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HIV-1 gp41 Six-Helix Bundle Formation Occurs Rapidly after the Engagement of gp120 by CXCR4 in the HIV-1 Env-Mediated Fusion Process

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ABSTRACT: The onset of cell fusion mediated by HIV-1 IIIB Env is preceded by a lag phase of 15−20 min. Fusion mediated by the CD4-independent HIV-1 Env 8x, which is capable of interacting directly with CXCR4, proceeds with a greatly reduced lag phase. We probed the intermediate steps during the lag phase in HIV-1 IIIB Env-mediated fusion with Leu3-a, an inhibitor of attachment of gp120 to CD4, AMD3100, an inhibitor of attachment of gp120 to CXCR4, and C34, a synthetic peptide that interferes with the transition of gp41 to the fusion active state. Inhibitions of fusion as a function of time of addition of C34 and of AMD3100 were equivalent, indicating that engagement of gp120 by CXCR4 and formation of the gp41 six-helix bundle follow similar kinetics. The initial steps in fusion mediated by the CD4-independent Env 8x are too rapid for these inhibitors to interfere with. However, when 8x Env-expressing cells were incubated with target cells at 25 °C in the presence of AMD3100 or C34, prior to incubation at 37 °C, these inhibitors were capable of inhibiting 8x Env-mediated fusion. To further examine engagement of gp120 by CXCR4 and exposure of binding sites for C34, we have reversibly arrested the fusion reaction at 37 °C by adding cytochalasin B to the medium. We show that CXCR4 engagement and six-helix bundle formation only occur after the release of the cytochalasin arrest, indicating that a high degree of cooperativity is required to trigger the initial steps in HIV-1 Env-mediated fusion.

Animal-enveloped viruses enter susceptible cells by means of fusion of viral and cellular membranes mediated by their envelope glycoproteins (*1*), which are organized on the viral surface as oligomeric spikes (*2*). In the case of HIV, the surface of the spike, gp120, is associated by noncovalent interactions with each subunit of the trimeric gp41 (*3*). Host cell surface CD4 interacts with the gp120−gp41 molecules and causes conformational changes in gp120 that enables it to interact with a coreceptor, generally either CCR5 (R5) or CXCR4 (X4) (*4*−*6*). Coreceptor binding then triggers a battery of additional conformational changes in the envelope glycoprotein (*7*, *8*) eventually resulting in the formation of a coiled coil in gp41, which is composed of three NH_2-terminal leucine/isoleucine zipper regions, one contributed by each subunit of the Env trimer (*9*−*12*). The exterior surface of the coiled coil contains grooves which bind a second heptad repeat membrane proximal region of gp41 (C helix). According to a widely accepted "viral hairpin" model for HIV-1 Env-mediated membrane fusion, the gp41 core forms a stable six-helix bundle in which the fusion peptide and transmembrane domain of gp41 are now oriented at the same end of the molecule (*13*, *14*). The six-helix

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bundle presumably represents the terminal conformation of a fusogenic Env. Synthetic C peptides (peptides corresponding to the C helix) potently inhibit membrane fusion by both laboratory-adapted strains and primary isolates of HIV-1 (*15*, *16*) presumably by binding to the prehairpin groove and thereby interfering with the formation of the viral hairpin (*17*, *18*).

Given that in the presumed prehairpin state of the intact gp41 the NH_2-terminal leucine/isoleucine zipper and C-helical domains are present at very high concentrations, it is somewhat surprising that the synthetic C peptides are capable of inhibiting the formation of the six-helix bundle at relatively low (nanomolar) concentrations (*8*, *19*, *20*). In fact, the C-terminal peptide, DP178, is still capable of inhibiting cell−cell fusion even when added 15 min after coculture of cells expressing HIV-1 Env with cells bearing CD4 and an appropriate coreceptor (*20*). In the case of acid-activated viruses, triggering occurs rapidly and synchronously so that all viral envelope proteins are activated within a short period, leading to rapid fusion kinetics (*21*, *22*). By contrast, the CD4 and coreceptor-induced triggering events leading to HIV-1 Env-mediated fusion are presumably stochastic, leading to much slower fusion kinetics. This lack of synchrony therefore provides an opportunity for the C-terminal peptides to bind to the prehairpin grooves which become transiently exposed following CD4 and coreceptor-induced triggering of HIV-1 Env. Thus, the action of these peptides provides a way of studying structural intermediates of the fusion process.

Melikyan and co-workers (*23*) showed that preincubation of cells expressing HIV-1 Env with target cells at 23 °C, the "temperature-arrested state" (TAS), results in a relatively rapid rate of fusion when the cells were warmed to 37 °C. They found that the TAS intermediate is sensitive to DP178, indicating that the triple-stranded coiled coil is accessible under these conditions. Moreover, they provide evidence that formation of the six-helix bundle is coincident with membrane fusion. It has been shown previously that treatment of target cells with cytochalasin B (*24*, *25*) and D (*26*) blocks HIV-1 envelope glycoprotein-mediated fusion, presumably by inhibiting actin polymerization. We find that the fusion activity was completely recovered after washing the cells with a cytochalasin-free medium.

Since the rate of Env triggering will determine the ability of inhibitors to interfere with the formation of structural intermediates, we explored intermediates in the fusion cascade mediated by the CD4-independent virus derived from HIV-1 IIIB, termed 8x, whose chemokine receptor binding site is exposed without prior interaction with CD4 (*27*, *28*). In this study, we use C-terminal peptides which bind to the prehairpin grooves formed by the NH_2-terminal leucine/isoleucine zipper of gp41 and AMD3100, a small molecule (830 Da) which blocks HIV-1 fusion by interfering with the engagement of gp120 with CXCR4 (*29*, *30*) as probes for IIIB, and 8x Env conformations at intermediate states.

MATERIALS AND METHODS

HIV-1 IIIB Env was transiently expressed on the surface of HeLa cells using the recombinant vaccinia constructs VSC60 and vPE16 (*31*, *32*). To express the 8x Env, HeLa cells were infected with recombinant vaccinia virus vTF7-3 expressing T7 polymerase (*32*) and transfected with pSP73-8x (*28*) (kindly supplied by R. W. Doms) using lipofectamine (Gibco/BRL, Gaithersburg, MD). Target cells (either SupT1 or 3T3/CD4/X4) were labeled with calcein AM at a concentration of 10 μM, and HIV-1 Env-expressing HeLa or BJAB [TF228 (*33*)] cells were labeled with the cytoplasmic dye 5- and 6-{[(4-chloromethyl)benzoyl]amino} tetramethylrhodamine (CMTMR) at a concentration of 20 μM for 1 h at 37 °C. The dyes were purchased from Molecular Probes (Eugene, OR). Calcein-labeled target cells were cocultured with CMTMR-labeled effector cells for 2 h at 37 °C, and dye redistribution was monitored microscopically as described previously (*34*). The extent of fusion was calculated with the relationship percent fusion = 100 × number of bound cells positive for both dyes/number of bound cells positive for CMTMR. The inhibitors DP178 (*16*) [which is the same as T20 (*35*)] and C34 (*18*, *36*) were synthesized by Macromolecular Resources (Fort Collins, CO). The sequences of these peptides are YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF and WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL, respectively. Leu3-a (*37*) was purchased from BD Biosciences (San Jose, CA), and AMD3100 (*30*) was a kind gift from Anormed, Inc. For TAS experiments (*23*), cells are cocultured in RPMI medium for 2 h at 25 °C with or without inhibitor, washed, and replenished with 37 °C medium before being placed in the incubator at 37 °C. To reversibly arrest fusion at 37 °C, cells are cocultured in RPMI medium for 2 h at 37 °C with 10 μM cytochalasin B (Sigma Chemical Co., St. Louis, MO) in the presence or absence of inhibitor, washed, and replenished with 37 °C medium before being placed in the incubator at 37 °C.

RESULTS

Kinetics of Fusion. It has been shown that HIV-1 IIIB Env-expressing cells cocultured with $CD4^+$/$CXCR4^+$ target cells start to fuse after a lag phase at 37 °C of 15−20 min (*20*, *23*), whereas no fusion is observed at <28 °C. However, preincubation of HIV-1 IIIB Env-expressing cells with target cells at 23 °C, the temperature-arrested state (TAS), results in a relatively rapid rate of fusion when the cells are warmed to 37 °C (*23*). Figure 1 shows the lag phase when HIV-1 IIIB Env-expressing cells are incubated with target cells at 37 °C, and a much shorter lag phase when the mixture is warmed to 37 °C following the TAS, in accordance with the results of Melikyan and co-workers (*23*). The times to reach half-maximal fusion ($t_{1/2}$) were 49 and 36 min for the coculture and TAS experiments, respectively. These observations indicate that the steps responsible for the lag phase occurred during the TAS. To examine the molecular attributes of these steps, we compared these data with the kinetics of cell fusion mediated by the CD4-independent HIV-1 Env derived from HIV-1 IIIB, termed 8x, which has been modified in such a way that it does not require CD4 to expose coreceptor binding sites of HIV-1 (*27*). The levels of gp120 expression were similar for the IIIB and 8x Env-expressing cells. Figure 1 shows that the lag phase is much reduced even during coculture at 37 °C. The $t_{1/2}$ was very similar to that for IIIB Env following the TAS (34 min). These results indicate that the 8x Env is primed to trigger the fusion reaction without requiring the transitions through which the IIIB Env must go during the TAS. However, 8x

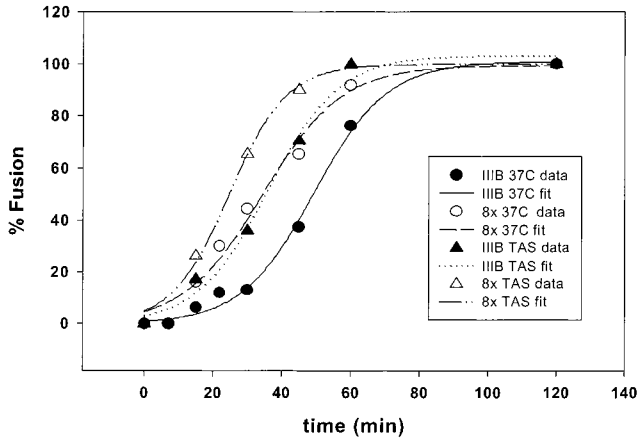

FIGURE 1: Kinetics of fusion mediated by IIIB and 8x Env. HeLa effector cells were plated on 12-well tissue culture plates at a density of 10^5 cells per well, and were infected either with the vaccinia recombinant vSC60 to express HIV-1 IIIB Env (black symbols) or with vTF7-3 and transfected with pSP73-8x to express HIV-1 8x Env (white symbols) on the cell surface. The next day, they were labeled with the cytoplasmic dye CMTMR (20 μM, excitation and emission at 550 and 565 nm, respectively). SupT target cells were labeled with the fluorescent cytosolic probe Calcein AM (10 μM, excitation and emission at 492 and 516 nm, respectively). Target cells (5×10^5) were added to the wells containing effector cells and incubated for various amounts of time in a humidified incubator and 5% CO_2. At the end of the incubations, images were collected using a 20× objective and fusion was scored using the assay described in the text. Cells were coincubated directly at 37 °C (○ and ●) or at 25 °C for 2 h (TAS), and the temperature was then increased to 37 °C (△ and ▲). The lines represent fits to the sigmoidal equation $f = a/\{1 - \exp[-b(t - t_{1/2})]\}$. The parameter fits yield $t_{1/2}$ (the time for half-maximal fusion) values of 49, 34, 36, and 25 min for IIIB at 37 °C, IIIB TAS, 8x at 37 °C, and 8x TAS, respectively. Parameters *a* and *b* represent the maximal extents and rates of fusion, respectively.

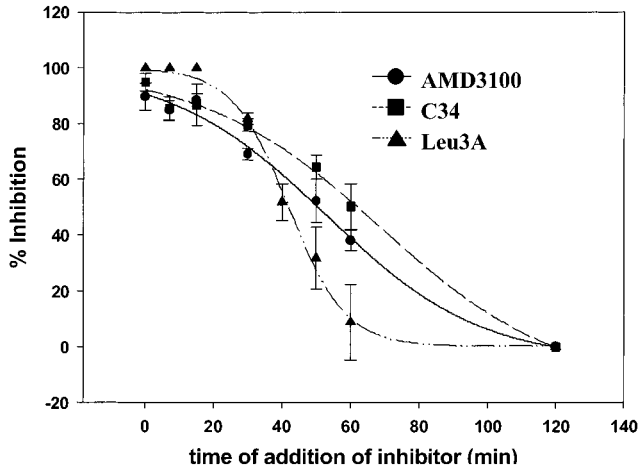

FIGURE 2: Time dependence of the inhibitory effect of Leu-3a, AMD3100, and the C34 peptide. TF228 effector cells and 3T3/CD4/X4 target cells, labeled with fluorescent dyes as described in the legend of Figure 1, were cocultured at 1:1 ratios. Then, 3 μg/mL Leu-3a (▲), 40 μg/mL AMD3100 (●), or 1 μM C34 (■) was added to effector/target mixtures at time 0 and at different times after the initiation of coculture at 37 °C. After total incubation for 2 h at 37 °C, the percentage of cell−cell fusion was calculated as described in Materials and Methods. Data are means ± standard deviation of three independent experiments.

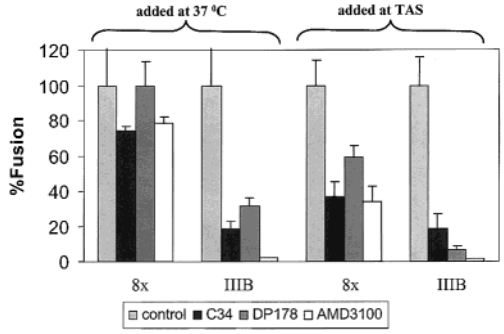

FIGURE 3: Fusion mediated by IIIB and 8x Env in the presence of inhibitors. HeLa cells expressing HIV-1 8x Env (first and third sets of bars) or HIV-1 IIIB Env (second and fourth sets of bars), labeled with CMTMR, were cocultured at 1:1 ratios with SupT target cells, labeled with calcein, at 37 °C (first and second sets of bars) for 2 h or at 25 °C for 2 h (TAS) followed by 2 h at 37 °C (third and fourth sets of bars). The percentage of cell−cell fusion was scored as described in Materials and Methods. Inhibitors were added at the beginning of the 37 °C coincubation or at the beginning of the TAS: (gray bars) no inhibitor, (black bars) 400 nM C34, (dark gray bars) 3 μM DP178, and (white bars) 10 μg/mL AMD3100.

Env-mediated fusion was accelerated following the TAS ($t_{1/2}$ = 25 min).

To analyze the intermediates in the HIV Env fusion cascade, we examined the kinetics of the inhibitory effects of gp41 C-terminal peptides, Leu3-a, the anti-CD4 antibody which blocks gp120−CD4 binding, and AMD3100, which blocks gp120−CXCR4 binding. Figure 2 shows that fusion was nearly completely inhibited when either Leu3-a, C34, or AMD was added to the fusion reaction mixture during the first 15 min of coculture. When the inhibitors were added at later time points, the inhibitory effect was reduced. However, fusion as a function of time of addition of C34 and AMD3100 was equivalent, indicating that engagement of gp120 by CXCR4 and exposure of the gp41 prehairpin groove follow similar kinetics. The kinetics of inhibition by adding Leu-3a after coculture were markedly different. Figure 2 shows that Leu3-a lost its ability to block fusion after 60 min, at which time only ~50% fusion was inhibited in the presence of AMD3100 or C34.

Exposure of Inhibitory Sites in HIV-1 Env. Because of the ability of 8x Env to bypass steps in the fusion reaction, which are required for IIIB Env-mediated fusion, the kinetics of exposure of inhibitory sites may be altered. We therefore examined inhibition of 8x Env-mediated fusion by C34 and DP178, and by AMD3100. Figure 3 shows that 8x Env-mediated fusion was not inhibited by these reagents when added at the beginning of coculture at 37 °C. These data indicate that both CXCR4 engagement by 8x gp120 and gp41 six-helix bundle formation are too rapid under these conditions for these inhibitors to interfere with. However, adding these inhibitors at the beginning of the TAS resulted in the inhibition of 8x Env-mediated fusion (Figure 3). We observed the same amount of inhibition whether the inhibitors were added at the beginning of the TAS (during the 2 h incubation at 25 °C) or at the end of the TAS just before heating to 37 °C (data not shown). The similar inhibition by AMD3100 at the beginning or end of the TAS indicates that gp120 has not engaged its coreceptor during the 2 h incubation at 25 °C.

Since addition of sCD4 to IIIB Env-expressing cells leads to the exposure of the prehairpin grooves in the N-terminal triple helix which can bind C-terminal peptides (*8*, *23*), it was of interest to examine whether the CD4-independent strain also had exposed prehairpin grooves. Figure 4 shows

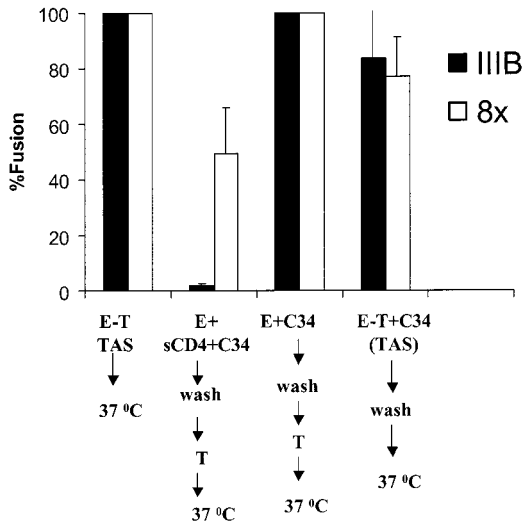

FIGURE 4: Exposure of the gp41 binding sites on IIIB and 8x Env. HeLa cells expressing HIV-1 IIIB Env (black bars) or HIV-1 8x Env (white bars) were labeled with CMTMR, and SupT cells were labeled with calcein. Effector and target cells were incubated at 37 °C for 2 h (first set of bars) following the TAS. Env-expressing cells were pretreated with 1 μM C34 with or without 50 μg/mL soluble CD4 at 37 °C (second and third sets of bars, respectively), washed, and incubated with target cells for 2 h at 37 °C. Effector and target cells were incubated at 25 °C for 2 h (TAS) in the presence of C34, and the cells were washed and incubated for 2 h at 37 °C (fourth set of bars). The percentage of cell−cell fusion was scored as described in Materials and Methods.

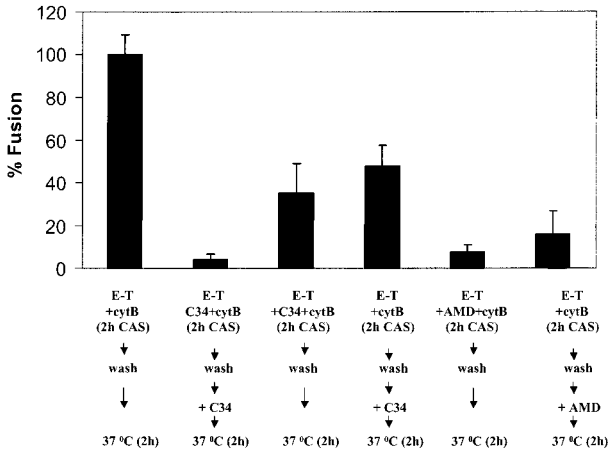

FIGURE 5: Exposure of the HIV-1 IIIB gp120 and gp41 binding sites in the cytochalasin-arrested state (CAS). HeLa cells expressing HIV-1 IIIB Env, labeled with CMTMR, were cocultured at 1:1 ratios with SupT cells, labeled with calcein, for 2 h at 37 °C in the presence of 10 μM cytochalasin B. The cells were washed and incubated for an additional 2 h at 37 °C (first bar). C34 (1 μM) was added during the CAS incubation, and added again after washing (second bar). C34 was added during the CAS incubation but not after washing (third bar). No C34 was added during the CAS incubation, and C34 was added after washing (fourth bar). AMD3100 (40 μg/mL) was added during the CAS incubation, but not after washing (fifth bar). CAS incubation without AMD3100, which was added after washing (sixth bar). The percentage of cell−cell fusion was scored as described in Materials and Methods.

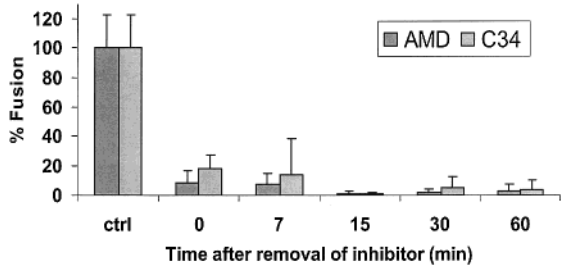

FIGURE 6: Inhibition by C34 and AMD3100 at CAS is irreversible. HeLa cells expressing HIV-1 IIIB Env, labeled with CMTMR, were cocultured at 1:1 ratios with SupT cells, labeled with calcein, for 2 h at 37 °C in the presence of 10 μM cytochalasin B and 40 μg/mL AMD3100 (dark gray bars), 1 μM C34 (gray bars), or control (no inhibitor). The cells were washed with a medium containing cytochalasin B, incubated for various amounts of time, washed with a cytochalasin-free medium, and incubated for 2 h at 37 °C. The percentage of cell−cell fusion was scored as described in Materials and Methods.

that incubation of IIIB or 8x Env-expressing cells with C34 alone followed by washing did not result in inhibition of fusion, indicating that the prehairpin grooves are not exposed in parental or CD4-independent strain. In contrast, incubation of IIIB Env-expressing cells with sCD4 and C34 followed by washing did lead to inhibition (Figure 4). Treatment of HIV-1 Env-expressing cells with 50 μg/mL sCD4 for 1 h at 37 °C followed by washing did not inhibit fusion, consistent with previous results (*38*). These results indicate that although 8x Env and sCD4-primed IIIB are similar in that their gp120 binding sites for CXCR4 are exposed, they differ in their exposure of gp41 prehairpin grooves. Incubation of effector and target cells with C34 during the TAS followed by washing also resulted in inhibition of fusion (Figure 4), albeit not to the same extent as that reported by Melikyan and coworkers (*23*).

Cytochalasin Arrested State. It has been shown previously that treatment of target cells with cytochalasin B (cytB) (*24*) and D (*26*) blocks HIV-1 envelope glycoprotein-mediated fusion, presumably by inhibiting actin polymerization. We observed that HIV-1 IIIB Env-mediated fusion, which was completely inhibited in the presence of 10 μM cytochalasin B, totally recovered after washing the cells with a cytochalasin-free medium (Figure 5). The reversibility of the cytochalasin-arrested state (CAS) then provides an opportunity to probe for intermediates which involve engagement of gp120 with CXCR4 and exposure of gp41 prehairpin grooves. Figure 5 shows that incubation of HIV-1 Env-expressing cells with target cells in the presence of cytochalasin and C34 followed by washing led to significant inhibition of fusion, indicating that the prehairpin grooves were available for binding to C34 at the CAS. The incubation during the CAS in the presence of cytB and AMD3100 followed by washing also resulted in inhibition. Interestingly adding AMD3100 at the end of the CAS also led to inhibition indicating that the gp120 had not engaged CXCR4 during the CAS.

To allow the inhibitors to dissociate during the CAS, we washed the cells with a medium containing cytochalasin but no C34 or AMD3100 and waited for various periods of time before removing the cytochalasin block by washing with medium (Figure 6). Surprisingly, fusion was inhibited even 60 min after removal of C34 or AMD3100. This is in contrast to results with the TAS where recovery of fusion was observed 1 h after washing out the peptides (*23*).

DISCUSSION

In this study, we show that fusion mediated by the CD4-independent HIV-1 Env 8x exhibits kinetics different from those of the parental CD4-dependent Env (Figure 1). In the

case of 8x, the normal sequence of events is short-circuited since 8x Env is capable of interacting directly with CXCR4. Since we use the same $CD4^+$ target cells in these experiments, the fusion reaction is not limited by the binding of Env-expressing cells to target cells. In the case of IIIB Env, the cells start to fuse after a lag phase of 15−20 min (Figure 1). During the lag time, the gp120−gp41 molecules on the Env-expressing cells are presumably preparing to bind to CD4, undergo conformational changes, attach to CXCR4, and trigger the fusion reaction. This process is described in terms of the metaphor presented by Doms and Moore (*39*): "In fishing terms, CD4-binding may be analogous to the cocking of the harpoon gun, but coreceptor-binding is the targeting radar that enables the shooter to pull the trigger only when a fish is actually in sight". Since fusion mediated by 8x Env exhibits a much shorter lag phase, it would appear that the CD4-induced activation ("cocking") of gp120 is rate-limiting. The potency of the inhibitor Leu3a, which blocks gp120−CD4 interactions, rapidly decreases after this lag phase, also implicating gp120−CD4 binding as a rate-limiting step (Figure 2). This notion is at odds with kinetic data regarding the interaction of purified monomeric gp120 with the coreceptor. Doranz and co-workers (*40*) found that preincubation of gp120 with sCD4 does not affect the rate of binding to the coreceptor, indicating that the conformational changes induced in gp120 by CD4 occur rapidly. Although those data were gathered with JFRL gp120 binding to CCR5, we do not expect qualitative differences in the case of IIIB gp120−CXCR4 interactions.

Why then is the CD4-induced activation of gp120 rate-limiting when IIIB Env and CD4 are expressed on opposing cell surfaces? In contrast to sCD4, which has little restriction upon the orientation it is able to adopt, membrane-anchored CD4 does not have similar accessibility to cell surface gp120−gp41 molecules. Therefore, the CD4 attachment may occur at a few contact sites leading to conformational changes in very few gp120−gp41 molecules, which need to find a cluster of coreceptors (*41*), presumably recruited into membrane rafts (*34*, *42*). Thus, in the case of IIIB Env, the rare occurrences of coreceptor engagement leading to fusion events will give rise to the relatively extended waiting time before fusion is observed. In the case of 8x, however, the gp120−gp41 molecules are already activated and will not have trouble engaging a patch of CXCR4 molecules on the target membrane, leading to fusion events with much shorter lag times (Figure 1). Preincubation of IIIB Env-expressing cells at room temperature with target cells for an ample amount of time (TAS) will result in a sufficient activation of gp120−gp41 molecules to render the kinetics mediated by IIIB Env more similar to those mediated by 8x Env (Figure 1). The fact that the $t_{1/2}$ values are similar for fusion under conditions of IIIB TAS and 8x coculture is consistent with this notion.

The lag times in IIIB Env-mediated fusion afforded us an opportunity to examine the temporal relationship between coreceptor engagement and formation of the gp41 six-helix bundle. Previously, we had shown that the C-terminal peptide, DP178, is still cable of inhibiting cell−cell fusion even when added 15 min after coculture of IIIB Env-expressing cells with target cells (*20*). Failure to inhibit fusion upon addition of the inhibitor at later times indicates that a given population of gp41 molecules has already formed the six-helix bundle, which is coincident with membrane fusion (*23*). By the same token, failure to inhibit fusion upon addition of AMD3100 after 15 min indicates that a given population of gp120 molecules has already engaged CXCR4. Fusions as a function of time of addition of C34 and AMD3100 were equivalent to each other but slower than that of Leu3-a (Figure 2), indicating that engagement of gp120 by CXCR4 and formation of the gp41 six-helix bundle follow similar kinetics but occur after CD4 binding. This is in agreement with the Doms−Moore conjecture in that "coreceptor-binding is the targeting radar that enables the shooter to pull the trigger only when a fish is actually in sight" (*39*). The fact that Leu3-a lost its ability to block fusion after 60 min, at which time only ∼50% fusion was inhibited in the presence of AMD3100 or C34, indicates that gp120 became completely anchored to CD4 while the search for CXCR4 binding and subsequent six-helix bundle formation was still proceeding. The rapid engagement of 8x Env with CXCR4 leading to six-helix bundle formation precludes interference by C peptides and AMD3100, when the reagents are added at the time of coculture at 37 °C (Figure 3). However, adding these inhibitors during the coculture of cells at room temperature did leave sufficient time for these inhibitors to interfere with CXCR4 engagement and six-helix bundle formation. Although the coreceptor binding site is exposed in the untreated 8x, the gp41 prehairpin groove only becomes exposed during the TAS (Figure 4). There appeared to be no difference whether the inhibitors were added at the beginning or end of the TAS or CAS (Figure 5), indicating that the association with their binding sites was not rate-limiting.

Derdeyn and colleagues (*43*) reported that X4 viruses are significantly more sensitive to T-20 inhibition than R5 viruses. We can explain this finding on the basis of our data regarding the relative kinetics of coreceptor engagement and six-helix bundle formation. According to Doranz and colleagues (*40*), binding of X4 gp120s to CXCR4 in the presence of CD4 is not as robust as binding of R5 gp120s to CCR5. Since six-helix bundle formation rapidly follows coreceptor engagement, the presumably more rapid CCR5 receptor engagement would require larger amounts of T20/DP178 to interact rapidly with the prehairpin grooves in gp41 before the six-helix bundle is formed.

Incubation of Env-expressing cells with target cells in the presence of C34 during the TAS followed by washing resulted in inhibition of fusion, indicating exposure of gp41 prehairpin grooves during the TAS (*23*). However, we only observed ∼20% inhibition under these conditions (Figure 4). We therefore made use of the observation that incubation of HIV-1 Env-expressing cells with target cells at 37 °C in the presence of cytochalasin B leads to a reversible inhibition of fusion (Figure 5). Incubation of Env-expressing cells with target cells in the presence of C34 during the CAS followed by washing resulted in a more significant inhibition of fusion, indicating that exposure of gp41 prehairpin grooves at 37 °C was more pronounced (Figure 5). The observation that HIV-1 Env-mediated fusion was inhibited even 1 h following removal of C34 or AMD3100 indicates that HIV-1 Env may have been irreversibly inactivated, due to its failure to proceed along the pathway to fusion. In terms of the Doms−Moore conjecture, "the trigger has been pulled without the fish actually being in sight".

Cytochalasin blocks HIV-1 Env-mediated fusion presumably by blocking polarized cocapping of CD4 and CXCR4 with subsequent pseudopod formation (*26*). Since the gp120 molecules had not engaged CXCR4 during the CAS (Figure 5), we surmise that a high local concentration of CXCR4, presumably recruited into rafts (*34*, *42*), is required to engage CXCR4 molecules and trigger the fusion event. This would indicate a high degree of cooperativity in the gp120−gp41−CD4−coreceptor interactions leading to HIV-1 entry.

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