Functional impact of HIV coreceptor-binding site mutations

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Abstract

The bridging sheet region of the gp120 subunit of the HIV-1 Env protein interacts with the major virus coreceptors, CCR5 and CXCR4. We examined the impact of mutations in and adjacent to the bridging sheet region of an X4 tropic HIV-1 on membrane fusion and entry inhibitor susceptibility. When the V3-loop of this Env was changed so that CCR5 was used, the effects of these same mutations on CCR5 use were assayed as well. We found that coreceptor-binding site mutations had greater effects on CXCR4-mediated fusion and infection than when CCR5 was used as a coreceptor, perhaps related to differences in coreceptor affinity. The mutations also reduced use of the alternative coreceptors CCR3 and CCR8 to varying degrees, indicating that the bridging sheet region is important for the efficient utilization of both major and minor HIV coreceptors. As seen before with a primary R5 virus strain, bridging sheet mutations increased susceptibility to the CCR5 inhibitor TAK-779, which correlated with CCR5 binding efficiency. Bridging sheet mutations also conferred increased susceptibility to the CXCR4 ligand AMD-3100 in the context of the X4 tropic Env. However, these mutations had little effect on the rate of membrane fusion and little effect on susceptibility to enfuvirtide, a membrane fusion inhibitor whose activity is dependent in part on the rate of Env-mediated membrane fusion. Thus, mutations that reduce coreceptor binding and enhance susceptibility to coreceptor inhibitors can affect fusion and enfuvirtide susceptibility in an Env context-dependent manner.

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Introduction

The envelope protein (Env) of HIV mediates entry into target cells. Env is composed of a surface subunit, gp120, and a transmembrane subunit, gp41, which assemble as trimers on the surface of virions (Center et al., 2002). HIV enters cells following sequential interactions with the cell surface receptor CD4 and a coreceptor molecule, usually CCR5 or CXCR4 (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996; Trkola et al., 1996; Wu et al., 1996). The coreceptor-binding site in gp120, together with the third variable loop (V3), mediate coreceptor binding (Choe et al., 1996; Cocchi et al., 1996; Kwong et al., 1998; Trkola et al., 1996; Wu et al., 1996). Coreceptor binding triggers conformational changes in gp41, likely involving insertion of the hydrophobic fusion peptide into the target cell membrane, then reorganization of gp41 to bring about fusion between the cell and viral membranes (Chan et al., 1997; Weissenhorn et al., 1996).

The coreceptor-binding site in gp120 is centered around an anti-parallel β-sheet structure, termed the ‘bridging sheet domain’, that is formed from conserved, discontinuous regions of gp120 (Kwong et al., 1998; Rizzuto and Sodroski, 2000; Rizzuto et al., 1998; Speck et al., 1997; Wu et al., 1996). Coreceptor binding triggers conformational changes in gp41, likely involving insertion of the hydrophobic fusion peptide into the target cell membrane, then reorganization of gp41 to bring about fusion between the cell and viral membranes (Chan et al., 1997; Weissenhorn et al., 1996).

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domain can reduce the efficiency of coreceptor binding (Reeves et al., 2002, 2004; Rizzuto and Sodroski, 2000; Rizzuto et al., 1998; Suphaphiphat et al., 2003) as can changes in V3 (Reeves et al., 2002; Suphaphiphat et al., 2003). In addition, the sequence of V3 is the major determinant of coreceptor specificity (Choe et al., 1996; Cocchi et al., 1996; Hoffman et al., 2002; Hwang et al., 1991; Speck et al., 1997; Wu et al., 1996). For example, replacement of the V3 loop of a CXCR4 utilizing Env (X4 tropic) with that from a CCR5 tropic virus (R5) can confer R5 tropism (Hwang et al., 1991).

Of the few Env s that have been examined carefully, R5 Env s interact with CCR5 with higher affinity (4–15 nM) (Doranz et al., 1998; Wu et al., 1996) than X4 tropic Env s interact with CXCR4 (200–500 nM) (Babcock et al., 2001; Hoffman et al., 2000), with the important caveat being that these measurements have been done with monomeric gp120. The interplay between coreceptor specificity and coreceptor-binding efficiency is not well understood. From mutagenesis studies, a whole range of CCR5 affinities are compatible with Env fusion and infection (Reeves et al., 2002, 2004). However, reduced coreceptor affinity can result in reduced fusion and infection efficiency, which has consequences for entry inhibition (Reeves et al., 2002, 2004). For example, reduced CCR5 affinity can increase susceptibility to entry inhibitors that target CCR5 and to the fusion inhibitor enfuvirtide (ENF), as a consequence of reduced rates of fusion (Reeves et al., 2002, 2004). ENF is a peptide based on the HR2 region in gp41 and competes with HR2 for HR1 binding to prevent fusion (Greenberg et al., 2004). HR1 is only exposed following receptor binding (Furuta et al., 1998; Gallo et al., 2001; He et al., 2003; Melikyan et al., 2000); thus, there is a window of opportunity between receptor binding and six-helix bundle formation during which ENF can act. Therefore, mutations that reduce coreceptor affinity and that delay fusion kinetics can result in enhanced ENF susceptibility (Reeves et al., 2002, 2004).

In this study, we compared the impact of mutations in and adjacent to the bridging sheet domain on CCR5- and CXCR4-dependent binding, fusion and entry inhibitor susceptibility, as well as the consequence of these mutations on utilization of two alternative HIV coreceptors, CCR3 and CCR8. We found that CXCR4-mediated fusion was more sensitive to coreceptor-binding site mutations, resulting in a greater reduction in fusion efficiency compared to equivalent mutations in a CCR5 tropic Env. Coreceptor-binding site mutations also impacted CCR3 and CCR8 use to varying degrees. Additionally, mutations increased susceptibility to the CCR5 and CXCR4 inhibitors, TAK-779 and AMD-3100, but had minimal impact on ENF susceptibility, consistent with minimal impact on fusion kinetics.

Results

Mutagenesis of the coreceptor-binding site

Crystallization of a deglycosylated, loop-deleted gp120 protein revealed the presence of two highly conserved regions: the recessed CD4 binding site and a coreceptor-binding domain, composed of regions encompassing and adjacent to the bridging sheet domain (Kwong et al., 1998; Rizzuto and Sodroski, 2000; Rizzuto et al., 1998). The effects of mutations in the coreceptor-binding site on Env function have only been examined in a few Env proteins (Reeves et al., 2002, 2004; Suphaphiphat et al., 2003). To investigate the role that this region plays in coreceptor-binding and Env-mediated membrane fusion with both major and alternative coreceptors, we introduced single or double point mutations in the bridging sheet and adjoining regions of the X4 Env HXB and the R5 Env HXB V3BaL. These Env s are identical save for their V3 loops. Some mutations were selected based on previous work which showed that mutations in this region can modulate the affinity of gp120 binding to CCR5 (T123D, K207D, R419D, K421D, P437A, P438A, G441V, and Q442L) (Reeves et al., 2002, 2004; Rizzuto and Sodroski, 2000; Rizzuto et al., 1998), while others were selected based on their location within this domain and their highly conserved nature (T202D, F423D/I, I423D, I439D, S440D) (Fig. 1). Mutations were placed in the full-length Env s as well as in gp120 expression constructs, so that their effects on fusion and coreceptor binding could be measured.

Effect of coreceptor-binding site mutations on receptor binding

Changes in the coreceptor-binding site could potentially affect not only coreceptor binding, but CD4 binding as well. To investigate this, we produced gp120 HXB V3BaL proteins and examined their ability to interact with CD4 using a cell surface binding assay. Human 293T cells were infected with a vaccinia virus encoding T7 polymerase and transfected with gp120-
encoding plasmids under the control of a T7 promoter and the media collected 24 h post-transfection. The amount of gp120 in each sample was measured by an ELISA, and the samples were diluted as needed such that each contained equivalent amounts of gp120 as judged by ELISA and by Western blot (data not shown). To define the linear range of the binding assay, serial dilutions of wild-type HXB V3BaL gp120 were incubated on NP2 or NP2/CD4 cells for 2 h at room temperature, after which the cells were washed, and bound gp120 was detected by immunostaining and flow cytometry. Once the linear range of the assay was defined, the entire panel of gp120 mutants was then tested for the ability to bind to CD4 expressed on the surface of NP2 cells. The coreceptor-binding site mutations had no significant effect on the efficiency of CD4 binding (Fig. 2A). Likewise, when the panel of HXB gp120 proteins was tested, no significant differences in CD4 binding efficiency were observed (data not shown).

A similar equilibrium binding assay was used to measure gp120 binding to CCR5. gp120 preparations were incubated with or without soluble CD4 (sCD4) to trigger the conformational changes needed for coreceptor binding. gp120-sCD4 complexes were then added to T-REx/CCR5 cells, induced to express high levels of CCR5, and bound gp120 was detected by immunostaining and flow cytometry (Fig. 2B). We found that HXB V3BaL containing either the P437A or Q442L mutations exhibited sCD4-induced CCR5 binding similar to WT, while T123D, T202D, and S440D showed low levels of CCR5 binding. The other gp120 mutants exhibited little or no detectable binding to CCR5 under these conditions. Interestingly, Q442L exhibited a slight degree of sCD4-independent binding to CCR5, while parental HXB V3BaL and all of the other mutants required sCD4 for binding to CCR5. Our results are in good agreement with the results of Rizzuto and Sodroski (2000) and Rizzuto et al. (1998) who examined receptor binding of coreceptor-binding site mutations in the context of YU-2 gp120 core proteins.

Fig. 2. Binding efficiencies of HXB V3BaL mutants to CD4 and CCR5. (A) Equivalent amounts of the indicated gp120 proteins were incubated with either NP2 cells (open bars) or NP2/CD4 cells (closed bars), after which the cells were washed and the amount of bound gp120 detected by immunostaining and flow cytometry analysis. The amount of HXB V3BaL gp120 used for the binding assay was empirically determined so as to fall within the linear range of the assay, ensuring that either increased or decreased binding by the mutants could be detected. The amount of HXB V3BaL bound was set to 100%. (B) Equivalent amounts of the indicated gp120 proteins were incubated with T-REx/CCR5 cells in the absence (open bars) or presence (closed bars) of sCD4. After washing, bound gp120 was measured by immunostaining and flow cytometry analysis. The results in panels A and B are the average + SD of at least three independent experiments with at least two independent gp120 preparations.
that lacked 82 residues from the N-terminus and the V1/V2 loops and with a subset of mutations that we examined in the context of intact YU-2 gp120s (Reeves et al., 2002, 2004). Thus, mutations placed in the highly conserved coreceptor-binding site can impair the ability of Env to bind to CCR5 while not affecting CD4 interactions. Unfortunately, the HXB gp120 proteins bound to CXCR4 so weakly that we were unable to quantitate gp120 binding in this equilibrium-binding assay. This is consistent with other studies that employed more sensitive binding assays and that showed that T-cell line adapted HIV-1 gp120 binds to CXCR4 with affinities equal to or greater than 200 nM (Babcock et al., 2001; Hoffman et al., 2000). We therefore assessed the CXCR4 affinity of the HXB mutants indirectly by assaying their AMD-3100 susceptibility (see below).

**Effects of coreceptor-binding site mutations on Env-dependent membrane fusion**

Although the effects of mutations in the coreceptor-binding site on interactions between monomeric gp120 and its receptors have been described (Basmaciogullari et al., 2002; Reeves et al., 2002, 2004; Rizzuto and Sodroski, 2000; Rizzuto et al., 1998), the effects of coreceptor-binding site mutations on Env function have only been examined in detail with one Clade B (Reeves et al., 2002, 2004) and one Clade C R5 tropic Env (Suphaphiphat et al., 2003). To analyze the effect of our expanded panel of coreceptor-binding site mutants on Env function, we first compared Env expression and processing. We found that the Env proteins were expressed at similar levels and exhibited similar levels of gp160 processing and gp120 shedding as judged by Western blot and flow cytometry analyses (data not shown). We then determined the effect of coreceptor-binding site mutations on Env function in a quantitative cell–cell fusion assay. As shown in Fig. 3A, all of the HXB V3BaL mutants elicited cell–cell fusion, despite the fact that some exhibited undetectable CCR5 binding. The T123D, I439D, S440D, and G441V mutants yielded the lowest fusion levels at about 60–70% compared to WT. K421D/F423I, F423D, P438A, and Q442L fused with approximately 70–80% efficiency, while T202D, R419D/F423D, and

![Fig. 3.](image-url)
P437A exhibited essentially wild type fusogenicity. Previous studies with a subset of the mutations examined here in the context of YU-2 Env revealed a greater differential impact on fusion efficiency, ranging from WT levels for P437A and P438A, intermediate levels of about 25% for T202G and G441V and markedly reduced levels for K421D and I423S to approximately 6% of WT (Reeves et al., 2004). Thus, the effects of mutations in this region of gp120 on fusion activity via CCR5 can depend on the Env background.

The panel of HXB Env mutants were also examined in a cell–cell fusion assay to examine the effects of coreceptor-binding site mutations on CD4/CXCR4-dependent membrane fusion. In this context, all of the mutations resulted in reduced fusion compared to WT Env, with T123D and Q442L exhibiting the highest level of fusion at approximately 60% of WT (Fig. 3B). Every other mutation showed fusion levels lower than 30% of WT, with K421D displaying the lowest fusion level at about 10% of WT. Taken together, these results indicate that coreceptor-binding site mutations can reduce the efficiency of Env-mediated cell–cell fusion. In the contexts studied here, mutations were more detrimental on CD4/CXCR4-dependent membrane fusion than on CD4/CCR5-dependent membrane fusion, despite markedly reducing the ability of gp120 proteins to interact with CCR5.

Effect of HXB V3BaL mutations on CCR3 and CCR8 mediated fusion

In addition to CCR5 and CXCR4, several other seven transmembrane domain coreceptors can support Env-mediated membrane fusion and virus infection, particularly when expressed at high levels (Berger et al., 1999). The role of the bridging sheet domain and adjacent regions of Env in mediating interactions with these alternative coreceptors is not known. To investigate this, cells expressing HXB V3BaL Envs were incubated with cells expressing CD4 and either CCR3 or CCR8, and the extent of cell–cell fusion was determined. WT HXB V3BaL fused with CD4/CCR3- and CD4/CCR8-positive cells at approximately 35 and 20% efficiency, respectively, compared to CD4/CCR5-positive cells. A range of different activities was observed for mutant Env fusion via these alternative coreceptors (Fig. 4). In general, most mutations decreased the efficiency of CCR3- and CCR8-dependent membrane fusion, with the exception of T202D, P437A and Q442L for CCR3, and T202D and P437A for CCR8-dependent fusion. Of the remaining mutations, some reduced fusion to approximately the same extent as they reduced fusion on CCR5-positive cells, including T123D (approximately 60% of WT Env function), while others reduced fusion to a greater extent, ranging from about 15 to 50% of WT for CCR3 for S440D and P438A and 20 to 65% of WT for CCR8-dependent fusion by R419D/F423I and Q442L. Thus, mutations in the coreceptor-binding site affect the ability of Env to support membrane fusion that is dependent upon either the major or alternative coreceptors. However, the effects of these mutations on CCR3- and CCR8-dependent membrane fusion were more variable than they were upon CCR5- and CXCR4-dependent membrane fusion. Additionally, the impact of individual mutations on fusion via CCR5, CXCR4, CCR3, or CCR8 was variable, suggesting that Envs rely on differential interactions with various coreceptors for fusion.

Inhibition of fusion by a CCR5 antagonist

Several mutations in the coreceptor-binding site reduced the affinity of HXB V3BaL gp120 for CCR5 to the point that binding could not be detected in our equilibrium binding assay. Despite this significant change in coreceptor binding, these mutants mediated cell–cell fusion as efficiently, or nearly as efficiently, as the parental HXB V3BaL. This apparent discrepancy could be explained if the mutations in gp120 affected coreceptor binding to a greater extent in the context of monomeric gp120 than in the context of the native Env trimer.

Fig. 4. Fusion efficiencies of HXB V3BaL mutants via CCR3 and CCR8. Relative fusion levels via CCR3 and CCR8, assessed by a cell–cell fusion luciferase reporter assay using Q16 effector cells expressing the indicated Envs and T7 RNA polymerase, and QT6 target cells expressing CD4, coreceptor and a luciferase reporter gene under the control of the T7 promoter. Relative fusion levels are shown for HXB V3BaL mutants on CD4+ cells (open bars), CD4/CXCR3+ cells (black bars), and CD4/CCR8+ cells (gray bars). Fusion is expressed as a percent of HXB V3BaL fusion on CD4/CCR5+ cells (set to 100%). Results represent the average + SD of at least five independent experiments.
We sought to address this possibility indirectly by testing a subset of the HXB V3BaL mutants for their susceptibility to TAK-779, a small molecule CCR5 inhibitor. We reasoned that Env proteins that bind to CCR5 with reduced affinity would exhibit enhanced susceptibility to TAK-779 as shown previously (Reeves et al., 2002, 2004).

We found that TAK-779 susceptibility (Fig. 5A) largely correlated with CCR5 binding (Fig. 2B). The TAK-779 susceptibility of Q442L was similar to HXB V3BaL, suggesting that Q442L Env binds CCR5 in a WT manner as seen with Q422L gp120. Furthermore, T202D increased TAK-779 susceptibility by about 3-fold, consistent with the ability of this mutant Env to bind to CCR5 at 35% of WT gp120. The gp120 mutants that bound undetectably to CCR5 exhibited increased susceptibility to TAK-779 from approximately 10- to 25-fold. Slight differences in relative orders obtained in the binding assay versus those found in the inhibition assay may be due to the fact that in the binding assay, the monomeric interaction of gp120 to CCR5 is measured, while in the inhibition assay, multimeric interactions of trimers interacting with multiple CCR5 molecules are possible.

Inhibition of fusion by a CXCR4 antagonist

The impaired ability of the HXB coreceptor-binding site mutants to elicit membrane fusion via CXCR4 (Fig. 3B) suggested that they would show increased susceptibility to AMD-3100. This was the case for five of the six point mutations selected for this study. T123D and T202D were most susceptible to AMD-3100 inhibition, exhibiting approximately a 7- to 8-fold reduction in IC_{50} values, while R419D, I423D, and P438A were only reduced by 2.25- to 3.5- fold (Fig. 5B). Q442L, on the other hand, showed a slight increase in IC_{50} over HXB (1.4-fold), which may indicate an enhanced ability to utilize CXCR4. This was not reflected by an increase in fusion efficiency, however, as Q442L elicited fusion to only 60% of HXB values.

Inhibition of fusion by enfuvirtide

Given that the affinity for CCR5 was reduced by many of these mutations, it might be expected that this would increase their susceptibility to enfuvirtide since fusion may now proceed more slowly (Reeves et al., 2002, 2004). However, when enfuvirtide IC_{50}’s were compared across a subset of six R5 and X4 Env mutant viruses, less than a 3-fold range was noted—differences that are not likely to be significant (Table 1). We found this result surprising, since in other Env reductions in coreceptor affinity has been associated with reduced fusion kinetics and enhanced susceptibility to enfuvirtide (Reeves et al., 2002, 2004). However, when we examined the rate of cell fusion elicited by these mutants, we found only minimal effects on fusion kinetics (data not shown), consistent with minimal differences in ENF susceptibility. Differences were not observed when cells expressing lower levels of coreceptor were used. Thus, the effect of reduced coreceptor affinity in the context of this laboratory-adapted Env had relatively little impact on fusion kinetics and ENF susceptibility compared to the effect of equivalent mutations in the context of the R5 tropic

![Fig. 5. Entry inhibitor susceptibility of HXB and HXB V3BaL mutants. IC_{50} values for fusion inhibition by AMD-3100 and TAK-779 were determined in a cell–cell fusion luciferase reporter assay using QT6 effector cells expressing the indicated Envs and T7 RNA polymerase, and QT6 target cells expressing CD4, coreceptor and containing a luciferase reporter gene under the control of the T7 promoter. The IC_{50} value was defined as the concentration of inhibitor needed to reduce fusion activity by 50%. (A) TAK-779 IC_{50} values for HXB V3BaL mutants on CD4/CCR5+ QT6 cells. (B) AMD-3100 IC_{50} values for HXB mutants on CD4/CXCR4+ QT6 cells. Results represent the average IC_{50} value + SEM of at least four independent experiments.](image-url)

Table 1

<table>
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<tr>
<th>Mutation</th>
<th>ENF IC_{50} fold change^a</th>
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ENF inhibition of HXB V3BaL and HXB mutant fusion.

^a ENF susceptibility of mutant Envs expressed as fold change over WT Env ENF IC_{50}. Results are derived from at least 3 independent experiments.
primary YU-2 Env (Reeves et al., 2002, 2004). As detailed in the discussion, we speculate that laboratory-adapted Envs may be triggered more efficiently than at least some primary Envs, with the result being that reduced coreceptor affinity may not significantly impact fusion kinetics.

**Fusion triggering by soluble CD4**

A number of HXB V3BaL mutants mediated cell–cell fusion as efficiently or nearly as efficiently as the wild-type protein despite the fact that we were unable to detect binding of their monomeric gp120 proteins to CCR5 (Figs. 3A and 2B). The most striking of these, the combination mutant R419D/F423I, fused as efficiently as the wt protein without binding detectably to CCR5 in the context of the monomeric gp120-binding assay. The fact that this mutant could elicit fusion via CCR5 demonstrates its ability to bind to CCR5. Thus, in the HXB V3BaL native trimer, the consequences of reduced affinity on fusogenicity can be masked. Alternatively, the coreceptor-binding site mutations may alter the conformation of Env in a way that can compensate for reduced affinity, perhaps by lowering the energy threshold required for Env triggering and ultimately, fusion. To examine the effect that these mutations had on CD4 triggering efficiency, we measured the ability of envelopes to mediate fusion on CD4−/CCR5+ cells in the presence of increasing amounts of sCD4. Fusion levels in this assay would also likely be more reliant upon CCR5 binding affinity. In cell–cell fusion assays using CD4/CCR5+ target cells, cells are “tethered” together through the interaction of CD4 and envelope. This “tethering” potentially conceals the

**Fig. 6. sCD4 induced fusion of HXB and HXB V3BaL mutants.** Relative fusion levels assessed by a cell–cell fusion luciferase reporter assay using QT6 effector cells expressing the indicated Envs and T7 RNA polymerase, and QT6 target cells expressing coreceptor alone and containing a luciferase reporter gene under the control of the T7 promoter. To assess dose-dependent soluble CD4 (sCD4) triggering of Env-mediated fusion, conventional membrane-bound CD4 was replaced with increasing concentrations of sCD4 (white—0 μg/ml, light gray—0.1 μg/ml, dark gray—1 μg/ml, black—5 μg/ml). (A) Fusion of HXB V3BaL on CCR5+ cells in the presence of 5 μg/ml of sCD4 was set to 100%. (B) Fusion of HXB on CXCR4+ cells in the presence of 5 μg/ml of sCD4 was set to 100%. Results represent the average ± SD of at least three independent experiments.
effects of lowered affinity to CCR5 by holding the cells together, while enough coreceptor-binding events occur to allow the cells to fuse. Thus, the sCD4-triggering assay takes away this bridge, placing an emphasis on the envelope–coreceptor interaction, and allows examination of ability of the sCD4-triggered trimer to interact with coreceptor and cause fusion.

sCD4 triggered the HXB V3BaL Q442L mutant to fuse with somewhat better than WT efficiency (Fig. 6A). gp120 binding and TAK-779 susceptibility (Figs. 2B and 5A) would indicate that Q422L interacts with CCR5 with WT efficiency, however, unlike WT Env, this mutant can bind and fuse inefficiently with CCR5+ cells in the absence of CD4 triggering (Fig. 6A). Therefore, Q422L may alter the conformation of Env to allow coreceptor binding in the absence of sCD4 triggering, which may reflect a reduced requirement of sCD4 to trigger fusion compared to WT Env. sCD4 triggered near WT fusion of T202D and P437A mutants (Fig. 6A), consistent with their CCR5 binding and TAK-779 susceptibility profiles (Figs. 2B and 5A). P438A, I439D, and S440D exhibited intermediate levels of fusion following sCD4 triggering, while R419D/F423L, F423D, and G441V triggered inefficiently. T123D and K421D/F423I exhibited minimal or no sCD4-induced fusion, though both utilized CCR5 for fusion in the presence of membrane bound CD4. The lack of sCD4-induced triggering for the T123D mutant was surprising since this Env binds detectably to CCR5 (Fig. 2B). However, this mutant was also more susceptible to TAK-779 inhibition than would have been expected (Fig. 5A), indicating that CCR5 interactions of the trimeric Env may be compromised. Conversely, P438A, I439D and G441V triggered more efficiently than might have been expected (Fig. 6A) in comparison to other mutants, from their undetectable CCR5 binding profiles (Fig. 2B). In the context of YU-2 Env, the P438A and G441V mutants were also found to fuse and/or exhibit sCD4-induced triggering more efficiently than would have been expected from low CCR5 binding (Reeves et al., 2004).

In the context of HXB, sCD4 induced fusion via CXCR4 was reduced for all mutants, consistent with cellular CD4-induced fusion (Figs. 3B and 6B). Again the Q442L mutant triggered more efficiently than the other mutants (Fig. 6B) consistent with reduced AMD3100 susceptibility (Fig. 5B). Additionally, in contrast to relatively efficient cellular CD4-induced fusion of T123D, sCD4 induced fusion was reduced more than might have been expected, which is consistent with enhanced AMD3100 susceptibility compared to the other mutants tested.

Discussion

Binding of coreceptors to HIV-1 gp120 is mediated by the V3 loop and the coreceptor-binding site located in the bridging sheet of gp120, with the conserved β19 strand being of particular importance (Reeves et al., 2002, 2004; Rizzuto and Sodroski, 2000; Rizzuto et al., 1998; Suphaphiphat et al., 2003). The coreceptor-binding site and the adjoining base of the V3 loop are thought to interact with the amino terminal domains of the coreceptors (Hartley et al., 2005; Hung et al., 1999; Rizzuto and Sodroski, 2000; Rizzuto et al., 1998). Sulfated peptides based on the N-terminal domain of CCR5 bind directly to gp120, but only after gp120 binds soluble CD4 (Cormier et al., 2000, 2001; Farzan et al., 2002a, 2000b). Mutagenesis studies of gp120 also support the idea that the amino terminal domain of CCR5 interacts with the β19 strand and the base of the V3 loop (Cormier et al., 2001). Interestingly, a portion of the antigen combining region from a MAb that binds to the coreceptor-binding site can functionally mimic the N-terminus of CCR5 (Xiang et al., 2005). Thus, a CCR5 molecule containing this region in place of the native N-terminal domain functioned as an efficient coreceptor for R5 virus strains.

Less information is available on role of the coreceptor-binding site in binding to CXCR4. However, the functional conservation of the coreceptor-binding site is shown by the fact that, as in this study, simply switching the V3 loop between an R5 and an X4 virus can alter coreceptor choice. Thus, the coreceptor-binding site must be able to interact with either CCR5 or CXCR4, with coreceptor choice being dictated by the V3 loop. In addition, alanine substitutions for several residues in the β19 strand have been shown to reduce gp120 binding and CXCR4-dependent membrane fusion for both a clade C and a clade B Env (Rizzuto and Sodroski, 2000; Rizzuto et al., 1998; Suphaphiphat et al., 2003).

Our mutagenesis studies provide further support that the bridging sheet, and the β19 strand, are important not only for efficient CCR5 and CXCR4 utilization, but for CCR3 and CCR8 as well. In general, the mutations that we introduced universally suppressed fusion activity that was dependent upon CXCR4, CCR3, or CCR8. The mutations typically had more modest effects on CCR5-dependent cell–cell fusion. We suspect that this is due to the fact that HXB-V3BaL binds to CCR5 with higher affinity than HXB binds to CXCR4. Thus, for functional effects to become significant, HXB-V3BaL may have to suffer a greater loss in binding affinity than does HXB, which already binds to its coreceptor weakly. The coreceptor-binding site mutations in HXB-V3 BaL did increase the susceptibility of the resulting Env to inhibition by a small molecule CCR5 antagonist, and they did reduce binding affinity as judged by a cell-surface gp120-binding assay. We did not identify mutations that had a profound effect on utilization of one coreceptor, with little effect on the use of others. Perhaps the manner in which Env engages the N-termini of various coreceptors is quite similar. Both the CCR5 and CXCR4 N-terminal domains are negatively charged, and both contain one or more sulfated tyrosine residues, though sulfation appears to be more important for efficient utilization of CCR5 than for CXCR4 (Choe et al., 2003; Farzan et al., 2002a). Together, our results and previous studies indicate that the bridging sheet region of gp120 comprises a universal coreceptor-binding site that is important for efficient utilization of both major and minor coreceptors.

The most surprising finding in our study was that mutations in the coreceptor-binding site, while enhancing susceptibility of Env to coreceptor antagonists, had little discernable effect on susceptibility to fusion inhibition by ENF. We have found that some of the same mutations studied here, when introduced into primary R5 Env proteins, reduce coreceptor-binding affinity,
delay fusion kinetics, and enhance susceptibility to ENF (Reeves et al., 2002, 2004). However, coreceptor affinity is but one factor that governs fusion kinetics and ENF susceptibility. For example, a P438A change in the context of the R5 Env YU-2 markedly reduces binding to CCR5 and enhances susceptibility to CCR5 inhibitors but has little effect on fusion extent, fusion kinetics, and ENF susceptibility (Reeves et al., 2004). Thus, we concluded that this change, while reducing binding affinity, increases the efficiency with which this Env is triggered to undergo the fusion-inducing conformational changes that are induced by coreceptor binding. This highlights an area of HIV entry about which relatively little is known—how binding of coreceptor to gp120 induces structural alterations in gp41. Given the variability in Env, it would not be surprising if some Env s are easier to ‘trigger’ than others. Env s that are often referred to as being more ‘ fusogenic’ may well fall into this category, perhaps being triggered to cause fusion by single coreceptor-binding events. In fact, Berger and colleagues found that HXB is particularly easy to trigger by receptor binding, since HXB heterotrimers composed of some subunits that are not competent to bind CD4 and some that have a defective fusion peptide or mixed trimers of mutant HXB and wild-type SF162 can cause membrane fusion nonetheless (Salzwedel and Berger, 2000). Thus, coreceptor binding to one gp120 subunit in these Env trimers was sufficient to induce conformational changes in other subunits. Enhanced triggering may be a general property of laboratory-adapted isolates such as HXB, where in the absence of immune selection continual passage on CXCR4-expressing cell lines might be expected to select for variants that enter cells more quickly. If this speculation is correct, then reductions in coreceptor affinity in laboratory-adapted HIV isolates would be expected to have more modest effects on fusion rates than the same changes introduced into the context of primary virus isolates. Alternatively, it could be that binding CXCR4 triggers Env more efficiently than does binding to CCR5. If this is the case, then some of the changes described here might have differential effects on fusion kinetics when introduced into an R5X4 Env protein, depending on whether CCR5 or CXCR4 is introduced into the target cell. If so, their effects on entry inhibitor susceptibility, particularly that of ENF, might also be variable since the potency of enfuvirtide is linked in part to the rate at which membrane fusion occurs.

Materials and methods

Cells

Cell lines QT6, 293T, NP2 (Yamanaka et al., 1993), NP2/CD4 (Soda et al., 2000), T-REx/CCR5 (Reeves et al., 2002), U87/CD4/CCR5, and U87/CD4/CXCR4 (Bjorndal et al., 1997; Deng et al., 1997) were cultured in Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml of penicillin and 100 μg/ml streptomycin (GIBCO; DMEM/10/PS). CD4 expression in NP2/CD4 cells was maintained by adding 1 μg/ml of G418 (MEDIATECH) to the DMEM/10/PS. T-REx/CCR5 cells required the addition of blasticidin (5 μg/ml, Invitrogen) and zeocin (200 μg/ml, Invitrogen) to retain the tet-repressor and ccr5 genes, respectively. High-level CCR5 expression was induced on T-REx/CCR5 cells by addition of 10 ng/ml doxycycline (Sigma) to the culture media. CD4 expression was maintained in the U87 cell lines with 0.3 mg/ml G418, while 1 μg/ml puromycin (Sigma) maintained CCR5 or CXCR4 expression.

Plasmids

HXB and HXB V3BaL gp160s were cloned into the pSP73 (Promega) expression vectors as previously described (Hoffman et al., 1998, 1999). Amino acid substitutions T123D, T202D, K207D, R419D, K421D, I423D, P438A, I439D, G441V, and Q442L (Fig. 1) were introduced into the HXB backbone sequence and T123D, T202D, R419D/F423I, K421D/F423I, F423D, P437A, P438A, I439D, S440D, G441V, and Q442L (Fig. 1) amino acid substitutions were introduced into HXB V3BaL using the Quikchange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. In order to produce gp120 expression constructs, stop codons were introduced at the gp120-gp41 cleavage junction in Env for each of the mutants by site-directed mutagenesis (Quikchange; Stratagene). Env gene sequences were verified following mutagenesis.

The pGEM T7-luc expression plasmid was obtained from Promega. The expression plasmids for CD4, CCR5, CXCR5, CCR3, CCR8, and pNL-luc- were described previously (Connor et al., 1995; Deng et al., 1996; Hoffman et al., 1998).

Env receptor-binding assays

gp120 proteins were produced using a vaccinia-T7 polymerase-driven expression system (Alexander et al., 1992). Briefly, 293T cells were transfected with gp120 expression constructs by calcium phosphate transfection and cells were infected with a vaccinia virus encoding T7 polymerase (vTF1.1; Alexander et al., 1992) to boost protein expression via the T7 promoter present in these constructs. Culture media containing gp120 protein were harvested approximately 24 h post-transfection. Western blotting was performed to confirm gp120 production, and an ELISA was utilized to quantify gp120 protein concentrations as previously described (Reeves et al., 2002). Briefly, ELISA plates were coated overnight with 10 μg/ml Galanthus nivalis lectin (Vector Laboratories), then blocked with 2% milk powder in Tris-buffered saline (TBS). Serial dilutions of gp120 supernatants were then bound to the plate for 2 h at room temperature alongside a standard set of serial dilutions of purified HXB gp120 protein. Bound gp120 was detected with an Env-specific rabbit sera followed by a horseradish peroxidase conjugated anti-rabbit secondary (Amersham Pharmacia Life Science). Tetramethylbenzidine (TMB) substrate (Kirkegaard and Perry Laboratories) was then added, and the colorimetric signal quantitated in a microplate reader.

The relative binding efficiencies of gp120 proteins to CD4 and CCR5 were assessed as described previously (Reeves and
Equivalent amounts of gp120 proteins were incubated with NP2 neuroglioma cells and NP2-CD4 cells to determine relative CD4 binding or T-REX-CCR5 cells in the presence and absence of scCD4 to determine sCD4-induced CCR5 binding efficiencies. An Env-specific rabbit serum was used to detect bound gp120, followed by a phycocerythrin-conjugated anti-rabbit secondary antibody (Pharmingen). The samples were then fixed in paraformaldehyde and analyzed by flow cytometry. Importantly, the amount of gp120 needed to achieve binding within the linear range of this assay was empirically determined.

Cell–cell fusion assay

This assay has been previously described in detail (Rucker et al., 1997). Briefly, “target” QT6 cells were cotransfected with CD4 and a coreceptor or control expression plasmid as well as a luciferase reporter gene expression plasmid under the control of a T7 promoter (pGEM2 T7-luc; Promega). QT6 “effector” cells were transfected with Env expression plasmids and infected with a recombinant vaccinia virus expressing T7 polymerase (vTF1.1) (Alexander et al., 1992). Effector cells were added to target cells approximately 18 h post-transfection and the cells allowed to interact at 37 °C for a period of 7–10 h. Quantifiable fusion occurs following functional envelope–receptor interactions and subsequent content mixing, allowing T7 polymerase to drive luciferase expression by direct interaction with the T7 promoter (Rucker et al., 1997). Cells were lysed in 1% Triton X-100 in PBS, luciferase substrate added and luciferase activity measured in a luminometer.

To determine the ability of soluble CD4 (scCD4) to trigger cell–cell fusion, variable concentrations of scCD4 (0, 0.1, 1, and 5 μg/ml) were added to mixes of effector and target cells in the absence of membrane-bound CD4. The TAK-779 and inhibitors for 30 min prior to addition of effector cells. Fusion was determined by preincubating target cells with absence of membrane-bound CD4. The TAK-779 and

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