Early Steps of HIV-1 Fusion Define the Sensitivity to Inhibitory Peptides That Block 6-Helix Bundle Formation

Kosuke Miyauchi1, Michael M. Kozlov2, Gregory B. Melikyan1

1 Institute of Human Virology and Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland, United States of America, 2 Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Abstract

The HIV envelope (Env) glycoprotein mediates membrane fusion through sequential interactions with CD4 and coreceptors, followed by the refolding of the transmembrane gp41 subunit into the stable 6-helix bundle (6HB) conformation. Synthetic peptides derived from the gp41 C-terminal heptad repeat domain (C-peptides) potently inhibit fusion by binding to the gp41 pre-bundle intermediates and blocking their conversion into the 6HB. Our recent work revealed that HIV-1 enters cells by fusing with endosomes, but not with the plasma membrane. These studies also showed that, for the large part, gp41 pre-bundles progress toward 6HBs in endosomal compartments and are thus protected from external fusion inhibitors. Here, we examined the consequences of endocytic entry on the gp41 pre-bundle exposure and on the virus’ sensitivity to C-peptides. The rates of CD4 and coreceptor binding, as well as the rate of productive receptor-mediated endocytosis, were measured by adding specific inhibitors of these steps at varied times of virus-cell incubation. Following the CD4 binding, CCR5-tropic viruses recruited a requisite number of coreceptors much faster than CXCR4-tropic viruses. The rate of subsequent uptake of ternary Env-CD4-coreceptor complexes did not correlate with the kinetics of coreceptor engagement. These measurements combined with kinetic analyses enabled the determination of the lifetime of pre-bundle intermediates on the cell surface. Overall, these lifetimes correlated with the inhibitory potency of C-peptides. On the other hand, the basal sensitivity to peptides varied considerably among diverse HIV-1 isolates and ranked similarly with their susceptibility to inactivation by soluble CD4. We conclude that both the longevity of gp41 intermediates and the extent of irreversible conformational changes in Env upon CD4 binding determine the antiviral potency of C-peptides.

Introduction

HIV Env-induced fusion between the viral and cellular membrane progresses through a series of steps that begin with binding of the gp120 subunit to CD4. This step results in the formation of the gp120 bridging sheet which, along with the third hypervariable loop (V3 loop), forms the coreceptor binding site (reviewed in [1]). The recruitment of coreceptors, CCR5 or CXCR4, by Env-CD4 complexes initiates gp110 refolding that progresses through a pre-bundle intermediate, in which the gp41 N- and C-terminal heptad repeat domains (N-HR and C-HR, respectively) are exposed [2–5]. The heptad repeat domains ultimately coalesce into the stable post-fusion conformation referred to as the 6-helix bundle (6HB). The 6HB is formed by an antiparallel association of the trimeric N-HR domain (coiled coil) with three peripheral C-HR domains (reviewed in [6]). In a pre-bundle conformation, gp41 is susceptible to inhibition by synthetic peptides derived from its C-HR domain (hereafter referred to as C-peptides). These peptides bind to the complementary N-HR region and block HIV fusion by preventing the formation of 6HBs [6–8].

The kinetics of HIV fusion and the progression of gp41 pre-bundles to the 6HB has been studied in a cell-cell fusion model [4,9–13]. Biochemical studies using a tagged C-peptide showed that, depending on the virus strain, the gp41 coiled coils can be exposed as early as upon CD4 binding [2]. Once formed, the pre-bundles are thought to persist for a couple of minutes prior to converting into the 6HB [14]. Using a real-time cell-cell fusion assay, we observed that small fusion pores collapsed soon after the addition of C-peptides [13], indicating that the formation of 6HBs was not completed at this point. These findings show that Env remains vulnerable to inhibitors of 6HB formation and to antibodies targeting gp41 intermediates throughout the fusion reaction.

Our recent work revealed important differences between cell-cell and virus-cell fusion models [15]. Whereas HIV-1 Env can mediate cell fusion by merging two plasma membranes, the virus itself fails to release its content at the cell surface. Instead, HIV-1 fuses with endosomes, presumably after undergoing CD4- and coreceptor-mediated endocytosis. Time-resolved single virus imaging showed that fusion with the plasma membrane was blocked at a stage downstream of lipid mixing and did not progress to productive entry. Importantly, endosomal fusion was markedly delayed relative to virus internalization, demonstrating that the surface exposure of gp41 intermediates is limited by the relatively quick virus clearance from the cell surface. Endocytic HIV-1 entry
Author Summary

The human immunodeficiency virus (HIV) envelope glycoprotein (Env) mediates fusion between the viral and cell membranes. The fusion is initiated by Env-receptor interactions and is followed by coreceptor binding and refolding of the transmembrane gp41 subunit. The gp41 refolding proceeds through several distinct intermediates, culminating in the formation of a final helical bundle structure which is blocked by inhibitory peptides targeting the complementary domains of gp41. We have recently shown that the exposure time of gp41 intermediates on the cell surface is limited by productive HIV endocytosis leading to fusion with endosomes. Here, we measured the rates of progression of different HIV isolates through distinct intermediate steps accessible to fusion inhibitors and correlated these rates with the inhibitory potency of peptides against these viruses. Whereas the potency of peptides was proportional to the lifetime of gp41 intermediates on the cell surface, the baseline sensitivity of the virus was also Env context-dependent. Higher concentrations of these inhibitors were required to block fusion induced by glycoproteins that were more resistant to inactivation by the soluble receptor. Collectively, these findings imply that both the kinetic factors and the stability of Env-receptor complexes control the HIV sensitivity to inhibitory peptides.

could thus attenuate the effects of neutralizing antibodies and C-peptides that target intermediate conformations of Env.

Disparate HIV isolates are known to exhibit a broad range of sensitivities to C-peptides (e.g., [16]), but the mechanisms underlying this differential sensitivity are not well understood. Functional studies suggested a correlation between the potency of a 34-residue long peptide, C34, and the propensity of Env to expose the gp41 coiled coil domains upon binding to a soluble CD4 (sCD4) [2,17,18]. The efficacy of C-peptides is also modulated by their primary sequence and the sequence of complementary N-HR domains that determine the binding affinity [19,20]. However, the potency of C34 peptides derived from HIV-1, HIV-2 and SIV isolates poorly correlated with their propensity to form stable 6HBs with the complementary N-HR domains [17].

Another determinant of the efficacy of C-peptides is thought to be the window of opportunity for their binding to gp41 intermediates. This notion is based on correlation between the kinetics of cell-cell fusion and the HIV-1 resistance to C-peptides [9,10,17,18,21]. These findings led to a hypothesis that the gp41 residence time in pre-bundle conformations determines the HIV-1 sensitivity to C-peptides [9]. Implicit for this model is the inverse relationship between the rate of fusion and the longevity of pre-bundles and the slow, rate-limiting binding of C-peptides to these intermediate conformations. The slow peptide binding would require prolonged exposure of the gp41 coiled coil and would thus limit its ability to block the quickly-progressing fusion. However, since the HIV-1 fusion proceeds through intermediate steps at which the gp41 coiled coils are not exposed, the overall kinetics of fusion may not reflect the window of opportunity for the peptide binding. Thus, in order to meaningfully examine the kinetic determinants of the HIV resistance to C-peptides, one needs to determine the actual residence time of gp41 in pre-bundle conformations on the cell surface.

Endocytic entry of HIV-1 warrants careful examination of the relationship between the rates of surface-accessible pre-fusion steps and the sensitivity to C-peptides. Here, we employed inhibitors of distinct steps of HIV-1 fusion to monitor the progression through CD4 and coreceptor binding steps followed by productive endocytosis that protected the virus from fusion inhibitors employed in this study. Using a simple kinetic model of HIV fusion, we determined the rates of HIV-1 progression through key pre-fusion intermediates and thus the residence times of Env in these intermediates. Our results imply that multiple factors contribute to the potency of C-peptides. An important kinetic factor is the lifetime of gp41 pre-bundles which is defined by the rate of engagement of a requisite number of receptors and coreceptors on one hand and the productive virus endocytosis on the other. We also found that viral determinants, such as the extent of conformational changes in Env in response to the CD4 binding, significantly modulate the susceptibility of HIV-1 to C-peptides. The ability to evaluate the HIV-1 residence time in intermediate states permits rationalization of the complex mechanisms that define the resistance to C-peptides and other inhibitors targeting intermediate conformations of Env.

Results

Productive HIV-1 entry occurs through sequential CD4 and coreceptor binding followed by virus endocytosis

In order to directly monitor HIV-1 fusion with target cells, we measured the cytosolic activity of the beta-lactamase-Vpr (BlaM-Vpr) chimera packaged into the viral core [22]. Pseudoviruses containing the reporter enzyme were bound to target cells in the cold, and their fusion was initiated by quickly raising the temperature to 37°C, as described in [15]. The BlaM activity originates exclusively from viral cores delivered into the cytosol through fusion, whereas cell-bound or internalized viruses do not contribute to the signal [22]. Due to the low number of fused viruses, an overnight incubation is required to accumulate detectable amounts of cleaved fluorogenic BlaM substrate loaded into the cytosol. In order to resolve the kinetics of HIV fusion that occurs within a few hours (e.g., [23]), high concentrations of fusion inhibitors were added at varied times of incubation at 37°C [15].

The acquisition of resistance to a membrane-impermeant inhibitor yields the kinetics of virus progression beyond the step targeted by that inhibitor. Alternatively, resistance to inhibitors targeting a late step of fusion can occur through virus internalization that protects it from external inhibitors and permits subsequent fusion with endosomes.

The availability of inhibitors blocking distinct steps of HIV entry enabled monitoring the progression through sequential surface-accessible stages of fusion. The kinetics of CD4 binding were monitored by time-of-addition experiments using a small-molecule inhibitor BMS-806 [24,25]. Since the binding to CD4 renders HIV-1 resistant to this compound, escape from BMS-806 indicates the progression of fusion beyond the receptor-dependent steps (Fig. 1B). The virtually complete inhibition of fusion by BMS-806 added at the beginning of incubation shows that cell-associated HIV-1 did not engage CD4 immediately following the receptor-dependent steps. The virtually complete inhibition of fusion by BMS-806 indicates the progression of fusion beyond the receptor-dependent steps. The rates of CXCR4 or CCR5 binding were measured by adding small-molecule inhibitors AMD3100 and AD101, respectively.

The acquisition of resistance to C-peptides blocking the gp41 6HB formation has been traditionally interpreted as Env-mediated fusion [3,4]. However, since HIV-1 fuses with endosomes of HeLa-derived target cells [15], escape from C-peptides must occur through virus uptake. The fact that HIV-1 escapes from coreceptor antagonists before escaping from C-peptides (Fig. 1B) implies that the fusion signal originates from viruses that engage both CD4 and coreceptors prior to undergoing endocytosis. Note
that in HeLa-derived target cells the majority of viruses is internalized and degraded through CD4- and/or coreceptor-independent pathways [26]. It is unlikely that HIV-1 acquires resistance to these peptides by forming 6HBs prior to undergoing endocytosis because: (i) fusion with the plasma membrane does not progress beyond the lipid mixing stage [15]; and (ii) 6HB formation occurs only after opening of a fusion pore [19]. Here, we measured the rate of HIV-1 escape from C-peptides using a recombinant 52-residue peptide, C52L, derived from the gp41 C-HR domain [27]. In control experiments (data not shown) HIV-1 escape from the C52L peptide occurred at the same rate as escape from the better characterized 34-residue peptide, C34.

In this work, we will be concerned only with the pre-fusion steps occurring at the cell surface, which are key determinants of the sensitivity to C-peptides and antibodies targeting intermediate conformations of Env [9,10,18,28,29]. Once different HIV-1 isolates form ternary complexes with CD4 and coreceptors and undergo endocytosis, subsequent fusion events appear to occur with similar rates and efficiencies [15]. Thus, the fusion signal measured by the time-of-addition protocol should reflect the rate of cell surface-accessible steps of fusion.

**A model for HIV-1 fusion**

We sought to determine the residence time of HIV-1 in key intermediate states and thus to evaluate the lifetime of gp41 pre-bundles that are accessible on the cell surface to C-peptides and neutralizing antibodies. To this end, we considered a minimal kinetic model (Fig. 1C) that describes the virus association with the cell surface followed by CD4 binding, coreceptor (CR) binding, and, finally, by productive endocytosis. We operationally define the following intermediate states the virus adopts sequentially along its entry pathway: (i) the state (V) of the membrane-associated virus which is sensitive to all three types of fusion inhibitors; (ii) the state (VCD4) resistant to an inhibitor blocking the HIV-C4 binding, but sensitive to inhibitors of coreceptor binding and 6HB formation; (iii) the state VCD4CR resistant to inhibitors of receptor and coreceptor binding, but still sensitive to inhibitors of 6HB formation; and (iv) the state VE resistant to all three inhibitor types. The effective rate constants of transitions between the successive states V, VCD4, VCD4CR and VE are denoted by k1, k2, k3, and k4. Our model can be readily modified to describe direct virus fusion with the plasma membrane by omitting the endosomal fusion step and treating VE as the fusion state.

The model does not consider the reverse rates of CD4 and coreceptor binding reactions. Moreover, the model makes no assumptions about the stoichiometry of the receptor and coreceptor binding. If several Env glycoproteins are involved in HIV-1 entry, the virus must engage more than one pair of receptor and coreceptor molecules in order to undergo fusion. In that case, interactions with coreceptors at the virus-membrane contact may start before the recruitment of a requisite number of CD4 is completed. Then the VCD4 is a state where the receptor binding is finalized while the coreceptor recruitment is still incomplete, so that the transition to VCD4CR consists in the recruitment of the missing coreceptors.

The model postulates that the viruses are subject to inactivation characterized by the inactivation rate constants. Whereas the general form of the model (see Fig. S4) accounts for different inactivation rates at the sequential steps of the HIV-1 progression along the fusion pathway, it is currently impossible to experimentally determine the individual inactivation rates. Thus, in order to evaluate the effective rate constants of fusion, we used a simplified version of the model that assumes equal inactivation rate constants, ki, for all stages of the fusion reaction. We also assume...
that the HIV-1 inactivation is primarily due to a non-productive endocytosis which is the predominant pathway of HIV-1 uptake by HeLa-derived cells [15,26]. In other words, the total virus uptake is assumed to reflect the rate of virus inactivation. This non-productive pathway likely includes both CD4-independent and CD4-mediated virus uptake which, in the absence of coreceptor binding, does not lead to endosomal fusion.

The differential equations describing the virus evolution through each of the states of the kinetic scheme (Fig. 1A, C) are given in Appendix S1. The solution of these equations for the number of viruses $V_E$ entering through productive endocytosis (leading to endosomal fusion) as a function of time is given by:

$$V_E = V_{ext} \cdot \frac{k_1 \cdot k_2 \cdot k_3 \cdot V_{tot}}{(k_2 - k_1) + (k_3 - k_1) + (k_1 + k_i)} \cdot \exp[-(k_1 + k_i) \cdot t]$$

$$+ \frac{k_1 \cdot k_2 \cdot k_3 \cdot V_{tot}}{(k_2 - k_1) + (k_3 - k_2) + (k_2 + k_i)} \cdot \exp[-(k_2 + k_i) \cdot t]$$

$$- \frac{k_1 \cdot k_2 \cdot k_3 \cdot V_{tot}}{(k_3 + k_i) + (k_3 - k_2) + (k_3 - k_1)} \cdot \exp[-(k_3 + k_i) \cdot t]$$

where $V_{ext} = \frac{k_1 \cdot k_2 \cdot k_3 \cdot V_{tot}}{(k_1 + k_2) + (k_2 + k_i) + (k_3 + k_i)}$

Unlike the previously proposed models of Env-mediated fusion [23,30], this model accounts for the effective lag before fusion. This lag is given by $1/(k_3 \cdot k_2 \cdot k_3 \cdot V_{tot})$, where $V_{tot}$ is the total number of cell-bound fusion-competent viruses at time $t = 0$. Note that $V_{tot}$ cancels out upon normalizing data to the final extent of fusion (see also Fig. S1), so that the kinetics of fusion do not depend on the multiplicity of infection (MOI). Thus, normalization eliminates the need to determine and/or control the exact number of fusion-competent particles bound per cell. Our model describes a wide variety of cell-cell and virus-cell fusion data using four free parameters: $k_1$, $k_2$, $k_3$, and $k_i$. These effective rate constants can be defined through the measurements of the kinetics of CD4 binding (escape from BMS-806), coreceptor binding (escape from AD101 or AMD3100), the rate of productive endocytosis (escape from C52L), and the rate of virus inactivation through non-productive endocytosis (p24 uptake).

The knowledge of the kinetics of virus inactivation is important because this process, along with the respective pre-fusion rate constants, determines the exit rates from $V_C$, $V_{CD4}$, and $V_{CD4CR}$. We thus obtained the $k_i$ by fitting a single exponential function to the HIV p24 uptake data (Fig. 1B). A separate set of equations (equations (10–12) of Appendix S1) was derived to describe the kinetics of virus escape from CD4 and coreceptor binding inhibitors and from C-peptides added at varied times of virus-cell incubation. Through fitting of the solutions of these equations to the respective data sets, we were able to determine the remaining three rate constants $k_1$, $k_2$, and $k_3$ for the surface-accessible steps of fusion (see the legend to Fig. 2 for details and Table 1).

The lifetime of Env-CD4 complexes can be extended by lowering the density or the affinity of coreceptors to Env.

We first examined the progression of the primary R5-tropic JRFL isolate through the surface-accessible fusion intermediates. Pseudoviruses were produced and the incorporation and proteolytic processing of JRFL Env was assessed by Western blotting (Fig. S2). Viruses were pre-bound to target cells in the cold, and their uptake and fusion were initiated by shifting to 37°C. Inhibitors of
CD4 or CCR5 binding were added at indicated times of incubation to obtain the kinetics of HIV-1 escape from these inhibitors. As discussed above, HIV-1 acquires resistance to the inhibitors of 6HB formation through receptor-mediated endocytosis as opposed to fusion at the cell surface. Productive endocytosis of this virus was thus measured by adding a high concentration of the recombinant C32L peptide at varied time points. In parallel experiments, the total virus uptake was measured by the intracellular accumulation of the HIV-1 p24. These measurements allowed us to determine the fusion and inactivation rate constants for JRFL, entry into different target cells.

When HeLa-derived TZM-bl cells expressing high levels of CD4 and CCR5 [31] were used as targets, JRFL engaged a requisite number of CD4 and coreceptors with the virtually identical kinetics (Fig. 2A). Thus, under these conditions, the effective rate of CCR5 binding was too fast to be resolved. We therefore sought to slow down the formation of ternary complexes by reducing the density of CCR5 on the cell surface or by diminishing its affinity to Env. A ~20-fold reduction of the average number of CCR5 molecules per cell did not noticeably affect the rate of coreceptor engagement (data not shown). However, the kinetics of CCR5 binding and the final extent of fusion were markedly diminished for JC.10 cells [31] expressing a ~100-fold lower number of CCR5 compared to TZM-bl cells (Fig. 2B). We were thus able to kinetically resolve the CCR5 binding step and measure its effective rate constant, \( k_2 \) (Table 1).

Next, we examined the impact of the coreceptor binding affinity on the rate of the ternary complex formation. JRFL pseudoviruses were bound and fused with JYN.2-15 cells [32] expressing high levels of wild-type CD4 and the Y14N CCR5 mutant similar to those present on TZM-bl cells. JRFL fusion with these cells was less efficient and was highly sensitive to inhibition by AD101 (Table 2), in agreement with the diminished affinity to gp120 caused by the loss of the critical Tyr14 residue in the CCR5 N-terminus. As expected, the Y14N mutation reduced the kinetics of virus escape from AD101, whereas the rate of CD4 binding remained unchanged (Fig. 2C and Table 1). Together, these results demonstrate that the lifetime of Env-CD4 complexes on the cell surface can be manipulated by changing the density of coreceptors or their binding affinity to gp120.

Increased coreceptor binding affinity in the context of X4 Env accelerates the coreceptor engagement but not HIV-1 endocytosis

Next, we compared the kinetics of surface-accessible steps of fusion induced by JRFL Env and by the laboratory adapted HXB2 Env. These glycoproteins differ in many aspects, including the coreceptor tropism and sensitivity to neutralizing antibodies and C-peptides [18,33,34]. HXB2 pseudoviruses engaged CD4 faster than JRFL (Figs. 2A and 3A), in agreement with the enhanced receptor binding affinity associated with the HIV-1 adaptation to growth in culture [5,35,36]. By contrast, HXB2-CD4 complexes became protected from the coreceptor binding inhibitor at a much slower rate than JRFL. The different kinetics of coreceptor binding are consistent with the vastly different coreceptor binding affinities of laboratory adapted X4 and primary R5 Env glycoproteins [37–39]. The ~10-fold higher CCR5 expression on TZM-bl cells compared to the CXCR4 expression [31,40] did not seem to be responsible for the more rapid CCR5 engagement, since the rate of this coreceptor binding was not noticeably affected by the 10-fold reduction in its expression level (data not shown). Interestingly, HXB2-CD4-coreceptor complexes were internalized at a ~3-fold faster rate compared to the ternary JRFL complexes (\( k_3 \) constant, Table 1), whereas the rates of bulk endocytosis leading to virus degradation were close for these viruses (Figs. 2A and 3A, crosses). As a result of the above compensatory variations in the rate constants of pre-fusion steps, the overall kinetics of JRFL and HXB2 escape from C52L were close. This result is in contrast with the faster kinetics of cell-cell fusion induced by JRFL compared to HXB2 Env [18].

We next assessed the effect of coreceptor binding affinity in the context of X4 Env. Toward this goal, we used a chimeric HXB2 Env in which the V3 loop was substituted with that of the R5-tropic BaL isolate [41]. This chimera, hereafter referred to as V3BaL, exclusively utilizes CCR5 for fusion and appears to bind CCR5 with high affinity [21,41]. This notion is consistent with our data showing that V3BaL fuses with target cells expressing the low-affinity CCR5 mutants (Fig. S3A) and with cells expressing low density of CCR5 (data not shown). Moreover, V3BaL was even somewhat more resistant to AD101 than BaL and JRFL (Table 2).

After verifying that HXB2, V3BaL, and BaL Env were proteolytically processed and incorporated into pseudoviruses at similar levels (Fig. S2), we compared the kinetics of fusion mediated by these glycoproteins. As expected, the rates of CD4 binding by V3BaL and HXB2 were indistinguishable (Fig. 3A, B and Table 1), whereas the V3BaL-CCR5 binding was ~3-fold faster than HXB2-CXCR4 binding. This result is consistent with the high affinity of the chimera to CCR5 and with the higher expression of CCR5 on TZM-bl cells compared to endogenous expression of CXCR4. In spite of the faster rate of ternary complex formation, the subsequent internalization of V3BaL-CD4-coreceptor complexes was ~2-fold

| **Table 1.** Effective rate constants of HIV-1 fusion and inactivation. |
|---|---|---|---|---|
| Virus | Cells | \( k_1 \) (min\(^{-1}\)) | \( k_2 \) (min\(^{-1}\)) | \( k_3 \) (min\(^{-1}\)) |
| JRFL | TZM-bl | 0.06±0.01* | 0.03±0.01 | >100 |
| JYN.2-15 | | 0.04±0.02 | 0.04±0.01 | 0.07±0.02 |
| JC.10 | | 0.04±0.04 | 0.02±0.01 | 0.05±0.02 |
| HXB2 | TZM-bl | 0.05±0.01 | 0.09±0.01 | 0.15±0.02 |
| BaL | | 0.03±0.02 | 0.01±0.004 | 0.10±0.04 |
| V3BaL | TZM-bl | 0.05±0.01 | 0.09±0.01 | 0.48±0.1 |

*Standard error of the curve fit.

| **Table 2.** HIV-1 sensitivity to inhibition by AD101 and C34. |
|---|---|---|
| Viruses | Cells | \( IC_{50} \) AD101 (nM) | \( IC_{50} \) C34 (nM) |
| JRFL | TZM-bl | 390±87* | 15±3 |
| JYN.2-15 | | ND** | 7±1 |
| HXB2 | TZM-bl | <0.1 | 8±2 |
| JYN.2-15 | | 1.6±0.2 |
| BaL | | 5.1±0.2 |
| V3BaL | TZM-bl | 710±207 | 2.3±0.3 |
| JYN.2-15 | | 1.7±0.2 |

*Standard error of the curve fit.
ND, not determined.
***IC\(_{50}\) for the C34env, peptide.

slower than that of HXB2 (Table 1). These opposite trends in the kinetics of the HXB2 and V3BaL pre-fusion steps resulted in similar overall rates of escape from C52L.

To better understand the differences in the progression of HXB2 and V3BaL through surface-accessible steps of fusion, we examined the fusion of viruses pseudotyped with wild-type BaL Env. BaL exhibited markedly different kinetics of fusion compared to HXB2 and V3BaL (Fig. 3). First, BaL engaged CD4 ~3-fold slower than JRFL and almost 9-fold slower than HXB2 (Table 1). By contrast, BaL escaped from AD101 and from C52L at rates that were indistinguishable from the rate of CD4 binding. The unusually quick BaL protection from C52L prompted us to examine whether this effect was due to the direct fusion with the plasma membrane as opposed to productive endocytosis demonstrated for JRFL and HXB2 viruses [15]. We therefore compared the rates of virus escape from C52L and from the temperature block applied at varied times of BaL incubation with TZM-bl cells (see [15] and Fig. S3B). A marked delay between the BaL escape from the temperature block compared to its escape from the peptide inhibitor strongly implies that this virus also fuses with endosomes. Collectively, these data show that BaL engages CD4 slowly but then completes the CCR5 binding and enters endocytic compartments at an unusually high rate.

The lifetime of gp41 pre-bundles can be obtained from the rates of coreceptor engagement and productive endocytosis

Having determined the effective rate constants of progression through the CD4 and coreceptor binding steps for different Env glycoproteins and target cells (Table 1), we used the equations (6–8) of Appendix S1 to calculate the probability of finding the virus in VCD4, VCD4CR and VE states as a function of time (Fig. 4A–D). We then determined the time averages of HIV-1 in these states by integrating the equations (6) and (7) from time t = 0 to the end of virus-cell incubation and dividing over this time interval (the resulting equations (13) and (14) are given in Appendix S1). The time averages of different HIV-1 in VCD4 and VCD4CR and the combined residence times in both states are shown in Fig. 4E.

The gp41 pre-bundles are formed upon the Env binding to CD4 or both CD4 and coreceptors [2–4] and are cleared from the cell surface by endocytosis [15]. Thus, the ability to determine the time spent in VCD4 and VCD4CR provided an opportunity to estimate the lifetimes of pre-bundles on the cell surface. The shortest apparent exposure of pre-bundle intermediates was observed for BaL, whereas the longest combined time in VCD4 and VCD4CR was observed upon JRFL fusion with cells expressing the low-affinity CCR5 mutant (Fig. 4E). Since C-peptides target the gp41 coiled coils, we asked whether their inhibitory potency correlated with the lifetime of these intermediates on the cell surface. It has been proposed that the longevity of pre-bundles determines the HIV-1 sensitivity to C-peptides [9]. However, this notion was based on correlation between the peptide's potency and the overall kinetics of HIV Env-mediated cell-cell fusion and not on measurements of the actual lifetime of gp41 coiled coils in the context of virus entry. In order to rationalize the kinetic determinants of HIV-1 sensitivity to C-peptides, we sought to compare the time spent in pre-bundle intermediates with the inhibitory potency of these peptides.

HIV-1 resistance to C-peptides does not correlate with the rate of productive endocytosis

To determine the susceptibility of different HIV-1 Env to inhibition by C-peptides, we measured virus-cell fusion in the

![Figure 3. Progression of the HXB2, V3BaL and BaL through surface-accessible stages of fusion.](image-url)
presence of the well-characterized C34 peptide [19]. JRFL exhibited the highest resistance to this peptide while BaL was somewhat more susceptible to inhibition (Fig. 5A, Table 2). By comparison, HXB2 and V3BaL were much more potently inhibited by C34 with the chimera being only marginally more resistant than the wild-type HXB2. So, in the context of HXB2 Env and under our experimental conditions, the coreceptor tropism and the coreceptor binding affinity had a modest effect on the virus’ resistance to C34. Similar results were obtained with the C52L peptide (data not shown).

The 10-fold difference between the IC50 for JRFL and HXB2 by C34 is in stark contrast with the comparable rates of their escape from high doses of C-peptides due to productive endocytosis (Figs. 2A and 3A). Plotting the IC50 values against the half-times of HIV-1 escape from C52L, as determined from the measurements shown in Figs. 2 and 3, confirmed that the C34 potency did not correlate with the rate of virus protection from this inhibitor (Fig. 6A). In contrast to these results, the kinetics of cell-cell fusion appears to inversely correlate with the potency of C-peptides [9]. These seemingly discrepant findings likely stem from the fact that HIV-1 escape from inhibitory peptides does not reflect virus-cell fusion, but rather corresponds to productive endocytosis.

Another possibility is that the C34 peptide used in our experiments had different affinity to the gp120 N-HR domains of HIV-1 isolates examined in this work. The conventional C34 peptide is derived from the gp120 C-HR of the IIIB clone [17,19].
which is identical to the respective domains of HXB2 gp41 but not of JRFL gp41 (Fig. 5D). Since the C34 sequence has been shown to affect its potency against HIV and SIV isolates [17], we asked if the JRFL-derived C34 (designated C34JRFL) is a more potent inhibitor of fusion than the C34IIIB. Both C34IIIB and C34JRFL potently inhibited HXB2 fusion at comparable concentrations (Fig. 5B). Even though JRFL was approximately 2-fold more sensitive to its own C34 than to C34IIIB, still higher doses of the former peptide were required to block JRFL fusion compared to HXB2 fusion. Thus, neither the kinetics of virus escape from C-peptides nor the sequences of C34 can fully account for the greater resistance of JRFL to this inhibitor. The above results highlight the importance of measuring the actual lifetime of gp41 pre-bundles (Fig. 4) in order to better rationalize the kinetic factors controlling the HIV-1 resistance to C-peptides.

**Kinetic determinants of HIV-1 resistance to C-peptides**

We asked whether the time averages of VCD4 and VCD4CR states are predictive of the virus’ sensitivity to C-peptides. When the potency of C34 was plotted against the time spent in VCD4 (Fig. 6B), the points clearly fell into two groups – JRFL fusion with different target cells and HXB2/BaL/V3BaL fusion with TZM-bl cells. Within these two groups, the IC_{50} correlated well with the time average in VCD4. Thus, in the context of JRFL Env or HXB2/BaL Env, the diminished rate of coreceptor engagement and thus the increased lifetime of early gp41 intermediates were associated with enhanced sensitivity to C34. In contrast, we saw no apparent correlation between the lifetime of VCD4CR and the potency of this peptide (Fig. 6C). This was surprising because the gp41 coiled coils should be better exposed to the peptide at VCD4CR compared to VCD4 [2]. Since the HXB2 (and likely V3BaL) coiled coils are exposed as early as upon CD4 binding [2–4] and should thus persist throughout the surface-accessible steps of fusion, a more meaningful parameter for characterizing the window of opportunity for the C34 binding is the total time spent in VCD4 and VCD4CR states. This parameter should also adequately describe the
coiled coil exposure on JRFL and BaL gp41. Even though the JRFL coiled coils are optimally exposed only after engaging both CD4 and CCR5 [2], the total time in VCD4 and VCD4CR states is dominated by the latter intermediate (Fig. 4C and E). We found that the combined residence time in these states inversely correlated with the IC50 for C34 (Fig. 6D). As in the Fig. 6B, this correlation was more apparent when fusion of the V3 loop-swapped constructs (BaL, HXB2 and V3BaL) and JRFL fusion with different cells were considered separately. These data suggest that, in the context of the same Env backbone, the total time spent in VCD4 and VCD4CR is a good predictor of the C34's potency.

The extent of Env inactivation by soluble CD4 correlates with its sensitivity to C-peptides

The lifetime of the gp41 coiled coils did not fully account for the differences in the inhibitory potency of C34. Indeed, the quick progression of BaL through the surface-accessible pre-fusion steps suggests that the gp41 coiled coils are very briefly exposed prior to entering into endosomal compartments. However, whereas BaL was much more resistant to C34 than HXB2 (Table 2), it was more susceptible to this inhibitor than JRFL, which spent much longer time in pre-bundle intermediates than BaL. Thus, virus strain-specific factors appear to contribute to the baseline sensitivity of HIV-1 to C-peptides. In addition, the modest effect of the HXB2 V3 loop substitution on the potency of C34 indicates that critical determinants of the virus’ resistance reside outside the V3 loop. Envelope glycoproteins of laboratory adapted strains are generally less stable and tend to inactivate upon binding to CD4 [42–44]. It is thus possible that the degree to which different Env glycoproteins refold in response to the CD4 binding could determine the sensitivity to C-peptides [17]. Recent study implies that this inactivation occurs via CD4-induced conformational changes in Env and not due to the gp120 shedding [45]. This work also demonstrates the strain-dependent differences in the exposure of the gp41 coiled coils caused by sCD4 binding.

To assess the extent of irreversible Env refolding in response to receptor binding, we measured to degree of virus inactivation...
after pre-treatment with sCD4. Viruses were pre-incubated with varied concentrations of sCD4, and the remaining fusion activity was determined after an additional 90 min-incubation with TZM-bl cells. These experiments revealed that JRFL was the most resistant to sCD4, BaL showed an intermediate sensitivity, whereas V3BaL and HXB2 were both strongly inactivated under these conditions (Fig. 3C). The similar effect of sCD4 on HXB2 and V3BaL viruses was as expected for Env glycoproteins sharing the same backbone.

Notably, the extents of sCD4-induced inactivation and inhibition by C-peptides ranked similarly (Fig. 5A and C). This result is consistent with the notion that the stability of Env-CD4 complexes determines the extent of exposure of the gp41 coiled coil. In other words, for the same time spent in a pre-bundle conformation, the sensitivity to C-peptides appears to depend on the extent of conformational changes in Env occurring in response to the receptor binding. This model is supported by the different efficacies of C34 against HXB2 fusing with TZM-bl and JRFL fusing with JC.10 cells in spite of the similar lifetimes of gp41 pre-bundles (Fig. 6D). Together, our results reveal a complex interplay between the Env stability (viral determinants) and the rate of progression through surface-accessible intermediates (viral and cellular determinants) in defining the HIV-1 resistance to C-peptides.

Discussion

In the present work, we characterized the HIV-1 progression through surface-accessible steps of entry prior to virus uptake and fusion with endosomes. Through measuring the rates of escape from membrane-impermeant inhibitors blocking distinct pre-fusion steps, we were able to determine the residence times in intermediate states in which HIV-1 recruited a requisite number of CD4 or both CD4 and coreceptors. This analysis revealed the viral strain-dependent and target cell-dependent differences in the average times the HIV-1 spent in distinct intermediates on the cell surface. The knowledge of the HIV-1 progression through the pre-fusion steps permitted the rationalization of the inhibitory potency of C-peptides targeting the gp41 coiled coils. We found that, unlike the Env-mediated cell-cell fusion, the time-course of HIV-1 escape from inhibitors of 6HB formation poorly correlated with the resistance to C34 (Fig. 6A). This could be due to the different rates of productive virus endocytosis that, in addition to the kinetics of CD4 and coreceptor engagement, control the time of exposure of the gp41 coiled coils on the cell surface.

Quantitative analysis of the HIV-1 fusion kinetics revealed correlation between the total residence time in pre-bundle conformations and the potency of C34. Interestingly, the lifetime of the V\text{CD4}, but not of V\text{CD4CR}, was predictive of the virus’ sensitivity to this inhibitor. This result was unexpected because the gp41 coiled coils should be better exposed to C-peptides at the latter state [2]. The previously proposed model [9] posits that the C-peptide binding to the complementary coiled coil domain is slow and thus occurs optimally upon prolonged exposure of these domains. However, our data suggest that the peptides can effectively bind to relatively short-lived gp41 intermediates (Fig. 6B). The observation that C-peptides cause closure of nascent fusion pores shortly after their addition to fusing cells [13] also supports the notion that the peptide binding occurs on a shorter time scale than the average lifetime of a pre-bundle intermediate (several minutes). We surmise that the quick peptide binding to the stably exposed coiled coils can account for the lack of correlation between the lifetime of ternary complexes and the potency of C34 (Fig. 6C). On the other hand, the enhanced potency of C34 associated with the longer-lived V\text{CD4} indicates that the coiled coil exposure at this point is not completed.

Our findings are in agreement with the previous reports that multiple factors control the HIV-1 resistance to C-peptides [17,20]. First, the baseline sensitivity of diverse Env glycoproteins appears to be determined by their propensity to undergo irreversible conformational changes upon engaging CD4. The exceptionally high resistance of JRFL to C-peptides in spite of the long time spent in CD4/coreceptor-bound state could be due in part to a restricted exposure of its coiled coils [2]. Second, the C-peptide’s primary sequence, which affects their binding affinity to the gp41 coiled coil, appears to modulate their inhibitory potency. Third, our results show that kinetic factors determine the longevity of gp41 pre-bundles and thus control the potency of C-peptides.

The window of opportunity for the C-peptide binding depends on cellular factors, such as the density of coreceptors and their affinity to Env, as well as on the rate of receptor-mediated virus endocytosis. We surmise that the faster rate of productive endocytosis in certain cell types may diminish the gp41 pre-bundle exposure and protect the virus from C-peptides and antibodies targeting Env intermediates.

Clearly, a full quantitative description of HIV-1 fusion awaits the determination of the fusion stoichiometry, as well as the identification of intracellular steps and factors involved in virus entry. However, experimental strategies developed in this work provide a means to evaluate the kinetics of surface-accessible steps of HIV-1 fusion. Our data show that the rate of progression through pre-fusion steps is a critical determinant of the virus’ susceptibility to C-peptides and likely to neutralizing antibodies targeting CD4-induced epitopes. Further studies involving a larger set of primary and laboratory adapted HIV strains are needed to substantiate the conclusions of this work and to define the viral and cellular determinants of resistance to peptide inhibitors.

Materials and Methods

Cells and reagents
HeLa-derived [JC.10] [31], JYN.2-15, and JGR.H11 [32] cells were a gift from Dr. D. Kabat (OHSU, OR). TZM-bl cells were obtained from NIH AIDS Research & Reference Reagent Program and grown in Dulbecco modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT) and penicillin-streptomycin (Invitrogen). 293T/17 cells (ATCC, Manassas, VA) were obtained in DMEM/10% FBS, 0.5 mg/ml Geneticin (Invitrogen), and penicillin-streptomycin. The pCAGGS plasmids encoding JRFL or HXB2 Env [46] were provided by Dr. J. Binley (Torrey Pines Institute, CA). The pCAGGS plasmids encoding HXB2-BaL chimera Env in V3 loop, V3BaL, were constructed by replacing of Env coding sequence of pSIV7D HXB BaL, a gift from Dr. R. Doms (University of Pennsylvania). HIV-1 BaL.01 [47] Env expression vector was obtained from NIH AIDS Research & Reference Reagent Program. The HIV-1 based packaging vector p8R8Env lacking the env gene was from Dr. D. Trono (University of Geneva, Switzerland). The C52L recombinant peptide [27] was a gift from Dr. Min Lu (Cornell University). BMS-806 [24,25] was synthesized by ChemPacific Corp. (Baltimore, MD), AMD3100 [48] and pronase were purchased from Sigma (St. Louis, MO), and AD101 [49] was a gift from Dr. J. Strizki (Schering Plough, Kenilworth, NJ). The C34\text{inh} peptide was synthesized by Dr. L-X. Wang (HIV, University of Maryland) and the JRFL gp41-derived C34 peptide (Ac-WMEWEREID-NYSEIYTLIESQONQKEKNEQELL-NH\text{2}) was kindly provid-
ed by Dr. W. Lu (IHV, University of Maryland). The purity of these peptides was >90%, as determined by HPLC.

Virus preparation

Pseudoviruses containing the β-lactamase-Vpr (BlaM-Vpr) were produced as described in [15]. Briefly, 293T/17 cells on a 60 mm dish were transfected by Ca-phosphate protocol with 10 μg pR8ΔEnv, 5 μg pMM510 vector expressing BlaM-Vpr [50], 4 μg pRev [51], and 15 μg pCAGGS encoding JRFL, HXB2, or V3BaL Env, or pHIV1 Bal.01 (expressing Bal. Env). The infectious titer was determined by a β-Gal assay in TZM-bl cells [52].

Virus-cell fusion assay

Measurements of HIV-1 fusion with target cells were carried out essentially as described previously [15]. Briefly, viruses bearing the BlaM-Vpr chimera were bound to TZM-bl cells by centrifugation at 2095 ×g, 4°C for 30 min. After washing off unbound viruses, cells were incubated at 37°C for 90–120 min. At indicated times of incubation, the fusion reaction was stopped by adding specific inhibitors of CD4 binding (10 μM of BMS-806), coreceptor binding (5 μM of AMD3100 or 7 μM of AD101), or inhibitors of 6-helix bundle formation (1 μM of C52L). The concentration of a given entry inhibitor used in these time-of-addition experiments exceeded the fully inhibitory concentration (determined in separate experiments) by at least 3-fold. Samples were then loaded with the BlaM substrate CCF2-AM (Gene-BlAzer in vitro detection kit, Invitrogen) on ice and incubated at 20°C for 12 hr. The resulting fluorescence signals at 460 nm (blue) and 528 nm (green) were measured using the Synergy HT plate reader (Bio-Tek Instr., Germany). The dose-response dependence for HIV fusion in the presence of inhibitors was obtained by pre-binding viruses to cells, as above, and incubating at 37°C for 90 min with various concentrations of C34 prior loading cells with the BlaM substrate.

Virus internalization assay

Cells (3 × 10^5 cells/well in 96-well plates) were grown overnight in regular medium. Vero cells were added to 96-well plates (MOI 0.7) and centrifuged as described above. After washing to remove unbound viruses, cells were incubated at 37°C for 3 h for varied times in the presence of 1 μM of C52L to prevent fusion. At defined time points, virus uptake was stopped and the external virus was stripped off by treatment with 2 mg/ml pronase on ice for 10 min. After washing with DMEM/10% serum, cells were lysed and the amount of p24 in cell lysates was determined using a p24 ELISA Kit (PerkinElmer Life Sciences Inc, Boston, MA).

Virus inactivation by soluble CD4

Twenty μl aliquots of 10-fold concentrated viral preparations were pre-incubated for 30 min at 37°C with different concentrations of sCD4. Following the incubation, the mixture was diluted 10-fold with the growth medium and added to TZM-bl cells (final MOI = 0.8 in the absence of sCD4). Virus binding to cells was augmented by centrifugation at 4°C, as described above. Cells were washed to remove unbound viruses, and fusion was initiated by shifting to 37°C for 90 min, after which time the process was stopped by reducing the temperature. The BlaM signal was normalized to that obtained for mock-treated viruses.

Supporting Information

Figure S1 The normalized kinetics of fusion is independent of the number of cell-bound viruses. JRFL viruses were added to TZM-bl cells at the multiplicity of infection (MOI) 1 or 0.2. After pre-binding the viruses at 4°C, cells were washed and shifted to 37°C to induce fusion. (A) The kinetics of BlaM signal for the two different MOI is shown. (B) The kinetics of fusion after normalizing the data to the last time point. Even though the overall BlaM signal is proportional to the MOI, the rate of fusion is independent of the virus input.

Figure S2 Processing and incorporation of various HIV-1 Env glycoproteins into pseudoviruses assessed by Western blotting. Viral lysates were prepared with RIPA lysis buffer (0.05 M TrisCl, 0.15 M NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and 1% sodium deoxycholate) that contained the Complete protease inhibitor (Roche Applied Science, Mannheim, Germany). The samples were separated on a 10% SDS-PAGE gel and transferred to PVDF membrane (Millipore, Billerica, MA). The viral proteins were then detected with anti-β-lactamase monoclonal antibody (QED Bioscience Inc, San Diego, CA), anti-p24 sera (from AIDS Research and Reference Reagent Program), and anti-gp120 polyclonal antibody (Fitzgerald, Concord, MA). The blot was treated with biotinylated anti-mouse or rabbit immunoglobulin (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), or anti-goat immunoglobulin (Sigma) as the secondary antibody and then incubated with streptavidin-horseradish peroxidase conjugate (GE Healthcare Bio-Sciences). Chemiluminescence from the protein bands was visualized on the Fluor Chem SP (Alpha Innotech, San Leandro, CA) using the LumiLightPlus (Roche) substrate.

Figure S3 Fusion with target cells expressing mutant CCR5 and escape from C52L and the temperature block. (A) The efficacy of JRFL, HXB2, BaL and V3BaL fusion with HeLa cells expressing similar levels of CD4, CXCR4 and wild-type (TZM-bl) or mutant (JYN.2-15 and JGR.H11) CCR5. In these experiments, the viral input was normalized by inoculating the cells with the same amount of virus (an equivalent of MOI 0.7 for TZM-bl cells). Note that BaL failed to fuse with cells expressing the Y14N CCR5 and fused inefficiently with cells expressing the G163R mutant. The ability of V3BaL to utilize the Y14N CCR5 mutant is consistent with the less pronounced dependency of X4-tropic viruses on the N-terminal segment of their cognate coreceptor. (B) BaL or JRFL pseudoviruses were bound to TZM-bl cells in the cold and induced to fuse by shifting to 37°C. The fusion was stopped at indicated times either by adding 1 μM C52L or by briefly placing cells on ice. Cells were then loaded with the BlaM substrate and incubated overnight at 13.5°C, a temperature that prevented HIV fusion but allowed the cleavage of the BlaM substrate.

Figure S4 Simulation of the effect of changes in k2 and ki on the fusion kinetics. (A) A model of HIV fusion in which the equality requirement for the inactivation rate constants at different stages of fusion has been relaxed. Thus, in addition to ki2, k2, and ki3 constants, the reaction is described by three inactivation rate constants, ki0, ki1 and ki2. (B) Modeling the fusion kinetics using the rate constants similar to those obtained for V3BaL fusion (see Table 1). (C, D) Changes in the kinetics of the escape from C-peptides due to an 8-fold increase in ki4 or ki2, respectively.

Appendix S1

Found at: doi:10.1371/journal.ppat.1000585.s003 (0.46 MB TIF)
Acknowledgments

We wish to thank Dr. D. Kabat for the gift of HeLa-derived cell lines, Dr. R. Doms for providing the V3BαL expression vector, Dr. M. Lu for the C52L peptide, Drs. W. Lu and L.-X. Wang for synthesizing C34 peptides, Dr. J. Strizki for the gift of AD101, and the NIH AIDS Research & Reference Reagent Program for the TZM-bl cells and BaL01 expression vector. We also thank Dr. Mariana Marin for performing the control experiments comparing the kinetics of HIV-1 escape from the C34 and C52L peptides. We are grateful to Drs. Leonid Chernomordik, Olga Latinovic and Mariana Marin for critical reading of the manuscript and helpful discussions and to Patrick Millet for his editorial help.

Author Contributions

Conceived and designed the experiments: MMK GBM. Performed the experiments: KM. Analyzed the data: KM MMK GBM. Wrote the paper: GBM.

References

1. Wyatt R, Sodroski J (1998) The HIV-1 envelope glycoproteins: fusogens, receptors, and immunogens. Science 280: 1884–1892.
2. Furuta RA, Wald CT, Weng Y, Weiss CD (1998) Capture of an early fusion-active conformation of HIV-1 gp120. Nat Struct Biol 5: 276–279.
3. Gallo SA, Puri A, Blumenthal A, Hemmati H, Alfayyag GP, Wild CT (2003) Evidence that the transition of HIV-1 gp120 into a six-helix bundle, not the bundle configuration, induces membrane fusion. J Cell Biol 151: 413–424.
4. Latunovic O, Moore JP (1993) The role of CD4 in HIV binding and entry. Philos Trans R Soc Lond B Biol Sci 349: 59–66.
5. Eckert DM, Kim PS (2001) Mechanisms of Viral Membrane Fusion and Its Inhibition. Annu Rev Biochem 70: 777–810.
6. Kuhmann SE, Platt EJ, Kabat D (2000) Cooperation of multiple coreceptor-binding site mutations in the human immunodeficiency virus type 1 Env glycoprotein gp120 with C34 peptides derived from the coiled coil of HIV type 1 gp41 is an attractive drug target. Proc Natl Acad Sci U S A 97: 11215–11220.
7. Dornaz BJ, Baik SS, Doms RW (1996) Binding of gp120 to peptide fusion inhibitors. J Mol Biol 259: 433–444.
8. Devreye CA, Decker JM, Stikmanos JN, Wu X, O’Brien WA, et al. (2000) Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J Virol 74: 8338–8347.
9. Gallo SA, Sackett K, Ravat SS, Shi Y, Blumenthal A (2004) The HIV Env-mediated fusion reaction. Biochim Biophys Acta 1614: 36–50.
10. Markosyan RM, Cohen FS, Melikyan GB (2003) HIV-1 envelope protein completes its folding into six-helix bundles immediately after fusion pore formation. Mol Biol Cell 14: 926–938.
11. Chan DC, Kim PS (1998) HIV entry and its inhibition. Cell 93: 681–684.
12. Sattentau QJ, Madani N, Miamidian JL, Harvey PE, et al. (2002) Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. Proc Natl Acad Sci U S A 99: 16294–16295.
13. Platt EJ, Dornaz BJ, Kabat D (2000) Evidence that the transition of HIV-1 gp120 into a six-helix bundle, not the bundle configuration, induces membrane fusion. J Cell Biol 151: 413–424.
14. Latunovic O, Moore JP (1993) The role of CD4 in HIV binding and entry. Philos Trans R Soc Lond B Biol Sci 349: 59–66.
15. Eckert DM, Kim PS (2001) Mechanisms of Viral Membrane Fusion and Its Inhibition. Annu Rev Biochem 70: 777–810.
16. Kuhmann SE, Platt EJ, Kabat D (2000) Cooperation of multiple coreceptor-binding site mutations in the human immunodeficiency virus type 1 Env glycoprotein gp120 with C34 peptides derived from the coiled coil of HIV type 1 gp41 is an attractive drug target. Proc Natl Acad Sci U S A 97: 11215–11220.
17. Dornaz BJ, Baik SS, Doms RW (1996) Binding of gp120 to peptide fusion inhibitors. J Mol Biol 259: 433–444.
18. Devreye CA, Decker JM, Stikmanos JN, Wu X, O’Brien WA, et al. (2000) Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J Virol 74: 8338–8347.
19. Gallo SA, Sackett K, Ravat SS, Shi Y, Blumenthal A (2004) The HIV Env-mediated fusion reaction. Biochim Biophys Acta 1614: 36–50.
20. Markosyan RM, Cohen FS, Melikyan GB (2003) HIV-1 envelope protein completes its folding into six-helix bundles immediately after fusion pore formation. Mol Biol Cell 14: 926–938.
21. Chan DC, Kim PS (1998) HIV entry and its inhibition. Cell 93: 681–684.
22. Sattentau QJ, Madani N, Miamidian JL, Harvey PE, et al. (2002) Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. Proc Natl Acad Sci U S A 99: 16294–16295.
23. Platt EJ, Dornaz BJ, Kabat D (2000) Evidence that the transition of HIV-1 gp120 into a six-helix bundle, not the bundle configuration, induces membrane fusion. J Cell Biol 151: 413–424.
46. Binley JM, Cayanan CS, Wiley C, Schulke N, Olson WC, et al. (2003) Redox-triggered infection by disulfide-shackled human immunodeficiency virus type 1 pseudovirions. J Virol 77: 5678–5684.
47. Li Y, Svehla K, Mathy NL, Voss G, Mascola JR, et al. (2006) Characterization of antibody responses elicited by human immunodeficiency virus type 1 primary isolate trimeric and monomeric envelope glycoproteins in selected adjuvants. J Virol 80: 1414–1426.
48. Donzella GA, Schols D, Lin SW, Este JA, Nagashima KA, et al. (1998) AMD3100, a small molecule inhibitor of HIV-1 entry via the CCR5 co-receptor. Nat Med 4: 72–77.
49. Trkola A, Kuhmann SE, Strizki JM, Maxwell E, Ketas T, et al. (2002) HIV-1 escape from a small molecule, CCR5-specific entry inhibitor does not involve CXCR4 use. Proc Natl Acad Sci U S A 99: 395–400.
50. Tobiume M, Lineberger JE, Lundquist CA, Miller MD, Aiken C (2003) Nef does not affect the efficiency of human immunodeficiency virus type 1 fusion with target cells. J Virol 77: 10645–10650.
51. Malim MH, Hauber J, Fenrick R, Cullen BR (1988) Immunodeficiency virus rev trans-activator modulates the expression of the viral regulatory genes. Nature 335: 181–183.
52. Kimpton J, Emerman M (1992) Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. J Virol 66: 2232–2239.