Temperature-Dependent Intermediates in HIV-1 Envelope Glycoprotein-Mediated Fusion Revealed by Inhibitors that Target N- and C-Terminal Helical Regions of HIV-1 gp41

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Received January 5, 2004; Revised Manuscript Received April 8, 2004

ABSTRACT: Peptides derived from the N- (N-HR) and C- (C-HR) terminal heptad repeat regions adjacent to the fusion peptide and transmembrane domains, respectively, of human immunodeficiency virus (HIV)-1 gp41 inhibit HIV-1 viral envelope glycoproteins (Env)-mediated cell fusion specifically. The mechanism of HIV-1 Env-mediated cell fusion and its inhibition by agents that target the N- and C-HR regions was investigated. Priming experiments with Env-expressing cells indicate that the N-HR region but not the C-HR region is exposed by treatment with sCD4 at 31 °C, whereas both the N- and C-HR regions are exposed at 37 °C.

The binding of the trimeric human immunodeficiency virus (HIV)-1[1] envelope (Env) glycoprotein gp120/gp41 to the cell surface receptor CD4 and chemokine coreceptor CXCR4 or CCR5 triggers a series of conformational changes in the envelope proteins that ultimately leads to the formation of a six-helix bundle, comprising the N- (N-HR) and C- (C-HR) terminal heptad repeat regions of the gp41 ectodomain (*1*−*5*) and membrane fusion (*6*). A prehairpin state, which precedes the six-helix bundle (viral hairpin) formation, can be detected as the N-terminal regions of the ectodomain are revealed to the extracellular milieu upon CD4 binding (*7*). These epitopes then become inaccessible at approximately the same time as the occurrence of membrane fusion (*8*), and during the length of this exposure, HIV-1 Env-mediated fusion is susceptible to inhibition by peptides derived from the C-HR region of gp41 (*9*−*11*).

Several peptides that mimic the sequence of the N- and C-HR regions have been found to inhibit fusion by blocking the interaction between the C- and N-HR regions, respectively, and thus, preventing the formation of the six-helix bundle fusogenic state of gp41 (Table 1) (*12*−*18*). C-HR-derived peptides such as C34 and T20 (see Table 1) inhibit HIV-1 Env-mediated cell fusion, through binding to the hydrophobic grooves that line the internal N-terminal trimeric coiled-coil core of the gp41 ectodomain (*14*). C34 comprises residues that interact specifically with the deep hydrophobic pocket on the surface of the N-terminal internal trimeric coiled coil of gp41. Using a monoclonal antibody directed specifically against the six-helix bundle of gp41 together with isolate-restricted Env reactivity, direct evidence has been provided that, in receptor-activated viral Env, C-peptide entry inhibitors bind to the N-terminal internal trimeric coiled coil to form a peptide/protein hybrid structure and, in doing so, disrupt the native six-helix bundle formation (*19*). T20 (also referred to as DP178, enfuvirtide, and fuzeon) has extensive overlap with C34. Although T20 does not contain residues that interact with the deep hydrophobic pocket, it is still found to be effective in vitro and has been recently granted FDA approval as an anti-HIV therapeutic (see http://www.fuzeon.com/). It has been proposed, however, that T20 also targets the membrane-proximal domain of HIV-1 gp41 preventing clustering of gp41 trimers required for fusion pore formation (*20*, *21*).

Table 1: Peptide Sequences and Inhibition

inhibitor name	sequence[a]	IC_{50} (μM)
T20	YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF (638–673)	0.78
C34	WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL (628–661)	0.28
$N36^{Mut(e,g)}$	SGIDQEQNNLTRLIEAQIHELQLTQWKIKQLLARIL (546–581)	4.4
N_{CCG}-gp41	N35-Linker-C28-N34[b]	2.6
N35	SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARI (546–580)	ND[c]
Linker	SGGRGG	ND[c]

[a] Numbers denote position of peptide in context of the $HIV\text{-}1_{IIIB}$ gp41 sequence. [b] N34 = N35 without ILE at the C terminal, and C28 = C34 without NEQELL at the C terminal. [c] ND = not determined.

Synthetic peptides corresponding to residues of the N-HR region of gp41, such as DP107 and N36, are much less-effective inhibitors with IC_{50} values in the micromolar range (*12*). Coimmunoprecipitation of HIV-1 gp41 with HA-tagged DP107 indicates that this peptide (or an aggregate thereof)

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[1] Abbreviations: HIV, human immunodeficiency virus; Env, viral envelope glycoproteins; CMTMR, 5-(and-6) ((((4chloromethyl)benzoyl)amino)tetramythylrhodamine); HR, heptad repeat.

10.1021/bi049957v
Published on Web 06/03/2004

interacts with the C-HR region of gp41 (*22*). However, a multisite mutant known as $N36^{Mut(e,g)}$, which was designed to completely abolish any binding ability to the C-HR region, while maintaining the ability to self-associate into well-defined trimers, has a much higher inhibitory potency than the parent peptide, N36 (*17*). Thus, it appears that N-HR peptides corresponding to wild-type sequences may inhibit by binding either or both HR regions of HIV-1 gp41.

To design an inhibitor that targets the C-HR region of gp41 in an unambiguous way, an N-terminal construct, known as N_{CCG}-gp41, has been made in which the N helix of HIV-1 gp41 is grafted in a helical phase onto the N terminus of a minimal thermostable six-helix bundle of gp41 and stabilized by intermolecular disulfide bridges (*16*). N_{CCG}-gp41 presents a stable and exposed trimeric coiled coil of N helices that inhibits fusion at nanomolar concentrations. Thus, the availability of inhibitors that unambiguously target the N- and C-HR regions of HIV-1 gp41 enables us to dissect out distinct intermediates in the HIV-1 Env-mediated fusion reaction.

EXPERIMENTAL PROCEDURES

$HIV\text{-}1_{IIIB}$ envelope glycoproteins were expressed on HeLa or CV-1 effector cells (ATCC, Rockville, MD) by infection with a recombinant vaccinia virus, vpe16, obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Drs. Patricia Earl and Bernard Moss. Alternatively, we used as effector cells CHO cells expressing the $HIV\text{-}1_{IIIB}$ envelope (*23*), obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Drs. Carole Weiss and Judith White. The effector cells were labeled with CMTMR. Target cells consisting of either SupT1, obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. James Hoxie, or NIH 3T3 CD4/CXCR4 cells, obtained from Drs. Vineet Kewalramani and Dan Littman, were labeled with calcein in suspension (Molecular Probes, Eugene, OR). Cells were cocultured for 2 h at 37 °C, and dye transfer was monitored as described previously (*11*). The design and synthesis of N_{CCG}-gp41 and $N36^{Mut(e,g)}$ has been described previously (*16*, *17*). The C34 and T20 peptides were synthesized by SynPep (Dublin, CA). Sequences of these peptides are shown in Table 1. The peptides were generally added at the start of the incubation period, unless otherwise noted. In the priming experiments, effector cells were preincubated with sCD4 (ImmunoDiagnostics, Inc., Woburn, MA) at 15−20 μg/mL with or without the inhibitor for 60 min at different temperatures. Cells were then washed, and target cells were added. Fusion was observed after 2 h at 37 °C.

RESULTS AND DISCUSSION

Table 1 displays the concentrations of inhibitors at half-maximal inhibition (IC_{50}) of HIV-1 Env-mediated fusion of VPE16 infected CV-1 cells and SupT1 targets. C34 is the best inhibitor, followed by T20, N_{CCG}-gp41, and finally $N36^{Mut(e,g)}$. Although the absolute values of the IC_{50}'s are approximately 2 orders of magnitude higher in this paper, the similar relative potency of these compounds is entirely consistent with previous reports (*16*, *17*, *21*). Because it has been shown that sensitivity of HIV-1 to entry inhibitors correlates with fusion kinetics (*24*), we surmise that the relatively high IC_{50} values are due to our highly efficient fusion system. In addition, we have seen a 1−2 orders of magnitude difference in IC_{50} values between gene reporter assays and dye transfer assays when directly compared using the same cells (data not shown). This may be due to the prevention of pore expansion by C34, as well as by the other inhibitors, in which smaller pores still allow the transfer of small dye molecules but not large proteins such as T7 polymerase.

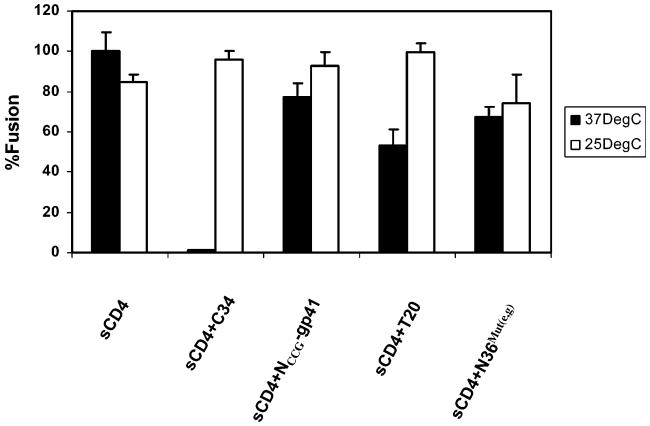

FIGURE 1: Exposure of gp41 N- and C-HR regions of sCD4-primed HIV-1 Env. Fusion of HIV-1 Env-expressing CHO cells with 3T3/CD4/CXCR4 target cells was monitored using a dye redistribution assay (*11*). The data are normalized to 100% fusion in the absence of the inhibitor. In these experiments, effector cells are preincubated with 20 μg/mL of sCD4 for 60 min at 25 or 37 °C, (white and black bars, respectively), with or without the inhibitor indicated on the *x* axis. These cells are then washed and incubated with target cells for 2 h at 37 °C. Inhibitors were used at concentrations 2−5 times the IC_{50} during the preincubation; 1.4 μM for C34, 3.9 μM for T20, 22 μM for $N36^{Mut(e,g)}$, and 5 μM for N_{CCG}-gp41, respectively. The error bars represent the standard error.

It has been shown that peptides derived from both the N- and C-HR regions preferentially bind receptor-activated Env and that CD4 binding is sufficient for triggering conformational changes that allow these peptides to bind Env (*8*, *9*, *22*, *25*, *26*). Additionally, peptides derived from N- and C-HR regions of the paramyxovirus SV5 F fusion protein, which also forms six-helix bundles, have been shown to bind to the F protein at different threshold temperatures (*27*). To examine the temperature-dependent exposure of the gp41 N- and C-HR regions, we primed HIV-1 Env-expressing HeLa cells with sCD4 with or without the peptide for 1 h at 25 or 37 °C. The peptides were added at concentrations 2−5 times the IC_{50} for fusion inhibition in the absence of sCD4 priming and washing. All peptides preincubated with effector cells in the absence of sCD4, followed by washing, did not significantly inhibit HIV-1 Env-mediated fusion (data not shown). Incubation of effector cells with sCD4 at 25 or 37 °C, followed by washing and coculture with SupT1 target cells for 2 h at 37 °C, resulted in little inhibition of HIV-1 Env-mediated fusion (first set of bars in Figure 1). Significant fusion inhibition was observed at 37 °C when sCD4-primed cells were incubated with C34 and then washed (second set of bars in Figure 1), although this inhibition was lost upon priming at 25 °C. Similarly, incubation of sCD4-primed HIV-1 Env-expressing cells with N_{CCG}-gp41 at 25 °C, followed by washing, resulted in the absence of fusion inhibition, whereas the same incubation at 37 °C led to

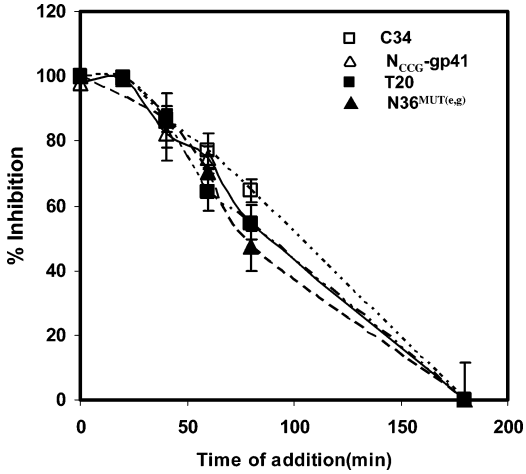

FIGURE 2: Fusion of HIV-1 Env-mediated cell fusion as a function of time of addition of fusion inhibitors. C34, T20, $N36^{Mut(e,g)}$, and N_{CCG}-gp41 were added at different times after coculture of HIV-1 Env-expressing HeLa cells with SupT1 targets at 31 °C, and the loss of inhibition with time was observed via a dye redistribution assay. The data are normalized to 100% fusion in the absence of the inhibitor. All inhibitors exhibited similar time courses and lost 50% of inhibitory potency after approximately 75 min. Inhibitors were used in high concentrations: 13 μM for C34 (□) and T20 (■), 75 μM for $N36^{Mut(e,g)}$ (▲) and 5 μM for N_{CCG}-gp41 (△).The error bars represent the standard error.

moderate fusion inhibition (third set of bars in Figure 1). This indicates that the sCD4-primed target for both C34 and N_{CCG}-gp41 is exposed at 37 °C but not at 25 °C. The "prime-wash" experiments with T20 showed similar results (fourth set of bars in Figure 1), inhibiting at 37 °C but not at 25 °C. Under the same conditions, sCD4-primed Env is only slightly susceptible to inhibition by $N36^{Mut(e,g)}$ at either temperature (fifth set of bars in Figure 1).

To examine whether the fusion intermediates that are revealed based on their thermal energy requirements also appear in a temporally distinct manner, we performed a series of time of addition studies. It has been shown previously that there is a lag phase between the binding of HIV-1 gp120 to CD4 and the engagement of gp120 with CXCR4 (*11*). However, time of addition studies with C34 showed that the formation of the six-helix bundle fusogenic state of gp41 occurs rapidly after the engagement of gp120 by CXCR4. The inhibitors were added at different time points after the initial coculture of HIV-1 Env-expressing HeLa cells and SupT1 cells, and the loss of inhibition was monitored over time (Figure 2). To enhance the time resolution for detection of temporal intermediates, the fusion reaction was slowed by incubation at 31 °C. Figure 2 shows that inhibition of Env-mediated fusion by T20, $N36^{Mut(e,g)}$, and N_{CCG}-gp41 is lost at approximately the same time as that by C34. This indicates that the transitions that expose the N- and C-HR regions of gp41 are fairly rapid following the engagement of gp120 with CXCR4.

To examine the correlation between the thermal requirements for HIV-1 Env-mediated fusion and the emergence of the N- and C-HR regions from their buried dispositions, we performed the fusion experiments and prime-wash experiments with C34 and N_{CCG}-gp41 at the same temperatures. Just these two inhibitors were used because they are the most effective pair of inhibitors with distinct targets on

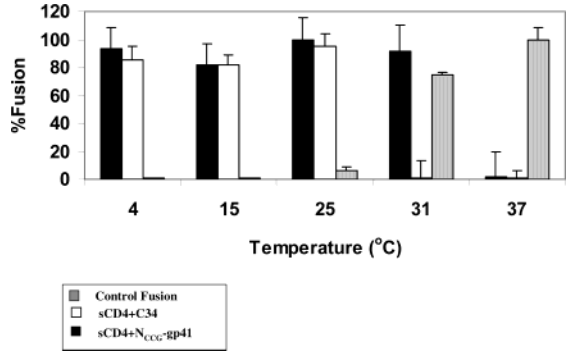

FIGURE 3: Temperature dependence of exposure of gp41 N- and C-HR regions and of HIV-1 Env-mediated fusion. Fusion of HIV-1 Env-expressing CHO cells with NIH 3T3 CD4/CXCR4 target cells was monitored at different temperatures using a dye redistribution assay (gray bars). The data are normalized to 100% fusion at 37 °C in the absence of the inhibitor. Fusion was also monitored following incubations of HIV-1 Env-expressing CHO cells with sCD4 (15 μg/mL) and C34 (1.4 μM, white bars) or N_{CCG}-gp41 (5 μM, black bars) for 60 min at different temperatures. Washing and incubation took place with NIH 3T3/CD4/CXCR4 target cells for 2 h at 37 °C. These data are normalized using this equation: $(F_I - F_{37})/(F_{CD4} - F_{37})$, where F_I is the fusion yield in the presence of sCD4 and the inhibitor at a given temperature, F_{37} is the fusion yield in the presence of sCD4 and the inhibitor at 37 °C, and F_{CD4} is the fusion yield in the presence of sCD4 alone at a given temperature. The uncertainty represents the standard error propagated through this equation. A student t test performed on the data in Figure 3 indicates only a significant difference ($p = 0.001$) at 31 °C between inhibition of sCD4-primed Env by C34 and N_{CCG}-gp41.

gp41 (the C-HR for N_{CCG}-gp41 and the N-HR for C34) and would, therefore, yield the most easily interpretable result. Each value from these priming experiments is normalized to both the maximal signal and to the CD4-alone signal. Figure 3 shows that fusion of HIV-1 Env-expressing CHO cells and 3T3/CD4/X4 target cells takes off beyond a threshold temperature of about 25 °C, as reported previously (*28*−*30*). The prime-wash experiments with C34 showed fusion inhibition at preincubation temperatures greater than 25 °C (Figure 3), indicating that the energetics of exposure of N-HR trimer in the prefusion state is quite similar to that of HIV-1 Env-mediated fusion. The prime-wash experiments with N_{CCG}-gp41, on the other hand, show fusion inhibition only at temperatures >31 °C (Figure 3). If the majority of gp41 were trimeric at 31 °C but shifts toward the monomeric form upon priming at 37 °C and if N_{CCG}-gp41 preferentially binds monomeric gp41, little inhibition by N_{CCG}-gp41 of the envelope primed at 31 °C would be seen in comparison to that primed at 37 °C. The monomeric pathway is likely nonproductive for fusion, which suggests that its promotion may represent a potential area for drug development.

ACKNOWLEDGMENT

We are grateful to the NIH AIDS Research and Reference Reagent Program for the supply of Sup-T1 cells, VPE16 recombinant vaccinia, and CHO-WT cells. We thank the members of the Blumenthal lab for their helpful suggestions. This work was supported by the AIDS Targeted Antiviral Program of the Office of the Director of the National Institutes of Health (to C.A.B., R.B., and G.M.C.).

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BI049957V