

HIV Envelope Functional Evolution During Coreceptor Switching

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ABSTRACT

Background: HIV-1 coreceptor switching involves envelope mutations that usually result in R5 to R5X4 to X4 transitions. We have examined the functional consequences of envelope mutations in four distinct switching pathways resulting in viruses with coreceptor preference ranging from R5>X4, R5=X4, X4>R5, to pure X4 to determine common and distinct features of each pathway. **Methods:** Site-directed mutagenesis was used to introduce most combinations of mutations in envelope previously shown to generate R5X4 or X4 variants of BaL or ADA isolates. For all combinations of mutations preserving infectivity on either CCR5- or CXCR4-expressing target cells, the sensitivity of the resulting envelope mutant to coreceptor inhibitors, soluble CD4, and the broadly neutralizing antibodies b12-IgG and 4E10 was determined. **Results:** Common features of Env mutations involved in coreceptor switching were decreased CCR5 binding, increased sensitivity to CCR5 inhibitors, and increased resistance to soluble CD4 that was confirmed by direct binding assays. Increased binding to CD4 preceded measurable increases in CXCR4 binding and increased resistance to CXCR4 inhibitors. Unique features of different coreceptor switch intermediates included varying sensitivity to antibody neutralization and the fraction of mutated envelopes with loss of entry function. Infection of CXCR4-expressing target cells was more sensitive to antibody neutralization than infection of CCR5-expressing target cells. **Conclusions:** Increased CD4 binding and decreased CCR5 binding are common features of coreceptor switch intermediates, even when the final virus utilizes CCR5 more efficiently than the parental R5 isolate. Increased CD4 binding may provide enough fitness advantage to allow survival of intermediates with poor binding to both CCR5 and CXCR4. Neutralizing antibody may slow the emergence of R5X4 or X4 variants.

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METHODS

Cell Lines - U87-CD4-CCR5 and U87-CD4-CXCR4 were used as target cells. **Mutagenesis** - ADA or BaL full length Env genes were PCR amplified from pNL4-3-ADA or BaL and single or multiple mutations introduced into the cloned Env by site-directed mutagenesis (QuikChange kit, Stratagene). Each mutant was sequenced. **Entry Assay** - Mutated Env clones inserted into the pSVIII plasmid were cotransfected with NL4-3 Luc+, E-, R- plasmids (Connor *et al.*, Virology 206:935, 1995) into 293T cells. The resulting pseudotyped viruses were used to infect target cells and luciferase activity measured at 48-72 hr. **Inhibitors** - The CCR5 inhibitors PSC-RANTES or TAK-779 were used to block single cycle infection of CCR5 target cells, the CXCR4 inhibitor AMD3100 was used to block infection of CXCR4 target cells, and IgG-b12 (D. Burton), 4E10 (AIDS Reagent Program) or CD4-IgG (Progenics) were used to block infection of both targets. IC₅₀ values are expressed as log reduction of ADA or BaL wt (CCR5 targets) or absolute values (CXCR4 targets). **Receptor binding assays** - gp120 proteins were expressed in 293T cells with a T7 promoter. Purified proteins were used in a cell binding assay using CD4-negative T-Rex/CCR5 cells + soluble CD4 for CCR5 or NP2/CD4 cells for CD4. Binding was detected by indirect immunofluorescence and flow cytometry (Reeves *et al.*, PNAS 99:16249, 2002).

Cell fusion assay - QT6 effector cells, transfected with Env expression plasmids and infected with vTF1.1, were added to QT6 target cells cotransfected with a luciferase reporter construct under the control of a T7 promoter (pGEM2 T7-Luc, Promega), plus CD4 and CCR5 or CXCR4 expression plasmids. Cell-cell fusion, resulting from a functional interaction between the Env-expressing effector cells and receptor-expressing target cells, was detected by assaying for T7 polymerase-driven luciferase expression within the linear range of the assay.

Env fusion kinetics - The fusion kinetics of ADA wild-type and mutant Envs were determined in a β-lactamase reporter cell-cell fusion assay, based on that described by Lineberger *et al.* (J. Virol. 76:3522, 2002). QT6 effector cells, cotransfected with Env and β-lactamase expression constructs and infected with vTF1.1, were added to U87-CD4-CCR5 target cells labeled with CCF2-AM. Cell-cell fusion was detected by assaying for a shift from green to blue fluorescence, indicating β-lactamase cleavage of CCF2.

RESULTS

We have previously reported the selection of coreceptor switch mutant viruses by changing target cells from U87-CD4-CCR5 to U87-CD4-CXCR4 and then MT-2 cells (Pastore, C *et al.*, J. Virol. 2004 Jul;78(14):7565-74.). Most of the mutations associated with coreceptor switching occurred in V1-V2 and V3 regions. The mutations in ADA and BaL needed for use of CXCR4 are shown in Table 1.

Table 1. Summary of Envelope Mutations

ADA-1 mutations	V2	V3
C2	NTSTSTQACPVVSPFEPVPIHCVTQPAQFALK	CTSPNNTRKSHIGPGRALVYTGRIIDDIQABC
	1-----2-----3-----4-----5-----6-----7-----8-----9-----10-----11-----12-----13-----14-----15-----16-----17-----18-----19-----20-----21-----22-----23-----24-----25-----26-----27-----28-----29-----30-----31-----32-----33-----34-----35-----36-----37-----38-----39-----40-----41-----42-----43-----44-----45-----46-----47-----48-----49-----50-----51-----52-----53-----54-----55-----56-----57-----58-----59-----60-----61-----62-----63-----64-----65-----66-----67-----68-----69-----70-----71-----72-----73-----74-----75-----76-----77-----78-----79-----80-----81-----82-----83-----84-----85-----86-----87-----88-----89-----90-----91-----92-----93-----94-----95-----96-----97-----98-----99-----100-----	1-----2-----3-----4-----5-----6-----7-----8-----9-----10-----11-----12-----13-----14-----15-----16-----17-----18-----19-----20-----21-----22-----23-----24-----25-----26-----27-----28-----29-----30-----31-----32-----33-----34-----35-----36-----37-----38-----39-----40-----41-----42-----43-----44-----45-----46-----47-----48-----49-----50-----51-----52-----53-----54-----55-----56-----57-----58-----59-----60-----61-----62-----63-----64-----65-----66-----67-----68-----69-----70-----71-----72-----73-----74-----75-----76-----77-----78-----79-----80-----81-----82-----83-----84-----85-----86-----87-----88-----89-----90-----91-----92-----93-----94-----95-----96-----97-----98-----99-----100-----
	197	301 306 313 321,2
ADA-3 mutations	V2	V3
C2	CSFNTTTRIKRKYKQALVYELVDPVPIHNDNTSYELIIC	CTSPNNTRKSHIGPGRALVYTGRIIDDIQABC
	1-----2-----3-----4-----5-----6-----7-----8-----9-----10-----11-----12-----13-----14-----15-----16-----17-----18-----19-----20-----21-----22-----23-----24-----25-----26-----27-----28-----29-----30-----31-----32-----33-----34-----35-----36-----37-----38-----39-----40-----41-----42-----43-----44-----45-----46-----47-----48-----49-----50-----51-----52-----53-----54-----55-----56-----57-----58-----59-----60-----61-----62-----63-----64-----65-----66-----67-----68-----69-----70-----71-----72-----73-----74-----75-----76-----77-----78-----79-----80-----81-----82-----83-----84-----85-----86-----87-----88-----89-----90-----91-----92-----93-----94-----95-----96-----97-----98-----99-----100-----	1-----2-----3-----4-----5-----6-----7-----8-----9-----10-----11-----12-----13-----14-----15-----16-----17-----18-----19-----20-----21-----22-----23-----24-----25-----26-----27-----28-----29-----30-----31-----32-----33-----34-----35-----36-----37-----38-----39-----40-----41-----42-----43-----44-----45-----46-----47-----48-----49-----50-----51-----52-----53-----54-----55-----56-----57-----58-----59-----60-----61-----62-----63-----64-----65-----66-----67-----68-----69-----70-----71-----72-----73-----74-----75-----76-----77-----78-----79-----80-----81-----82-----83-----84-----85-----86-----87-----88-----89-----90-----91-----92-----93-----94-----95-----96-----97-----98-----99-----100-----
	160	314 322
BaL-1B mutations	V2	V3
C2	NTCLLNATNGNDNTSTSRERMDGGGEMGNCSPKTTINIRKQVQETALFYELD	CTSPNNTRKSHIGPGRALVYTGRIIDDIQABC
	1-----2-----3-----4-----5-----6-----7-----8-----9-----10-----11-----12-----13-----14-----15-----16-----17-----18-----19-----20-----21-----22-----23-----24-----25-----26-----27-----28-----29-----30-----31-----32-----33-----34-----35-----36-----37-----38-----39-----40-----41-----42-----43-----44-----45-----46-----47-----48-----49-----50-----51-----52-----53-----54-----55-----56-----57-----58-----59-----60-----61-----62-----63-----64-----65-----66-----67-----68-----69-----70-----71-----72-----73-----74-----75-----76-----77-----78-----79-----80-----81-----82-----83-----84-----85-----86-----87-----88-----89-----90-----91-----92-----93-----94-----95-----96-----97-----98-----99-----100-----	1-----2-----3-----4-----5-----6-----7-----8-----9-----10-----11-----12-----13-----14-----15-----16-----17-----18-----19-----20-----21-----22-----23-----24-----25-----26-----27-----28-----29-----30-----31-----32-----33-----34-----35-----36-----37-----38-----39-----40-----41-----42-----43-----44-----45-----46-----47-----48-----49-----50-----51-----52-----53-----54-----55-----56-----57-----58-----59-----60-----61-----62-----63-----64-----65-----66-----67-----68-----69-----70-----71-----72-----73-----74-----75-----76-----77-----78-----79-----80-----81-----82-----83-----84-----85-----86-----87-----88-----89-----90-----91-----92-----93-----94-----95-----96-----97-----98-----99-----100-----
	137	316 322
BaL-2A mutations	V2	V3
C2	NTCLLNATNGNDNTSTSRERMDGGGEMGNCSPKTTINIRKQVQETALFYELD	CTSPNNTRKSHIGPGRALVYTGRIIDDIQABC
	1-----2-----3-----4-----5-----6-----7-----8-----9-----10-----11-----12-----13-----14-----15-----16-----17-----18-----19-----20-----21-----22-----23-----24-----25-----26-----27-----28-----29-----30-----31-----32-----33-----34-----35-----36-----37-----38-----39-----40-----41-----42-----43-----44-----45-----46-----47-----48-----49-----50-----51-----52-----53-----54-----55-----56-----57-----58-----59-----60-----61-----62-----63-----64-----65-----66-----67-----68-----69-----70-----71-----72-----73-----74-----75-----76-----77-----78-----79-----80-----81-----82-----83-----84-----85-----86-----87-----88-----89-----90-----91-----92-----93-----94-----95-----96-----97-----98-----99-----100-----	1-----2-----3-----4-----5-----6-----7-----8-----9-----10-----11-----12-----13-----14-----15-----16-----17-----18-----19-----20-----21-----22-----23-----24-----25-----26-----27-----28-----29-----30-----31-----32-----33-----34-----35-----36-----37-----38-----39-----40-----41-----42-----43-----44-----45-----46-----47-----48-----49-----50-----51-----52-----53-----54-----55-----56-----57-----58-----59-----60-----61-----62-----63-----64-----65-----66-----67-----68-----69-----70-----71-----72-----73-----74-----75-----76-----77-----78-----79-----80-----81-----82-----83-----84-----85-----86-----87-----88-----89-----90-----91-----92-----93-----94-----95-----96-----97-----98-----99-----100-----
	130	322
+ K490T in C5		
*numbering according to HxB2 convention		

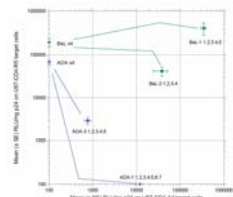


Fig. 1 - Final entry phenotype of ADA and BaL mutants. The 4-7 envelope (Env) mutations result in four unique phenotypes, ranging from X4 (ADA-1) to robust R5X4 (BaL-1).

Each of the mutations leading to ADA-1 were introduced singly or in combination. The changes in entry function and sensitivity to the CCR5 inhibitors PSC-RANTES or TAK-779 are shown below in Fig. 2A, B, & C.

Fig. 2 - ADA-1 mutations impact use of CCR5 and inhibition by CCR5 blocking agents.

Note that almost all Env mutations decreased use of CCR5 and increased sensitivity to both PSC-RANTES and TAK-779. Moreover, many combinations of V3 mutations abolished infection of both CCR5- and CXCR4-target cells. These lethal V3 mutations are compensated for by mutations in C2.

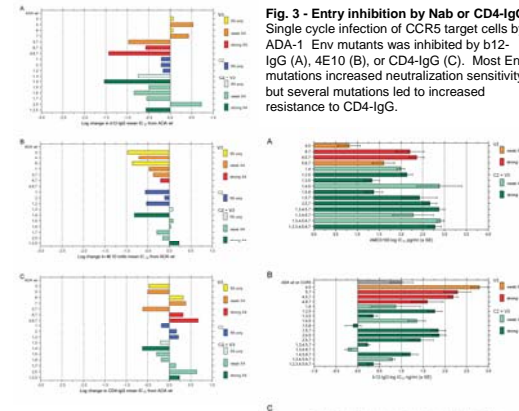
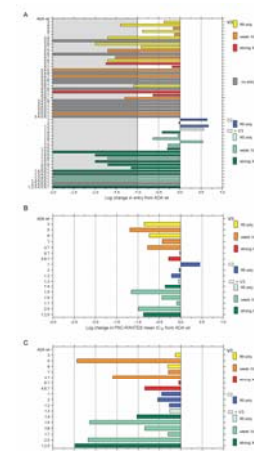


Fig. 4 - Inhibition of CXCR4-mediated infection. Most Env mutations were more sensitive to AMD3100 (A) than the final ADA-1 mutant. Neutralization sensitivity was variable, but tended to increase as CXCR4 use improved (B, C). Env mutants were more sensitive to CD4-IgG inhibition on CXCR4 target cells than CCR5 target cells (D).

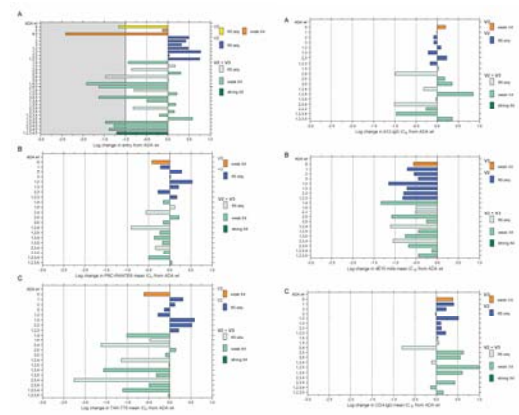


Fig. 5 (left) & 6 (right) - Entry characteristics and inhibitor sensitivity of ADA-3 Env mutants. Legends as in prior figures. No lethal mutations were observed in these 32 mutated Env's. V3 mutations were more deleterious for entry and inhibitor sensitivity, with the exception of CD4-IgG inhibition.

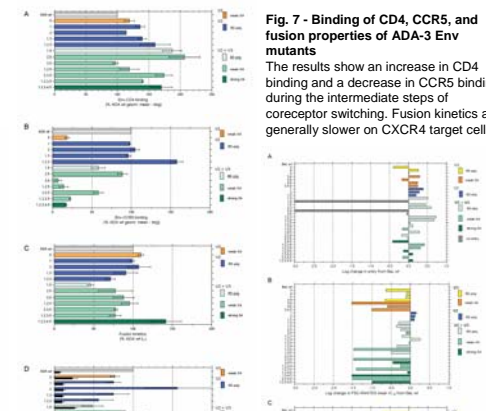


Fig. 7 - Binding of CD4, CCR5, and fusion properties of ADA-3 Env mutants. The results show an increase in CD4 binding and a decrease in CCR5 binding during the intermediate steps of coreceptor switching. Fusion kinetics are generally slower on CXCR4 target cells.

Fig. 8 (upper right) and 9 (lower right); Similar changes in Env function with BaL-1 Env mutations - The five Env mutations leading to the R5X5 BaL-1 also increase sensitivity to CCR5 inhibitors and neutralizing Ab, and also increase resistance to CD4-IgG.

CONCLUSIONS

Common features of coreceptor switch intermediates include:

- Reduction in CCR5 binding and correlated increased sensitivity to CCR5 inhibitors;
- Increased sensitivity to neutralizing antibody, particularly as CXCR4 use improves;
- Increased CD4 binding and correlated increased resistance to CD4-IgG inhibition.

IMPLICATIONS

- Coreceptor switch mutants with quite distinct final phenotypes nonetheless share common features of functional evolution.
- A high frequency of lethal mutations in some pathways reinforces that coreceptor switching is not a simple process, which is one reason it takes so long.
- Improved CD4 binding could provide a necessary survival advantage to coreceptor switch intermediates with poor binding to either CCR5 or CXCR4.
- Although V3 mutations are essential for coreceptor switching, they appear to be high risk mutations that are unlikely to survive in a competitive viral swarm in a patient unless additional compensatory mutations occur in a very short time frame.
- CCR5 antagonists are unlikely to rapidly select for resistance due to de novo coreceptor switching.