Characterization of the steric defense of the HIV-1 gp41 N-trimer region

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(RECEIVED August 13, 2008; FINAL REVISION September 12, 2008; ACCEPTED September 15, 2008)

Abstract

During viral entry, HIV gp41 adopts a transient conformation called the “prehairpin intermediate” in which a highly conserved therapeutic target, the N-trimer, is exposed. Despite extensive discovery efforts, potent and broadly neutralizing antibodies that target the N-trimer are elusive. We previously demonstrated the N-trimer is protected by a steric block that prevents large proteins, such as antibodies, from accessing it. Here we further characterize the steric block and identify its source. To study the N-trimer steric accessibility, we produced two sets of C-peptide inhibitors (a potent inhibitor targeting the N-trimer) fused to cargo proteins of increasing size facing either the virus or cell side of the prehairpin intermediate. Both bulky inhibitor sets show a steric block, but the effect is more pronounced with virus-side cargo. Additionally, both sets maintain their potencies in a modified entry assay that removes possible sources of target cell steric hindrance. These results implicate a viral source, likely gp120, as the primary component of the steric block. In addition, we studied the steric accessibility of the “pocket” region of the N-trimer, a highly attractive drug and vaccine target. We demonstrated a pocket-specific antibody, D5, is more potent as an scFv than as a full-length IgG, suggesting the N-trimer steric restriction extends to the pocket. This characterization will facilitate the design of sterically restricted antigens that mimic the steric environment of the N-trimer in the prehairpin intermediate and are capable of inducing potent and broadly neutralizing antibodies that circumvent the N-trimer steric block.

Keywords: gp41 N-trimer; steric block; gp41 pocket; prehairpin intermediate

Supplemental material: see www.proteinscience.org

The first step of the human immunodeficiency virus type 1 (HIV) life cycle, entry into the target cell, is mediated by the viral envelope glycoprotein (Env) located on the surface of the virus. Env is initially produced as a gp160 precursor, which is then cleaved by a host cellular protease into surface (gp120) and transmembrane subunits (gp41), which remain noncovalently associated.

gp120 recognizes the host cell, while gp41 provides the driving force for membrane fusion between the virus and host cell.

A large body of biochemical and structural data has led to a working model of HIV entry (Fig. 1A; reviewed in Eckert and Kim [2001]). To initiate infection, gp120 binds to the host cell, first to the primary HIV receptor (CD4) followed by the coreceptor (CXCR4 or CCR5). These interactions cause significant conformational changes in gp120 that are communicated to gp41 through the gp120/gp41 interface. gp41 adopts a transient conformation termed the “prehairpin intermediate,” which is embedded in both the host cell membrane (via an N-terminal hydrophobic fusion peptide) and the viral
membrane (via the transmembrane domain). Slow resolution of this intermediate (over minutes) into a highly stable trimer-of-hairpins structure brings the N- and C-terminal regions of the gp41 ectodomain into close proximity, providing the driving force for membrane fusion.

The gp41 ectodomain contains two helical heptad repeat regions, one near the N terminus and one near the C terminus (termed N- and C-peptide regions, respectively) (Wild et al. 1994; Lu et al. 1995). In the trimer-of-hairpins structure, the N-peptide region forms a central trimeric coiled coil (N-trimer) which is surrounded by three C-peptide regions that nestle into grooves on the N-trimer (Chan et al. 1997; Tan et al. 1997; Weissenhorn et al. 1997). Targeting the prehairpin intermediate and preventing formation of the trimer-of-hairpins structure inhibits membrane fusion and viral entry. Exogenous peptides derived from the N- and C-peptide regions inhibit formation of the trimer of hairpins in a dominant negative manner (Fig. 1A; Wild et al. 1992, 1993, 1994; Jiang et al. 1993; Lu et al. 1995).

Targeting Env is both an attractive prophylactic and therapeutic strategy since entry inhibitors have the potential to bind the viral surface and prevent the initiation and spread of infection. Specifically, the gp41 N-trimer is a promising target since it is highly conserved across all strains of HIV and presents an extensive binding surface (Chan et al. 1997; Tan et al. 1997; Weissenhorn et al. 1997; Root et al. 2001). Indeed, a potent C-peptide inhibitor, Fuzeron, that targets the N-trimer has been approved by the FDA and is currently used in patients that harbor viruses resistant to other available therapies (Wild et al. 1994; Rimsky et al. 1998). Potent D-amino acid peptides (D-peptides) have also recently been described that target a specific region of the N-trimer called the “pocket” and inhibit HIV entry (Welch et al. 2007).

The same properties that make the N-trimer a promising drug target also make it an attractive vaccine candidate. Extensive efforts have been undertaken to find potent broadly neutralizing antibodies against the N-trimer (Golding et al. 2002; Louis et al. 2003; Weiss 2003; Opalka et al. 2004). These efforts have produced large numbers of antibodies that bind tightly and specifically in vitro to their N-trimer targets, but do not have potent broadly neutralizing activity. The most notable antibody derived from these efforts, D5, binds to N-trimer mimics (e.g., IZN36) with high (sub-nM) affinity but is >1000-fold less potent in vivo (Miller et al. 2005).

Recently, we discovered that HIV employs a steric defense of the prehairpin intermediate N-trimer region that prevents large proteins (e.g., antibodies) from accessing it and likely explains the dearth of broadly neutralizing antibodies that target this region (Hamburger et al. 2005). Briefly, a C-peptide inhibitor, C37, was fused to cargo proteins of increasing sizes at its N terminus via a flexible linker. Increasing cargo size decreased the inhibitory potency of these C37 fusion proteins in viral infectivity assays, yet had no effect on binding to the N-trimer in vitro. Elongation of the flexible linker in between the C-peptide and the cargo proteins allowed the C-peptide to partially circumvent the steric block.
In this study, we explore the geometry and sources of the N-trimer steric block. In our previous study, with the cargo proteins fused to the N terminus of the C-peptide, the steric bulk faced the viral side of the prehairpin intermediate (Fig. 1B). In this study, we probe the steric environment of the cellular membrane side of the N-trimer by constructing an additional series of C-peptide fusion proteins in which the cargo is attached to the C terminus of the C-peptide. Our results demonstrate these cell-side bulky inhibitors are less sterically restricted than the original virus-side inhibitor panel. In addition, we explore the accessibility of the pocket, a binding hot spot on the N-trimer, by studying a pocket-specific N-trimer antibody, D5, in both scFv and IgG forms (Miller et al. 2005). Our data indicate the pocket is also protected by a steric defense. Finally, to further delineate sources of the steric block, we use a modified sCD4-assisted fusion assay that removes possible sources of steric hindrance from the target cell. We find that all of the cargo inhibitors experience similar steric restrictions in this fusion system when compared to the standard viral entry assay. These results indicate that a steric block still exists on sCD4-activated virus in the absence of a host cell.

Through these studies we show that the steric block surrounding the N-trimer is asymmetric, with the majority of the block derived from the viral side of the prehairpin intermediate. We propose the most likely source for the steric block is the Env surface subunit, gp120. By further dissecting the steric restriction of the N-trimer, we hope to be able to rationally design sterically restricted N-trimer antigens that accurately mimic the N-trimer and its environment in the prehairpin intermediate and can be used to elicit potent and broadly neutralizing antibodies that overcome HIV’s steric defenses.

Results

Previously, we discovered a steric block protecting the gp41 N-trimer from large molecules such as antibodies (Hamburger et al. 2005). In that study we fused cargo proteins of increasing size to the N terminus of a highly active C-peptide via a short flexible linker composed of Ser/Gly residues. In this orientation, the steric bulk of the cargo-bearing C-peptide approaches from the viral side of the prehairpin intermediate (Fig. 1B). In order to dissect the geometry of the N-trimer steric block, here we construct a new set of cargo inhibitors in which the cargo protein is attached to the C-terminal side of the C-peptide, and the cargo bulk approaches from the host cell side (Fig. 1B).

The recombinant C-peptide inhibitor, C37, is used as a reference inhibitor. Two cargo proteins used in our previous study were chosen as representative cargoes, the small (8.6 kDa) ubiquitin protein (Ub), which showed little effect on the inhibitory activity of C37 when attached to the N terminus, and the large (41 kDa) maltose-binding protein (MBP), which dramatically decreased the inhibitory activity of C37 when fused to the N terminus. These cargo were attached to the C terminus of C37 via a seven-residue Ser/Gly linker. This short flexible linker is designed to be long enough for the C-peptide to bind to the N-trimer without cargo interference, but short enough for the cargo to prevent access to a sterically restricted binding site.

The N-trimer steric block is asymmetric

The inhibitory potencies of our C37-cargo (“cell-side”) proteins were tested in a standard viral infectivity assay using Envs from both a laboratory-adapted strain (HXB2) and a primary isolate (JRFL) and were compared to the potencies of the corresponding cargo-C37 (“virus-side”) inhibitors (Fig. 2; Table 1). Similar to the effect of the previously studied virus-side cargoes, the cell-side C37-cargo proteins showed decreasing potency with increasing cargo size. The small cell-side cargo (C37-Ub) showed a modest decrease of inhibitory activity compared to C37 (approximately threefold), whereas the large cargo (C37-MBP) showed a more dramatic decrease in activity (~30-fold). Generally, the cell-side cargo inhibitors are more potent than their virus-side counterparts. For example, C37-MBP is approximately fourfold more potent against JRFL than is MBP-C37. In our previous study, extending the length of the flexible linker between the large MBP cargo and C37 partially restored the potency of the cargo inhibitor (Hamburger et al. 2005). Extending the linker of the cell-side C37-MBP had only a modest effect on the inhibitory potency against HXB2 and no effect against JRFL. Taken together, these data indicate that although the N-trimer of the prehairpin intermediate is sterically restricted from both the viral side and the cellular side, the restriction is more pronounced from the viral side.

As expected, all of the inhibitors are less potent against the primary isolate JRFL than the more easily inhibited laboratory-adapted isolate HXB2. However, the magnitude of the steric block, as demonstrated by the ratio of the cargo-inhibitor potency compared to the control C37, are virtually identical between HXB2 and JRFL, suggesting the steric block is a general trait exhibited by both laboratory-adapted and primary isolate Envs. It is interesting to note that although the absolute values of the IC50s of the virus-side inhibitors determined in this study differ slightly from those of our previous study (likely due to a small change in our infectivity assay protocol), the ratios compared to C37 are in good agreement.

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The N-trimer pocket is also protected by a steric defense

The pocket located near the C terminus of the N-trimer (Fig. 1B) is the target of potent D-peptide inhibitors of HIV entry as well as the neutralizing antibody, D5 (Miller et al. 2005; Welch et al. 2007). Our C-peptide inhibitor binds to the pocket as well as additional regions N-terminal to the pocket. To determine if the pocket region is specifically protected by a steric block, we compared the effectiveness of the D5 scFv (25 kDa) to the D5 IgG (150 kDa) in inhibiting HIV entry. The smaller scFv molecule is approximately four times more potent than the larger IgG (IC$_{50}$ of 61 nM vs. 254 nM in the standard viral infectivity assay using HXB2 pseudovirions) (Fig. 3). In contrast, in an in vitro binding assay where equimolar amounts of D5 scFv and IgG compete for binding to a limiting amount of the sterically accessible N-trimer mimic, IZN36, ~5–10-fold more IgG than scFv bound to the target (Supplemental Fig. 1). The disparity between the in vitro binding and anti-HIV potency indicates there is a steric block at the pocket region. Additionally, the smaller scFv likely already suffers from

Table 1. IC$_{50}$ (in nM) of fusion proteins in standard and sCD4-assisted viral infectivity assays

<table>
<thead>
<tr>
<th>Inhibitor type</th>
<th>Protein</th>
<th>Fusion partner molecular mass (kDa)</th>
<th>Standard assay HXB2</th>
<th>IC$_{50}$ ratio standard assay HXB2</th>
<th>Standard assay JRFL</th>
<th>IC$_{50}$ ratio standard assay JRFL</th>
<th>sCD4 assay JRFL</th>
<th>IC$_{50}$ ratio sCD4 assay JRFL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>C37</td>
<td>0</td>
<td>1.3 ± 0.15</td>
<td>1.0</td>
<td>21 ± 1.6</td>
<td>1.0</td>
<td>2.2 ± 0.33</td>
<td>1.0</td>
</tr>
<tr>
<td>Cell side</td>
<td>C37-Ub</td>
<td>8.6</td>
<td>3.4 ± 0.10</td>
<td>2.5</td>
<td>78 ± 14</td>
<td>3.7</td>
<td>12 ± 1.4</td>
<td>5.6*</td>
</tr>
<tr>
<td></td>
<td>C37-MBP</td>
<td>41.0</td>
<td>46 ± 4.5</td>
<td>35*</td>
<td>536 ± 13</td>
<td>26*</td>
<td>64 ± 1.6</td>
<td>29*</td>
</tr>
<tr>
<td></td>
<td>C37-MBP1</td>
<td>41.0</td>
<td>30 ± 0.58</td>
<td>22</td>
<td>515 ± 58</td>
<td>25*</td>
<td>83 ± 10</td>
<td>38</td>
</tr>
<tr>
<td>Virus side</td>
<td>Ub-C37</td>
<td>8.6</td>
<td>4.5 ± 0.85</td>
<td>3.3</td>
<td>39 ± 0.5</td>
<td>1.9</td>
<td>5.5 ± 0.53</td>
<td>2.5*</td>
</tr>
<tr>
<td></td>
<td>MBP-C37</td>
<td>41.0</td>
<td>117 ± 7.5</td>
<td>87*</td>
<td>2250 ± 122</td>
<td>108*</td>
<td>190 ± 5.8</td>
<td>88*</td>
</tr>
<tr>
<td></td>
<td>MBP1-C37</td>
<td>41.0</td>
<td>39 ± 10</td>
<td>29*</td>
<td>1030 ± 18</td>
<td>49*</td>
<td>82 ± 5.8</td>
<td>38</td>
</tr>
</tbody>
</table>

IC$_{50}$ ratios are relative to C37.
*Indicates significant difference between cell-side and virus-side IC$_{50}$ values (P < 0.05).
steric interference. In accordance with this concept, we observed a significant decrease in potency with our virus-side cargo inhibitor, GFP-C37, which is similar in size to the scFv, and surface plasmon resonance (SPR) of D5 IgG showed a sub-nM affinity for binding to an N-trimer mimic (Hamburger et al. 2005; Miller et al. 2005). Since the pocket is on the end of the N-trimer closest to the viral membrane, its steric block is consistent with the more pronounced restriction observed from the viral side of the prehairpin intermediate.

A viral component is the likely source of the steric block

To further define the steric block geometry, we tested our C-peptide cargo inhibitors in a modified fusion assay that removes potential sources of a steric block derived from the host cell. In this modified, sCD4-assisted fusion assay, CD4 is provided in trans prior to exposure to the host cell. Incubation of virus with sCD4 in the absence of target cell or coreceptor has been previously shown to expose the N-trimer of gp41 in both CXCR4- and CCR5-tropic HIV strains (Furuta et al. 1998; Melikyan et al. 2000; Reeves et al. 2002; He et al. 2003; Si et al. 2004; Henderson and Hope 2006). Therefore, in the sCD4-assisted fusion assay, inhibitors are able to bind to the N-trimer prior to virus binding to the host cell, thereby avoiding any steric clashes derived from host cell components, such as the cell membrane or coreceptor. All of the C-peptide inhibitors, including the C37 control, showed improved potency in the sCD4-assisted assay compared to the standard assay (≈6–12-fold), suggesting a longer kinetic window of N-trimer exposure (Table 1). The potency increase correlates positively with the length of sCD4 preincubation and reaches a maximum at 2–3 h (data not shown). This result confirms the exposure of the N-trimer on the virus upon sCD4 binding.

As in the standard infectivity assay, inhibitor potency negatively correlates with cargo size (for both virus-side and cell-side presentation) (Fig. 4; Table 1), indicating N-trimer steric restriction is still present on sCD4-activated virus. As in the standard assay, the steric block is weaker on the cell side, as C37-MBP is approximately threefold more potent than MBP-C37. Finally, the IC_{50} ratios of all of the cargo inhibitors relative to C37 in the sCD4-assisted assay are extremely similar to those in the standard assay (Table 1; cf. Fig. 2C to Fig. 4C). Taken together, these data show that the N-trimer steric block on sCD4-activated virus has a comparable geometry and magnitude to that on the virus activated by interaction with the host cell and implicate viral components as the major source of the steric block.

C-peptide in the cell-side cargo inhibitors remains accessible for N-trimer binding

To determine if cargo proteins linked to the C terminus of C37 interfered with C-peptide binding to the N-trimer, we assayed binding of these inhibitors to the N-trimer mimic, IZN36, using SPR. In these assays, biotinylated IZN36 (b-IZN36) was attached to a streptavidin surface, and the cell-side cargo inhibitors and control C37 were flowed over the IZN36 and control surfaces. Similar to the previous findings with the virus-side cargo inhibitors, the affinity of the cell-side cargo inhibitors showed modest deviations from that of C37 (Fig. 5). Compared to C37, the relative K_{D}s of C37-Ub, C37-MBP, and C37-MBP1 are 1.03, 2.4, and 3.2-fold higher, respectively. These small differences cannot explain the loss of potency for any of the C-peptide cargo inhibitors. If binding affinity was responsible for the loss of potency, we would have expected relative K_{D}s similar to the loss in potency (e.g., approximately threefold for C37-Ub and ~30-fold for C37-MBP and C37-MBP1). These results demonstrate that the difference in potency between C37 and the cell-side cargo inhibitors is primarily due to steric hindrance rather than differences in binding affinity.

Stability of cell-side cargo inhibitors during infectivity assays

To verify that the loss in potency of the cell-side cargo inhibitors was not due to loss of stability of the inhibitors during the course of the infectivity assays, we analyzed the inhibitors for aggregation and degradation (data not shown). The inhibitors were incubated under simulated infectivity assay conditions prior to analysis. To test for aggregation, inhibitors were centrifuged in media. No pellets were observed and no difference was seen in the Western blot signal between the media before and after

![Figure 3.](image-url)
centrifugation. Inhibitor degradation was visualized by Western blot, which allowed us to define a maximum possible degradation of <1%. Therefore, the cell-side cargo inhibitors are stable during the infectivity assays, and any decrease in potency cannot be attributed to either aggregation or degradation.

Discussion

Many anti-HIV vaccine efforts have focused on raising neutralizing antibodies to HIV Env (reviewed in Haynes and Montefiori [2006]). However, HIV has evolved several mechanisms to avoid the host cell immune response, and designing an antigen to elicit potent and broadly neutralizing antibodies has been challenging. In gp120, vulnerable epitopes are shielded by burial in the native structure, glycosylation (“glycan shield”), and/or steric restriction (Kwong et al. 1998; Saphire et al. 2001; Choe et al. 2003; Labrijn et al. 2003; Wei et al. 2003; McCaffrey et al. 2004). The discovery of the gp41 prehairpin intermediate as a conserved structure targeted by potent entry inhibitors raised hopes for discovering potent and broadly neutralizing antibodies capable of targeting the N-trimer. However, despite much effort, such antibodies remain elusive. We proposed that the N-trimer is protected by a strong steric defense, as potent C-peptide inhibitors lose potency with increasing size of attached cargo, and that molecules as large as antibodies have very poor access to this alluring target (Hamburger et al. 2005).

Figure 4. Inhibitory activity of cell-side and virus-side cargo inhibitors in sCD4-assisted infectivity assay. Representative inhibition curves for cell-side (A) and virus-side (B) cargo inhibitors against JRFL. (C) Calculated IC_{50} ratios for cell-side and virus-side cargo inhibitors relative to the control, C37. Data represent averages of quadruplicate measurements from at least two independent assays. Error bars represent the standard error of the mean (SEM).

Figure 5. Binding of cell-side cargo inhibitors to IZN36 measured by SPR. Binding responses (dots) were normalized to maximal response to facilitate comparison. Fits to the interaction model are shown as solid lines. Proteins were injected at four concentrations in a threefold dilution series starting at 11.1 nM (highest concentration data shown).
In this study we dissect the geometry of this N-trimer steric defense and find that the N-trimer is sterically protected from inhibitors approaching from both the cell side and virus side. The block from the cell side is less severe, however, as the increasing protein size on the cell-side cargo inhibitors did not decrease potency as severely as for virus-side inhibitors. In contrast to the virus-side steric block, the cell-side steric block is not relieved by extension of the flexible cargo linker. Interestingly, the cell-side block and the extended linker virus-side block are quite similar, implying a fundamental barrier to accessing the N-trimer that cannot be relieved by linker extension. Using a sCD4-assisted assay, we removed potential host cell factors that could contribute to a steric block. Both the virus-side and cell-side cargo inhibitors exhibited the same magnitude of potency decrease in the modified assay as they do in the traditional assay, indicating that the major source of the steric restriction is derived from viral factors.

We recently reported that the gp41 C-peptide and the neighboring disulfide loop (DSL) region constitute a major portion of the gp120-gp41 binding interface and proposed that gp120 sequesters the C-peptide region in the prehairpin intermediate, preventing it from forming the trimer-of-hairpins structure (Kim et al. 2008). The gp41 DSL and C-peptide are adjacent to the gp41 N-trimer, suggesting that gp120 is ideally positioned to sterically protect the N-trimer and is likely the major source of the N-trimer steric block.

Here, we also demonstrate that the pocket region of the N-trimer, a validated target for potent D-peptide inhibitors of HIV entry, is protected by the steric block. This region is adjacent to the DSL region of gp41, and is also likely blocked by gp120. This result suggests that efforts to "bulk up" pocket inhibitors to improve their pharmacokinetic properties will likely encounter reduced potency with increasing size.

The ultimate goal of dissecting the steric environment of the N-trimer is to apply this knowledge to the design of an N-trimer antigen that faithfully mimics the steric environment of the N-trimer in the prehairpin intermediate. Current N-trimer mimics are sterically open, and induce high affinity antibodies that bind to the N-trimer in vitro, but not in vivo. An N-trimer mimic with an appropriately engineered steric block could be used to elicit the much more rare antibodies capable of overcoming the steric restriction (e.g., antibodies with extended CDR H3 loops, as have been reported for binding to other sterically restricted targets). The discovery of gp120 as the likely source of the N-trimer steric block will greatly aid the design of N-trimer steric mimics. For example, previous studies showing that the DSL region of gp41 can be linked to gp120 via an engineered disulfide bond (Binley et al. 2000) suggest a strategy to attach gp120 to an N-trimer mimic to recreate the natural N-trimer steric environment. This design effort will also be greatly aided by the cell-side and virus-side bulky reagents, as well as the D5 antibody, employed in this study, which can be used to validate designs of sterically restricted N-trimer mimics (e.g., a designed steric block that freely admits D5 IgG binding is too lax, while blockage of C37 binding would indicate an overly restrictive block).

Materials and Methods

Reagents

Plasmids and cells were obtained from the indicated sources: pET20b vector (Novagen), pET9a-CD4<sub>1212</sub> (gift from R. Varadarajan [Varadarajan et al. 2005]), pEBB-HX2 and pEBB-JRFL (gifts from B. Chen [Chen et al. 1994]), pCANG-TAB-D5 (gift from Merck Research Labs) (Miller et al. 2005), BLR(DE3)pLysS Escherichia coli (Novagen), BL21(DE3) and BL21-Gold (DE3) E. coli (Stratagene), and Top10 E. coli (Invitrogen). The following plasmid and cells were obtained from the National Institutes of Health AIDS Research and Reference Program: pNL4-3.Luc.R-E- (N. Landau) (Connor et al. 1995; He et al. 1995), HOS-CD4-fusin/CCR5 cells (N. Landau) (Landau and Littman 1992; Deng et al. 1996), and CI2Th-synCCR5 cells (T. Mirzabekov and J. Sodroski) (Mirzabekov et al. 1999). Purified D5 IgG was obtained from Merck Research Labs (Miller et al. 2005). The mammalian cells were propagated in standard tissue culture medium, Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen). Amylose resin (New England Biolabs), HisTrap HP (Amersham Biosciences), and reverse phase HPLC with a C18 column (Vydac) were used for protein purifications. T7-Tag antibody agarose (Novagen) was used for inhibitor degradation studies.

Protein expression, purification, and characterization of inhibitors

C37 (with a C-terminal His<sub>6</sub> tag) and cargo-C37 proteins were produced as described (Hamburger et al. 2005). For C37-cargo proteins, all proteins were cloned into the NdeI/XhoI sites of the pET20b vector and expressed in either BLR(DE3)pLysS or BL21-Gold (DE3) E. coli. The cargo proteins used in this study were human ubiquitin (Ub) and E. coli maltose-binding protein (MBP; New England Biolabs). They were cloned onto the C-terminus of C37 with the following seven-residue linker sequence in between: Ser<sub>4</sub>Gly<sub>2</sub>Ser<sub>6</sub>Gly<sub>2</sub>Alep. C37-MBP1 had a 20-residue linker sequence of Ser<sub>4</sub>Gly<sub>2</sub>Ser<sub>6</sub>Gly<sub>2</sub>Ser<sub>6</sub>Gly<sub>2</sub>. All proteins contained an N-terminal T7 tag and a C-terminal His tag, His<sub>6</sub>, and were purified with two steps. MBP-containing proteins were first purified with amylose resin, confirming proper MBP folding, followed by Ni affinity chromatography. C37-Ub was first purified by Ni affinity chromatography followed by reverse phase HPLC. Purified proteins were lyophilized for long-term storage. C37-MBP and C37-MBP1 were shown to be >90% pure via SDS-PAGE and Simplyblue (Invitrogen) staining (with the major contaminant identified via mass spectrometry as a 20-amino acid N-terminal truncation of...
C37, which was present during protein expression), and C37-Ub was shown to be >98% pure. Protein masses were confirmed by electrospray mass spectrometry (University of Utah Core Facility). Working protein stocks were prepared by dissolving lyophilized protein in phosphate-buffered saline (PBS) and centrifuging at 18,000g for 10 min to remove any aggregates. For the majority of the experiments, the PBS used to make the protein stocks consisted of: 10 mM Na2HPO4, 2 mM KH2PO4, 2.7 mM KCl, and 137 mM NaCl, pH 7.4. The scCD4-assisted assay was more sensitive to PBS content, however, so protein stocks were prepared with a less buffered PBS: 1 mM KH2PO4, 3 mM Na2HPO4, and 155 mM NaCl, pH 7.4 (Invitrogen). Protein stocks were aliquoted and flash frozen. For each experiment, a fresh aliquot was thawed and centrifuged to remove aggregates, and the protein concentration was determined via absorption at 280 nm (Edelhoch 1967).

D5 scFv was expressed in Top10 *E. coli* and purified from an overnight culture supernatant. The protein was precipitated with ammonium sulfate and then purified using Ni affinity chromatography. The purified scFv was confirmed by size on SDS-PAGE, anti-His reactivity in a Western blot and specific binding to b-IZN36 precipitated by magnetic streptavidin beads. Due to extremely low expression, the purified scFv was contaminated with three additional proteins, none of which specifically bound to IZN36. The overall protein concentration was determined by absorbance at 280 nm, and then the percentage of scFv by weight (∼26%) was estimated by quantitating the Coomassie-stained bands on SDS-PAGE using an Odyssey Infrared Imager (LI-COR). The resulting concentration determination was confirmed by comparing a four-member twofold dilution series to that of a standard protein using SDS-PAGE and Krypton Infrared Protein Stain (Pierce). The purity of the D5 IgG provided by Merck Research Labs was shown via SDS-PAGE to be >95%, and the concentration was determined using absorbance at 280 nm.

scCD4 (CD412,3) was expressed and purified as previously described (Varadarajan et al. 2005). Briefly, using pET9a-CD412,3, scCD4 was expressed in BL21(DE3) *E. coli* and purified from inclusion bodies using Ni affinity chromatography. The protein was shown to be > 90% pure by SDS-PAGE and SimplyBlue staining.

**Control inhibitor**

To verify that the T7 tag on the N terminus of C37 had no effect on the antiviral inhibitory activity, a control C37 with an N-terminal T7 tag was made and compared to our standard C37 control peptide in the luciferase-based viral infectivity assay. No difference in inhibitory activity was seen (data not shown), so we continued to use C37 as the control inhibitor.

**Viral infectivity assays**

The standard luciferase-based viral infectivity assay was performed as described (Root et al. 2001), with minor modifications. Briefly, pseudotyped virions harboring a luciferase reporter gene were produced by cotransfecting the luciferase-containing genome, pNL4-3.Luc.R-E-, and an envelope plasmid, either pEBB-HXB2 or pEBB-JRFL, into 293T cells and collecting the viral supernatants 30 and 36 h later. The supernatants were filtered with a 0.45-μm membrane. To measure infectivity, the pseudovirions, either HXB2 or JRFL, were added to HOS–CD4–fusin or HOS–CD4–CCR5 cells, respectively, in the presence of a concentration series of each inhibitor and 8 μg/mL DEAE-dextran (to enhance fusion). Virus and inhibitor were removed 20–24 h after infection and replaced with standard media, and ∼24 h later the cells were lysed and measured for luciferase activity (Bright-Glo luciferase assay system, Promega). To calculate IC50 values, the data for each inhibitor concentration series was fit to a Langmuir equation $y = k/(1+[\text{inhibitor}]/IC_{50})$, where $y$ is the normalized luciferase activity and $k$ is a scaling constant (Kaleidagraph, Synergy Software). For the C-peptide cargo inhibitor data set, $k$ was set to 1, to represent the luciferase activity in the absence of inhibitor. For the D5 data set, $k$ was allowed to float because a slight overshoot was seen in the low concentration points (as has been previously described) (Hamburger et al. 2005). The curve fit was weighted by the standard error of each concentration point (with the minimum standard error set to 1%). Significance of cell-side vs. virus-side differences in Table 1 IC50 values was determined using unpaired Student t-test (Kaleidagraph).

The scCD4-assisted fusion assay was performed in a similar manner to the standard assay with a few modifications. JRFL pseudovirions and inhibitors were preincubated with 200 nM scCD4 for 2 h at 37°C before addition to C2THy-synCCR5 cells. All assays included 4 μg/mL DEAE-dextran to enhance fusion and 0.5% PBS (Gibco). To promote viral attachment, cells in 96-well plates were centrifuged at 2600 rpm at 37°C for 30 min using a Sorvall RT6000c centrifuge. The IC50 calculations were fit using the same Langmuir equation as above, with $k$ allowed to float to account for overshoot at the low inhibitor concentration points. The overshoot varied between different inhibitors and also correlated to media composition (e.g., PBS amplified the overshoot). HXB2 was not studied using scCD4-assisted fusion due to loss of infectivity under these conditions.

**Surface plasmon resonance analysis**

To compare the $K_{D58}$ of C37-cargo proteins and C37, SPR binding experiments were performed as previously described (Hamburger et al. 2005) on a Biacore 2000 optical biosensor at the University of Utah Protein Interaction Core Facility. Briefly, b-IZN36 was immobilized on a streptavidin-coated CM5 sensor chip (Biacore). Samples were injected at four concentrations (threefold dilution series starting at 11.1 nM) in duplicate. To facilitate comparisons, data were normalized to the highest point in the response curve. Binding data from all concentration points were analyzed using a 1:1 binding model in Scrubber2 (BioLogic software). Only highest concentration data is shown in Figure 5.

**Inhibitor degradation and aggregation assays**

To check for aggregation of the C37 fusion inhibitors, the inhibitors were incubated at 37°C overnight in a 96-well plate with HOS–CD4–fusin cells in standard tissue culture medium to simulate the conditions of the antiviral assay. The medium was then analyzed both before and after centrifugation (18,000g, 10 min) via Western blot with a polyclonal rabbit anti-His tag antibody (Abcam) and an Alexa Fluor anti-rabbit secondary antibody (Molecular Probes) imaged with an Odyssey Infrared Imager. To check for degradation of the C37 fusion inhibitors and the release of free C37, the inhibitors were again incubated in a 96-well plate as described above. Afterward, the inhibitors were purified with T7-Tag antibody agarose and analyzed via SDS-PAGE and Krypton staining imaged with the Odyssey.
imager. T7-tagged C37 was used as a positive control for purification and Krypton staining.

Electronic supplemental material

Details of the in vitro D5 binding assay are provided, including materials and methods and Supplemental Figure 1.

Acknowledgments

We thank Joseph Joyce (Merck) for the gift of D5 IgG and scFv expression plasmids, purified IgG, and for critical review of the manuscript; Raghavan Varadarajan for the CD4 expression plasmid; Ben Chen for Env expression plasmids; David Myszka for biosensor studies; and Shrawan Kumar for assistance with protein purification. This work was supported by NIH Grants GM082545 and AI076168 to M.S.K.

References


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