of the target membrane towards the viral membrane (see Fig. 2) rather than "push" models that couple this energy to the bulging of viral membranes as a result of fusion peptide self-insertion [121]. Mittal and Bentz discuss a variation of the latter model in a separate chapter of this volume.

Whatever mechanism drives the process forward, the essential role of fusion catalysts is to accelerate the rate of fusion, i.e. lower the activation energy for fusion. The destabilization of the lipid bilayer as a result of the interaction of segments of fusion proteins with membranes provides a way to lower these barriers. There have been many studies showing that synthetic peptides, which promote liposome fusion, are capable of destabilizing membrane bilayers [122]. One motif for destabilizing membranes that appears to be common to a number of diverse fusion systems is for the entry of the peptide into the membrane as an α-helix inserted at an oblique angle [123]. This motif has been demonstrated by polarized Fourier-transform infrared spectroscopy (FTIR) on viral fusion peptides and has been shown to correlate with the fusogenic activity of the intact virus [124]. It should be pointed out, however, that modeling the membrane-inserted fusion peptide as a rigid α-helix is likely to be an oversimplification. Han et al. [125] showed that the fusion peptide of influenza virus inserts into a membrane in a distorted helix with a kinked structure. A combination of ^{13}C-enhanced FTIR with molecular simulations has been used to study the secondary conformation of synthetic HIV-1 gp41 fusion peptide in aqueous, structure-promoting, lipid and biomembrane environments [126]. These studies indicate that at low peptide loading in human erythrocyte ghosts membranes, the central core of the fusion peptide (residues 5–15) assumed α-helical conformations. On the other hand, at high peptide loading, the central core of the fusion peptide assumed an antiparallel β-structure. It is tempting to speculate that the α-helical conformations at low peptide loading are associated with fusion and that the β structures at high peptide loading are associated with leakage. This is consistent with the observations by Haque and Lentz [127] that at low peptide/lipid ratios (<1:200), the gp41 fusion peptide enhances poly(ethylene glycol)-mediated fusion of highly curved small unilamellar vesicles, whereas at higher peptide/lipid ratios, the peptide induces rupture of the vesicles. The insertion of the peptide into the outer monolayer of the membrane presumably results in a rapid expansion of the area of the outer monolayer [128,129]. The bending stress of the outer monolayer may be relieved by rapid flip-flop of phospholipid [130] and peptide from outer to inner monolayer followed by the formation of nanometer-scale pores.

5. Fusion pore development

In Fig. 1, the fusion step has been depicted as a question mark since there is little information regarding this process in the case of HIV/SIV fusion. The concepts as to what happens at this juncture are based on the "stalk-pore" paradigm [131–135] (for a recent review, see Ref. [136]). According to this concept, lipid rearrangements during the fusion reaction proceed in at least two stages during fusion [132]. In the first, the contacting monolayer (referred to as "cis") leaflets have merged, but the distal (denoted "trans") leaflets have remained intact. The structures of these intermediates have been described as "stalks" that further evolve to form a hemifusion diaphragm. The transmonolayers making up the hemifusion diaphragm then rupture to form the complete fusion pore [133].

The stalk-pore paradigm is supported by a large body of evidence showing that fusion is facilitated by the presence

of lipids in the cis monolayer that have large intrinsic negative monolayer curvature propensity, and by lipids in the trans monolayer that have large intrinsic positive monolayer curvature propensity [137]. Conversely, fusion is inhibited by the presence of lipids in the cis monolayer that have positive curvature propensity and by lipids in the trans monolayer that have large intrinsic negative monolayer curvature propensity. In the case of HA-mediated fusion, it has been shown that addition to the cis monolayer of compounds that promote positive curvature (e.g. lysophosphatidylcholine) inhibit fusion, and that addition of compounds that promote negative curvature (e.g. oleic acid) facilitate fusion [138]. To complete the fusion process, the trans-monolayer has to assume positive curvature. Evidence for this requirement has been established by showing that fusion pore formation is promoted by adding chlorpromazine [139] or lysophosphatidylcholine [140] to the trans monolayer side.

This concept was further supported by the observation of stable lipid mixing intermediates induced by the glycosylphosphatidylinositol-linked ectodomain of HA, GPI-HA, lacking its transmembrane domain and cytoplasmic tail [141]. HA with truncations in its 27-amino acid TM domain [142] or with certain replacements of the fusion peptide [143,144] also display the hemifusion phenotype. Hemifusion phenotypes have also been observed with truncated [145] or GPI-anchored [146] paramyxovirus Env glycoproteins. Furthermore, substitution of two glycine residues in the TM domain of VSV Env by Ala or Leu resulted in a hemifusion phenotype [147].

The hypothesis is thus widely held that hemifusion is a key intermediate stage of membrane fusion [148]. However, a recent attempt to visualize the fine structure of the contact area between GPI-HA-expressing cells and RBC using transmission electron microscopy to image thin sections of rapidly frozen, freeze-substituted material did not reveal any such diaphragms with the limits of detection (70–100 nm) [149]. Presumably, diaphragms need to be about an order of magnitude larger in order to be revealed by transmission electron microscopy. However, a multiplicity of small fusion sites was observed within which either lipid redistribution or formation of non-enlarging fusion pores could ensue [150]. These contact sites appear in the form of dimples on both membranes. Such dimpling, which has previously been observed in the process of exocytosis in mast cells [151], has been put forward as an early step in the fusion reaction [152–156]. Frolov et al. [149] observed these dimples in both GPI–HA and HA, indicating that the transmembrane domain may not be essential for contact site formation. However, agents that increase the positive curvature of inner monolayers promote fusion pore expansion in the GPI–HA hemifusion intermediate [139]. Therefore, it seems likely that the TM domain of intact viral envelope glycoproteins promotes fusion pore expansion by perturbing the inner monolayers of the fusing membranes.

The stalk-pore hypothesis is supported in the case of HIV Env-mediated fusion by experiments showing that addition of lysophosphatidylcholine to the cis monolayer inhibited fusion [31]. However, other experiments indicate that lysophosphatidylcholine may affect viral protein–host cell binding rather than the formation of lipid intermediates required for fusion [157]. Although a number of mutations of fusion proteins from several different viruses have been shown to result in lipid dye spread without mixing of aqueous dye when the mutant proteins were expressed on cell surfaces, no mutants of HIV, SIV, Mo-MuLV Env created so far has been shown to produce hemifusion. GPI-anchored HIV-1 expressed in cells was incapable of mediating membrane fusion either by lipid or contents mixing [41]. Similarly, membrane anchor truncation mutants of Mo-MuLV [158] or SIV Env [158a] did not induce lipid or contents mixing. It is possible that the differences may be due to the fact that in the case of ortho and paramyxo viruses, red blood cells were used as targets whose membranes may stabilize the intermediate.

In general, no differences are observed in the kinetics of redistribution of lipid dyes versus small aqueous markers as a result of HIV-1 Env-mediated cell fusion [31,42]. However, in the presence of the C-terminal peptide T20 [106], different patterns of inhibition were seen when fusion was monitored by lipid versus aqueous dye redistribution [42,43], although not all observations are consistent with each other [31]. These differences may reflect different sensitivities of the assay to lipid and contents mixing, especially when the efficiency of pore formation is largely reduced when six-helix bundle formation is impaired.

Although the energy of interaction of the coiled coil is clearly important in the creation of a fusion pore [31], it is not the only consideration. In order to examine the role of regions outside the six-helix bundle, mutations were introduced into HIV-1 gp41 within a region immediately adjacent to the membrane-spanning domain. This region, which is predicted to form an α-helix, contains highly conserved hydrophobic residues and is unusually rich in tryptophan residues. In addition, this domain overlaps the epitope of a neutralizing monoclonal antibody, 2F5, as well as the sequence corresponding to a peptide, DP-178, shown to potently neutralize virus. Some mutations did not affect HIV-1 Env-mediated fusion whereas other rendered HIV-1 Env incapable of mediating fusion. However, two mutations in this region display a distinct phenotype that is restricted in fusion pore enlargement [159]. Presumably, the hinge region between the TM anchor and the six-helix bundle needs to be flexible enough to allow the viral and target membranes to be pulled together to closer proximity. The optimal effect will be achieved when the six-helix bundle is parallel with the plane of the membrane (Fig. 1). Removal of the hinge region totally abrogates this effect. However, a few gp41 hinge mutants are weakened in their ability to tilt the six-helix bundle. Therefore, the intermediate (parallel) structure will not be present for a sufficient amount of time

to create the appropriate fusion pore expansion resulting in a non-enlarging pore (NEP) phenotype. This phenotype has also been seen with SIV Env trans-membrane anchor truncation mutants [158a].

The data regarding the NEP phenotype in the absence of hemifusion, taken together with intermediates revealed by immunostaining and susceptibility to C-terminal inhibitors (see Sections 4.1 and 4.2) suggest the model shown in Fig. 2. Since the fusion sites are presumably on microvilli [67] that are naturally curved, the Env does not have to take care of this aspect of the fusion process. Insertion of the fusion peptide into the target membrane and formation of coil–coil provide the energy for moving the membranes in close proximity from which fusion can ensue. The tension created by binding of the C-terminal regions into the N-terminal grooves provides the driving force needed to pull viral and target membranes together. In the pre-fusion state, two of the three C-terminal helices are incorporated into the core bundle of three N-terminal helices. Having the third C-terminal helix "unzipped" allows for the binding of mimetic peptides to the bundle, which are known to inhibit the fusion mechanism beyond this stage. This situation may account for apparently contradictory data that gp41 is still sensitive to T20 [31] at a stage where six-helix bundles are detected by immunofluorescence [38]. The oblique angle of the fusion peptides destabilize the outer, apposed layer of the target cell membrane [160]. The close apposition in the pre-fusion state in the presence of T20 may allow the exchange of lipids [42,43]. However, once a fusion pore is formed (Fig. 2B), it rapidly expands leaving no time for observation of hemifusion [31]. Mutations in the hinge region [159] or transmembrane anchor [158a] (NEP mutants) produce unstable intermediates that give rise to transient pores. Only robust interactions of these crucial domains of the fusion proteins with the lipid membranes will ensure the completion of the reaction.

The multimerization and/or inter-envelope interaction of multiple trimers may be necessary to create a fusion pore. Considerable evidence has been found to support this hypothesis in the case of influenza HA. From the kinetics of dye transfer of cell–cell fusion pairs, a fusion pore has been estimated to have at least six HA trimers [161]. Analysis of pore conductivity data has yielded a value of three trimers necessary for fusion [162]. A similar estimation has been found from data involving cell fusion kinetics, measuring time lag versus envelope density [163]. Still other theoretical calculations posit that only a few trimers are needed to create the initial pore, and then multiple proteins outside the contact area are recruited for pore enlargement [164]. In contrast to these studies are reconstitution experiments that claim the lack of cooperativity [165], although this discrepancy may be due to strain differences.

In the case of HIV Env-mediated fusion, not much evidence is available regarding the multiplicity of Envs required for fusion pore formation. Self-aggregation of C-terminal peptides of the HR2 region of gp41 has been observed in liposome membranes [43]. These data have been interpreted in terms of a model, which is discussed by Shai in a separate article in this volume. According to this model, lipid membranes induce conformational changes in the six-helix bundle, which enable association of gp41 molecules leading to fusion pore formation. In another study, Freed et al. [117] have shown that the V2E mutant in the fusion peptide of gp41 results in an abrogation of

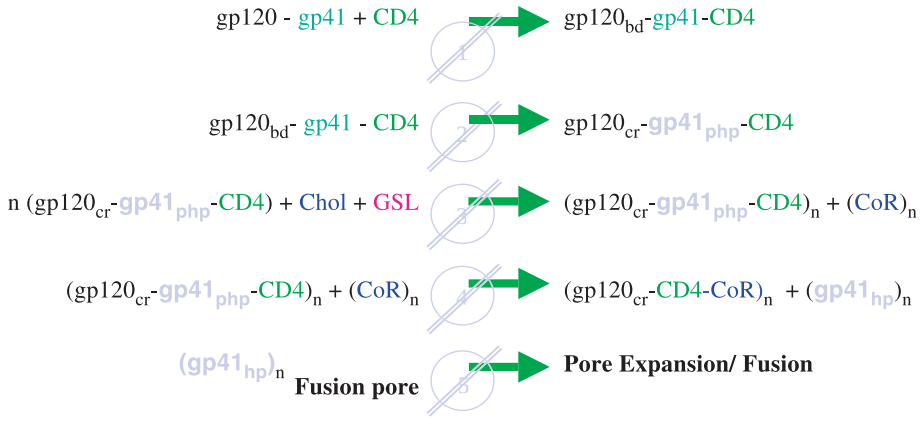

Fig. 3. Revised model of HIV-envelope-mediated membrane fusion. The first step is the initial binding and engagement with CD4 receptor (resulting in bound gp120 or $gp120_{bd}$), which can be blocked by sCD4, antibodies against the CD4 binding site on gp120, antibodies against the gp120 binding site on CD4, and small molecules (see Ref. [167] for a review). Secondly, the gp120/gp41 complex undergoes conformational changes (represented as $gp120_{cr}$, gp120 with exposed co-receptor binding site, and $gp41_{php}$, pre-hairpin gp41), which allow fusion to become sensitive to C-terminal peptides like T20 [34]. In the third step, co-receptors (CoR) are co-localized with CD4 molecules in lipid rafts composed of cholesterol (Chol) and glycosphingolipids (GSL), which can be blocked by lowering the temperature, cytochalasin, MβCD, and GSL biosynthesis inhibitors [18,67,168]. The fourth step is the engagement of the co-receptor by the envelope, which is blocked by antibodies against the gp120 binding site on the co-receptor, antibodies against the co-receptor binding site on gp120, and co-receptors agonists and antagonists [167]. Gp120 and gp41 then dissociate and the six-helix bundle configuration ($gp41_{hp}$) is formed concomitantly with co-receptor engagement [48]. This is also presumed to be the point at which the fusion peptide is inserted in to the target and/or host membrane. The final step, through the oligomerization of several (*n*) trimers, is pore expansion, which is impaired in gp41 NEP mutants (see text).

fusion phenotype and reduction in fusion when co-expressed with excess wild-type envelope. This effect was not seen when the mutant was co-expressed with HIV-2, suggesting specific interactions between multiple trimers are necessary for fusion. However, a threshold number of molecules have not yet been determined, although envelope density has clearly been shown to affect the fusion yield [44]. Due to the requirement of multiple CD4 and co-receptor molecules for fusion [52,53] and that the stoichiometry of trimer to CD4 molecule is 1:1 [166], it is reasonable to assume that multiple proteins are required for fusion pore formation.

6. Concluding remarks

The recent high-resolution determination of the structure of the gp41 core from HIV-1 provides well-defined landmarks in the terrain the viral envelope glycoproteins navigate following CD4 and co-receptor-induced conformational changes (Figs. 1 and 2). Further elucidation has been accomplished through the combined data covered in this review, resulting in a revision of the Chan/Kim model. The current HIV Env-mediated fusion reaction scheme we have is summarized in Fig. 3. The process leading to six-helix bundle formation has been split into CD4 and co-receptor dependent steps. In addition, we have supplemented it with the steps of co-receptor recruitment and oligomerization as well as pore enlargement. A number of steps in the fusion cascade are revealed by inhibitory conditions that block fusion at this step. The first step is the initial binding and engagement with CD4 receptor, which can be blocked by Leu3a antibody. The gp120/gp41 complex then undergoes conformational changes, allowing for the N-terminal heptad repeat region of gp41 to be exposed, sensitizing fusion to C-terminal peptide inhibition at this second step. This conformational change in gp41 is now defined as the pre-hairpin formation. Thirdly, co-receptors are recruited to the binding site, presumably into rafts rich in cholesterol and glycosphingolipids. Self-aggregation and/or interaction of several envelope/CD4 complexes may also occur at this step. This is blocked by cytochalasin and temperature, which may inhibit actin polymerization, or by MβCD, which inhibits raft formation. The fourth step is the engagement of the co-receptor, which is inhibitable by co-receptor agonists and antagonists. Concomitant with co-receptor engagement, gp120 and gp41 dissociate and the six-helix bundle configuration is formed, desensitizing fusion to C-terminal peptide inhibition. This is also presumed to be the point at which the fusion peptide is inserted in to the target and/or host membrane, leading to the establishment of a fusion pore. The final step is pore expansion, which is impaired by gp41 hinge and transmembrane mutations. Several critical areas in this model are in need of much additional data, for instance, fusion peptide insertion, oligomerization, and pore expansion. These uncertainties underscore the incompleteness of any present model of HIV entry without further studies. Many models based on current structural data, which includes neither fusion peptides and transmembrane anchors nor regions between those domains and six-helix bundles (Fig. 1), fail to give enough importance to regions which are crucial for fusion activity. Mutagenesis of these undetermined domains combined with sensitive assays for the activity of the modified proteins will lead to refinement of our thinking about the HIV-1 Env-mediated fusion reaction.

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