Published in final edited form as:

Biochemistry. 2013 May 21; 52(20): 3552–3563. doi:10.1021/bi400201h.

Significant Differences in Cell-Cell Fusion and Viral Entry Between Strains Revealed By Scanning Mutagenesis of The C-Heptad Repeat of HIV GP41

Barbara Diaz-Aguilar¹, Karen DeWispelaere¹, Hyun Ah Yi¹, and Amy Jacobs^{1,*}

¹Department of Microbiology and Immunology, School of Medicine and Biomedical Sciences, State University of New York (SUNY) at Buffalo, Buffalo, NY

Abstract

The transmembrane subunit, gp41, of HIV envelope mediates the viral fusion step of entry into the host cell. The protein consists of an extracellular domain, a transmembrane domain, and a cytoplasmic tail. The extracellular domain contains a fusion peptide, an N-terminal heptad repeat (NHR), a loop region, a C-terminal heptad repeat (CHR), and a membrane-proximal external region (MPER). For this study we examined each amino acid in the CHR (623-659), by alanine scanning mutagenesis in two HIV strains; one CCR5-utilizing (strain JRFL) and one CXCR4-utilizing (strain HXB2). We studied the functional importance of each amino acid residue by measuring mutational effects in both cell-cell fusion and viral entry and assessing envelope expression and gp120/gp41 proteolytic processing. The transmembrane subunit of HIV envelope, gp41, is very sensitive to subtle changes, like alanine substitution, which severely affect envelope function at multiple sites. Two important general findings are apparent when the entire data set from this study is taken into account: 1) strain HXB2 is much more stable to mutagenesis than is strain JRFL and 2) viral entry is much more stable to mutagenesis than is cell-cell fusion. These findings strengthen our notion that gp41 is a vulnerable target for therapeutic and prophylactic intervention. Further structural studies to gain a full understanding of the intermediate states that drive HIV membrane fusion are imperative.

Infection by human immunodeficiency virus type 1 (HIV-1) is initiated when the virus particle makes contact with the receptor cell surface and subsequently HIV envelope gp41 mediates membrane fusion, thereby allowing the viral genetic material to enter the cell. The HIV-1 envelope glycoprotein is synthesized as a precursor polyprotein, gp160, which is proteolytically processed by the cellular protease, furin, to generate two subunits, the surface subunit gp120 (sometimes referred to as SU) and the transmembrane subunit gp41 (sometimes referred to as TM). These subunits are noncovalently associated and form a trimer of heterodimers that is the envelope glycoprotein spike on the viral surface. Initial contact with the host cell is made when gp120 interacts with CD4. Conformational changes allow subsequent binding of gp120 to a chemokine co-receptor (CCR5 or CXCR4) found on the surface of target cells. These events trigger gp41 to undergo conformational changes that

* Address correspondence to: Amy Jacobs, 109 BRB, 3435 Main Street, Buffalo NY, Tel: (716), 829-2087; Fax (716) 829-2111, ajacobs2@buffalo.edu.

are crucial for activation of HIV-1 membrane fusion. HIV-1 fusion likely involves substantial conformational changes in gp41 from a metastable state to an energetically more stable conformation (1-4). The control of these structural rearrangements is thought to be central to HIV-1 entry and an important target for drug development, proven by the therapeutic use of the fusion inhibitor (T20/Fuzeon/Enfuvirtide) (5-7).

Structural studies have demonstrated that the NHR and CHR regions of the gp41 ectodomain associate to form a six-helix bundle in the post-fusion state with three N-terminal helices forming a trimeric coiled-coil in the center, and three C-terminal helices packing on the surface of the coiled-coil in a reverse orientation resembling a hairpin-like structure (8-14). This transition is concomitant with membrane fusion (15, 16). The transmembrane region is positioned in the viral membrane and the fusion peptide is embedded in the cellular membrane after gp120 contact with the cellular receptors. Intermediate states of the gp41 molecule are vital to viral function and the regions involved are highly conserved. Hence, they serve as a target for the development of HIV-1 inhibitors.

There is evidence that packing interactions between NHR and CHR are important determinants of HIV-1 entry (5, 17-19). The receptor-triggered conformational changes of the HIV-1 envelope glycoprotein that result in the formation of the stable gp41 core are suspected to drive the increasing proximity of the membranes and/or the subsequent lipid bilayer fusion (16). Therefore, it is of fundamental importance to understand the structural and mechanistic basis for the interactions in the core.

The CHR is a functionally important region in HIV gp41 that forms a part of the post-fusion 6-helix bundle (6HB) structure of gp41. The 6HB conformation of gp41 is a stable trimer in which the NHR forms an internal 3-helical bundle and the CHR wraps obliquely around the internal bundle to form the 6HB (8-10, 13, 20, 21). In this study, alanine substitutions at 37 positions within the CHR have been characterized in two different HIV-1 strains, one CCR5-utilizing (JRFL) and one CXCR4-utilizing strain (HXB2). We have studied these mutations for effects on membrane fusion, viral entry, envelope expression, furin cleavage, and gp41/gp120 association. We used alanine substitutions because alanine is considered one of the most subtle single amino acid changes that can be made with respect to chemical character and molecular structure but which also does not add backbone flexibility, as would be the case with glycine substitution. We present a systematic mutagenesis approach to study the importance of each amino acid residue of the CHR region to gp41 function in two different HIV-1 strains, one CCR5-utilizing (strain JRFL) and one CXCR4-utilizing (strain HXB2), with the long-term goal of identifying regions that are attractive sites for drug intervention and which are amenable to manipulation for labeling for structural studies.

The mechanisms of cell-cell fusion and viral entry have historically been considered to be substantially similar following the same steps of 1) attachment of gp120 to CD4 on the cell surface, 2) conformational changes in gp120 leading to co-receptor binding, 3) further conformational changes in gp41 mediating membrane fusion at the plasma membrane. Interestingly, however, recent studies have convincingly demonstrated that HIV entry occurs through the endocytic pathway (16, 22). This alteration to the paradigm of HIV viral entry suggests that the mechanistic difference between cell-cell fusion and viral entry might also

greater than originally thought. Membrane fusion is required for both phenomena, however, different membranes are now implicated with the plasma membrane being the site of cell-cell fusion and the endocytic membrane being the membrane that must fuse with the viral membrane for viral entry of the core. It is not yet clear what restricts the virion from fusing with the plasma membrane. It is known, however, that dynamin is required for HIV entry which implicates the clathrin-dependent pathway (16, 22). There is a difference in the amount of membrane curvature between the two mechanisms. The region of membrane fusion between two cells is often considered to have so little curvature that it can be estimated as a plane whereas the curvature of the viral membrane is much greater and more closely matches the curvature of the endosomal membrane than it would match the curvature of the plasma membrane. There is also a possibility that there are different concentrations of host cell proteins in the environment in which membrane fusion is taking place and this could have an effect on the two processes.

Interestingly, we found the effects of mutagenesis on cell-cell fusion versus viral entry to vary substantially in some cases. This would suggest that there are key differences in the mechanisms between these two phenomena. In fact, researchers had found evidence to suggest this in previous studies. For instance Konopka, *et al.*, found that there was a difference in monoclonal antibody binding between cell-cell fusion and virus-cell membrane fusion with murine monoclonal antibody (MAb F-91-55) raised against the complex of CD4 and gp120 (23). In another study, additional monoclonal antibodies were found to be relatively ineffective at inhibiting cell-cell fusion as well (24). Antibodies b12, m14 IgG, and 2G12 had moderate activity whereas 4E10 and 2F5 which bind the membrane proximal region of gp41 had no inhibitory effect (24). Further study to elucidate differences between the mechanisms of cell-cell fusion and viral entry in HIV-1 biology is warranted.

Materials and Methods

Plasmids and Mutagenesis

The plasmids bearing alanine substitutions were prepared from pHXB2 (NIH AIDS Research and Reference Reagent Program Cat. Number 526 (25)) and pJRFL which, in order to match the background of pHXB2, was cloned into the pSV7D background plasmid from pCAGGSJRFL; a gift from James M. Binley (26). Mutagenesis was performed using the Stratagene QuikChange II XL site-directed mutagenesis kit and changes were verified by DNA sequencing (Roswell Park Cancer Institute DNA Sequencing Laboratory). The plasmid expressing Rev was pCMV-rev (pRev-1) (NIH AIDS Research and Reference Reagent Program Cat. Number 1443 (27)) and the plasmid expressing Tat was pCEP4-Tat (NIH AIDS Research and Reference Reagent Program Cat. Number 4691 (28))

Cell-Cell Fusion

For the cell-cell fusion assay the envelope plasmid pHXB2 or pJRFL, WT or each mutant, were co-transfected, with plasmids for both Tat and Rev viral proteins, by calcium phosphate precipitation into 293T cells (ATCC), maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin. Twenty-four hours post-transfection the cells were removed from the plate and counted, then

seeded at 2×10^4 cells/well along with 2×10^4 cells/well of the receptor cell line, TZM-bl (NIH AIDS Research and Reference Reagent Program Cat. Number 8129 (29)), in to a 96 well plate. The plates were incubated overnight at 37 °C in a 5% CO₂ incubator. After 24 hours, membrane fusion levels were measured by a luciferase assay (One-glo, Promega) according to the manufacturer's protocol using the Spectra Max M5 (Molecular Devices) plate reader, and levels were normalized to the WT fusion levels. Experiments were performed in triplicate from the transfection step to the measurement of luciferase activity; thus, the uncertainties are inclusive of all steps of the experimental procedure.

Viral Entry

For the viral entry assay, plasmids pHXB2 or pJRFL, WT and each mutant, and pNL4-3.HSA.R-E- (NIH AIDS Research and Reference Reagent Program Cat. Number 3417 (30)) were co-transfected by calcium phosphate precipitation into 293T cells, which were maintained in DMEM with 10% FBS, 1% penicillin-streptomycin. Forty-eight hours post-transfection, the medium was harvested and filtered through a 0.45 µm filter and centrifuged at $55,000 \times g$ for 1 hour to make the virus stock. The infectious titer of wild type (WT) virus was measured by X-gal staining as previously described (31). The HIV-1 p24 antigen capture enzyme-linked-immuno-assay (AIDS & Cancer virus program, NCI at Frederick) was performed to determine equivalent amounts of mutant enveloped viral particles to WT virus at MOI 0.01. Then the amount of p24 at multiplicity of infection (MOI) 0.01 of WT was utilized to normalize for viral particle number and 100 ng/mL of p24 concentration of each mutated virus was used throughout the paper. The receptor cells, TZM-bl, which were maintained in DMEM with 10% FBS, and 1% penicillin-streptomycin, were seeded at 2×10^4 cells/well in a 96-well plate in a volume of 100 µl. The following day, the medium was removed and 100 µl of the virus stock (MOI 0.01, 100 ng/mL of p24) was added to each of the wells. The plates were centrifuged at $3000 \times g$ for one hour and then incubated at 37 °C in a 5% CO₂ incubator. After 48 hours, viral entry levels were measured by a luciferase assay as described above for the cell-cell fusion assay.

Protein Quantification

The virus pellet was prepared by ultracentrifugation at 55,000 rpm for 1 h using a Beckman SW55Ti rotor. The pellets were re-suspended in the lysis buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 0.1% SDS. The 293T cell lysates were collected using M-Per Mammalian protein extraction reagent (Thermo Scientific). Cell lysates and virus pellets were normalized for total protein concentration using the BCA protein assay kit (Pierce).

Western Blot Analysis

After electrophoresis, transfer, and blocking, the blots were probed with mouse anti-HIV-1 gp41 monoclonal antibody (Chessie 8; National Institutes of Health AIDS Research and Reference Reagent Program Cat. Number 526 (32)) and anti-HIV-1 gp120 polyclonal antibody (US Biological). To ensure that the virus stock contains equal amounts of virus particles, normalization was performed based upon western blots for p24 levels using the anti-HIV-1 p24 monoclonal antibody (National Institute of Health AIDS Research and Reference Reagent Program). Total protein concentration was determined using the BCA

assay as described above and 10 µg of total protein was loaded per lane. The secondary antibody used was IR800-conjugated Donkey anti-mouse IgG for gp41 and p24 and IR-700-conjugated Rabbit anti-goat IgG for gp120 and blots were scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR).

Results

Design of gp41 CHR Mutational Scanning

Based on structural studies and the HIV genome, the CHR consists of 37 residues numbered 623 to 659 in the HIV-1 HXB2 envelope sequence. In sequence from amino terminus to carboxy-terminus the gp41 subunit is divided into the fusion peptide, the N-heptad repeat region, the loop region, the C-heptad repeat (CHR) region, the transmembrane region and the cytoplasmic tail (Fig. 1). Of the 37 residues making up the CHR, 30 are conserved between the two strains tested in this study, HXB2 and JRFL, to yield an 85% overall sequence identity.

Cell-cell Fusion and Viral Entry of gp41 CHR Substitutions

The effects of scanning mutagenesis on cell-cell fusion were assayed by a luciferase-based assay in which the core containing the genetic material of the virus can only enter the cytoplasm upon membrane fusion of the plasma membrane of the receptor cell line with the plasma membrane of the cell line containing the envelope complex. Fusion of these two cellular plasma membranes is proportional to the observed luciferase activity. Likewise, the effects of scanning mutagenesis of gp41 on viral entry into receptor cells were assayed by a luciferase-based assay in which viral entry is proportional to the observed luciferase activity. As shown in Figures 2 and 3 and summarized in Tables 1 and 2, the effects of the mutations range from near complete abolishment to no effect on fusion activity to significantly enhanced activity (HXB2 only). Pictorial representations of the results of both of the functional assays, cell-cell fusion and viral entry, are depicted in Figure 10 to display the position in the helices colored for functional level.

Mutant Expression, gp160 Proteolytic Processing, and gp120/gp41 Association Effects

The effects of gp41 mutations on function could be the result of a variety of factors. For example, mutations could have deleterious effects on (a) gp160 expression or the trafficking of the gp160 protein through the endoplasmic reticulum, Golgi apparatus, to the plasma membrane, (b) proteolytic processing of the precursor gp160 to gp41/gp120 by furin cleavage, (c) gp41/gp120 association or (d) a defect in fusogenicity. We tested which of these steps was influenced using Western blot quantification of both the cell lysates and the virus stocks. The results of the Western blot analysis on cell lysates used for cell-cell fusion are summarized on the left half of Tables 1 and 2. On the right half of Tables 1 and 2, the results for protein levels on the virus are summarized. The protein levels for the cell lysates for both cell-cell fusion and for viral production are shown in Figures 4 - 7. The expression level of the virus-producing cells could have an effect on resulting protein levels on the virus surface. Finally, quantification of the western blot analysis for levels of gp120 and gp41 on the virus is shown in Figures 8 and 9.

Discussion

Comparison of cell-cell fusion and viral entry in HXB2

Our results reveal several differences between cell-cell fusion and viral entry levels in the case of strain HXB2. In general, viral entry is much less affected by mutation to alanine with 21 of the 37 mutation sites reduced to a much greater extent in cell-cell fusion than in viral entry levels (Fig. 2). In fact, there are no mutations in HXB2 that exhibit viral entry levels less than approximately 50%. Interestingly, many of the substitutions in HXB2 resulted in cell-cell fusion and viral entry levels that are higher than wild type. Enhanced cell-cell fusion in the case of HXB2 correlated with an increased amount of gp41 as compared to precursor gp160 or the binding partner gp120 in the following instances: N624A, T639A, E648A, Q653A, and E654A. This suggests the intriguing possibility that excess gp41 on the cell surface may facilitate fusion pore formation at the plasma membrane. Of this group of five mutations that are enhanced in cell-cell fusion, four of them are also substantially enhanced in viral entry as well: N624A, T639A, Q653A, and E654A. In the case of viral entry, however, the level of gp41 on the virus is not substantially different from the level of gp120 so it is unlikely that unbound gp41 would be the cause for enhancement of viral entry. One of the mutations with enhanced function, N624A, has a dramatic decrease in expression of gp160. In this case, there is only a slight decrease in the amount of gp41/gp120 complex that the virus incorporates despite gp160 expression being only 45% of the wild type level in the producer cell line. The levels of gp120 and gp41 in the producer cell line are much closer to wild type (80-100%). Similarly, for mutations W623A, H625A, and T626A, gp160 expression is ~45%, however, the amount of gp120 and gp41 on the virus is 80% and function is near wild type levels. Viral entry in the cases of T639A and E654A is enhanced although the gp120/gp41 levels are the same as wild type.

There is an interesting region in HXB2 (S640A through I646A) in which alanine substitutions result in cell-cell fusion that is dramatically decreased while viral entry remains either near wild type levels or is substantially increased. In the case of S640A and L641A, there seems to be loss of gp120 in both the cells used in the cell-cell fusion assay and the cells used for virus production (Fig. 4 & 6). I642A has a clear defect in proteolytic processing of gp160 to gp120/gp41 in both the cells used for cell-cell fusion and for virus production. H643A, S644A, L645A, I646A have partially decreased levels of gp120 and gp41 in both cells producing virus and those used for cell-cell fusion (Fig. 4 & 6). Despite differences in expression levels, the levels of both gp120 and gp41 on the virus are relatively stable with both being greater than 80% in all cases (Fig. 8). This dramatic difference between the effects of alanine substitution on viral entry versus cell-cell fusion is most likely due to the propensity for the virus to incorporate envelope complex with properly cleaved and associated gp120/gp41. Whereas, the cell surface will contain uncleaved gp160, along with any dissociated gp41 that has lost association with gp120 with the population of properly cleaved and associated gp120/gp41 being only a small part of the total envelope.

Mutations in the hydrophobic pocket binding domain

HXB2 is generally quite robust to mutation, however, this strain does seem to be particularly vulnerable in the region from T627A through N636A. This region encompasses a sequence

that is important to the association of the NHR with the CHR in formation of the core of the 6HB conformation of gp41 which facilitates membrane fusion (33, 34). In the CHR, residues W628, W631, and I635 represent three key hydrophobic residues that bind to an important region in the NHR called the hydrophobic pocket (35). There is also a critical salt bridge between D632 on the CHR and K574 on the NHR (36). Mutations in this region substantially decrease cell-cell fusion. Viral entry is also decreased although not as dramatically. This is the only sequence in HXB2 with more than one amino acid in a row that is affected in viral entry. For residues 627 through 632, it is apparent in the western blot analysis, that gp160 expression is increased over wild type levels but that very little cleaved gp120/gp41 is apparent (Fig. 4). This would suggest that the low level of cell-cell fusion and the decrease in viral entry in this region are caused by a defect in the proteolytic processing of precursor gp160 into the non-covalent active complex gp120/gp41. For residues T627-E630, despite the low level cleavage, there is approximately 40-50% incorporation of cleaved gp120/gp41 incorporated into the virus particle. Interestingly, this would suggest a highly functioning selection process for cleaved gp120/gp41. In the case of W631A and D632A, there is very little virus incorporation. However, for these two mutations, the viral entry level is relatively high between 60-80% suggesting that even a small amount of envelope can promote viral entry. It is also interesting to note that in the case of D632, the relatively conservative mutation to alanine only decreases viral entry to 78% whereas when this residue is switched to the opposite charge, viral entry is abolished (36). Even mutation to valine, which is more hydrophobic than alanine, causes abrogation of viral entry whereas alanine does not (36). In the case of alanine substitutions between 627 and 632, the major defect seems to be in proteolytic cleavage.

Comparison of cell-cell fusion and viral entry in JRFL

The difference between cell-cell fusion and viral entry was not as dramatic in the case of JRFL. Both cell-cell fusion and viral entry were affected to a much greater extent with all but 11 sites reduced to below 50% in both assays. Only 2 mutation sites in JRFL had wild type levels in the cell-cell fusion and viral entry assays and only 4 total sites were wild type level in cell-cell fusion. Although with JRFL, as opposed to HXB2, the difference between cell-cell fusion and viral entry was much less dramatic, cell-cell fusion was more often higher than viral entry when compared relative to the wild type levels in each case. In contrast, cell-cell fusion was less than viral entry in only two cases (S640A and Y643A). In fact, cell-cell fusion was much greater in 4 cases: E632A, N637A, T644A, and K655A. In comparison to this effect seen with JRFL, there is no case in which cell-cell fusion is dramatically higher than viral entry for HXB2. This suggests that HXB2 is markedly robust at assembly and produces functional virus despite defects in expression and/or proteolytic processing of gp160 to gp120/gp41. This observation is consistent with the fact that HXB2 is a highly lab-adapted strain (37, 38).

Another interesting issue with JRFL is that several mutational sites have a functional defect in cell-cell fusion whereas the upstream events including expression, proteolytic cleavage, and gp120/gp41 association don't appear to be affected. These residues are N624A, N625A, M629A, E630A, Y638A, T644A, I646A, Q653A, N656A, and E657A. Incorporation into

the virus is relatively low for all of these sites and therefore the defect in viral entry is likely due to a combination of effects on incorporation as well as six-helix bundle formation.

Comparison between strains HXB2 and JRFL

Our mutagenesis study is the first conducted using side-by-side scanning of two different viral isolates each with different coreceptor-usage: HXB2 is CXCR4-utilizing and JRFL is CCR5-utilizing. Our results show a distinct difference between the two strains. JRFL is much more vulnerable both in cell-cell fusion and in viral entry studies to single amino acid substitution with alanine than is HXB2.

Most mutagenesis studies to date have focused on strain HXB2. In the case of HXB2, it is most common to see a defect in incorporation that coincides with a cleavage defect (T627A, W628A, M629A, E630A, W631A, E632A, R633A, E634A, I635A, N636A, N637A, Y638A, T639A, and L641A).

One of the most surprising results of our study, however, was that many of the alanine substitutions in strain JRFL resulted in a cleavage defect that was nonetheless incorporated into the virus as full-length gp160. This is despite the fact that the region that we are scanning, namely the CHR, is at the opposite end of the ectodomain from the cleavage site, which is located at the extreme N-terminus of gp41. Long-range effects to function can occur when a mutation in one region affects the local secondary structure. This then in turn affects the region's ability to associate with other regions to form the tertiary or quaternary structure necessary for proper function. This type of long-range effect has been observed before for single amino acid substitutions (29, 39). However, it has not been commonly observed for the virus to incorporate uncleaved gp160 which is indeed what we see in JRFL for multiple residues (W623A, N624A, N625A, M626A, T627A, W628A, M629A, E630A, W631A, E632A, R633A, E634A, I635A, E641A, I642A, T644A, L645A, I646A, E647A, E648A, S649A, Q650A, N651A, Q652A, Q653A, E654A, K655A, N656A, E657A, Q658A, and E659A). This suggests that the viral process of selecting for functional cleaved envelope trimer is not as robust in JRFL as in the lab-adapted strain HXB2 for which we see robust levels of gp120/gp41 on the virus despite dramatic defects in expression and/or cleavage of gp160.

There could be many factors underlying the potential for differences between the strains HXB2 and JRFL. Miyauchi, *et al.*, in visualizing retrovirus uptake have found that JRFL has a much higher propensity to dissociate from cells and hence is less efficient at entering cells than is strain HXB2 (16). It is possible that this lowered efficiency contributes to the sensitivity of JRFL to alanine substitution. JRFL has also been found to have unusually efficient gp160 proteolytic processing (40). However, in our studies, both strains have a similar number of alanine substitution mutations with clear cleavage defects (7 in JRFL and 10 in HXB2). Therefore, efficient proteolytic processing of gp160 to gp120/gp41 in JRFL does not seem to make up for defects seen with these mutations.

Comparison with previously published alanine substitutions in CHR

A summary of previously published results for mutagenesis to alanine in this region is shown in Table 3. To our knowledge mutations to date have only been studied in strain HXB2. Mutation at W628A showed impaired precursor cleavage in agreement with our results (12), however, viral entry was completely abrogated whereas in our case viral entry was reduced to ~50%. W628A was also studied by another group but only in syncytia formation (11). In this case, cell-cell fusion was abrogated which is in agreement with our results (<10%). This study found a high level of gp120 shedding whereas the biggest defect of this mutation from our western blot analysis would seem to be a defect in precursor cleavage. Surprisingly despite the cleavage defect, the virus is able to incorporate almost 40% of the wild type level of both gp41 which seems to be in a one-to-one stoichiometry with gp120 on the virus surface. W631A exhibited the same results in the syncytium formation assay with abrogated cell-cell fusion and gp120 shedding. Again, our results suggest that there is a defect in furin cleavage. In this case, however, the virus has levels of gp41 and gp120 that are less than 20%. The viral entry level, however, is higher at 68%. I635A was decreased in syncytium formation in agreement with our results. Indeed we also see a decrease in the amount of gp120 as compared to gp41. On the virus, however, this effect is diminished suggesting that the virus preferentially incorporates associated gp41/gp120 as opposed to unbound gp41. Hence, viral entry levels are near wild type. With Y638A, researchers found lowered gp120, however syncytium formation was near wild type (11). In comparison, our results for both cell-cell fusion and viral entry were at or above wild type levels. With this mutation, we found levels of both gp41 and gp120 near wild type levels in all western blot analyses. In the case of T639A, previous research revealed no expression on the cell surface and lowered syncytium formation (41). We saw partially lowered gp160 expression; however, the virus had wild type levels of both proteins. Both cell-cell fusion as measured by luciferase assay and viral entry were near wild type levels. I642A similarly showed decreased syncytia formation with gp120 shedding (11). With this mutation, we found a very dramatic cleavage defect with gp41 and gp120 levels at much less than 10%. Again strikingly, the virus had wild type levels of both proteins and viral entry was near wild type. Cell-cell fusion, however, was drastically reduced with the envelope protein on cells being made up overwhelmingly of uncleaved gp160. It is likely that this overwhelming concentration of uncleaved gp160 interferes with any fusion capacity of the very small amount of cleaved protein on the cell surface. For mutations L645A, S649A, and Q652A, the results are similar between our study and previous results (11). However, we see a diminution in cell-cell fusion with L645A at 20% and Q652 at 55%. Otherwise, these mutations exhibit protein levels that are near to wild type and viral entry levels greater than wild type.

Structural Implications

Molecular models of the CHR domains in the presence of the N-helical inner bundle of the 6HB structure of gp41 are shown in Figure 10. Most surprisingly, mutations that abrogate fusion and/or entry are not isolated to the hydrophobic pocket region nor are the deleterious mutations isolated to one face of the helix as might be expected because of interference with the CHR/NHR association. This could simply suggest the vulnerability of HIV gp41 to long-range effects of manipulation. However, it also suggests that there are other structural

conformations upstream of the post-fusion structure that have different interactions and potentially also secondary structure and cannot be depicted by models of the 6HB of gp41. Other observations are that mutations that enhance infectivity in strain HXB2 are generally not located at the ends of the CHR domain. In the case of strain JRFL, it is readily apparent in this depiction how vulnerable this strain is to mutation. Also, both of the CHR termini are diminished and the results for cell-cell fusion in comparison to viral entry are much more similar than in strain HXB2. Finally, the amino acid positions that are unchanged (light grey) can be considered as reasonable candidates for manipulation to facilitate labeling for structural studies.

The alanine scanning mutagenesis reported herein reveals new insights into the individual contributions of the amino acids of the CHR of gp41 and intriguing differences between strains and between the phenomena of cell-cell fusion as compared to viral entry. Most importantly, these data give us a starting point to develop tools to better characterize the different conformations of gp41 by a combination of mutagenesis, biochemistry, and structural biology techniques.

These studies suggest that the HIV envelope is sensitive to mutation in a strain-dependent manner. Some strains are dramatically more sensitive than others proven by the differences in sensitivity to mutation between strains HXB2 and JRFL. The dramatic differences between cell-cell fusion and viral entry also lend support to the possibility that there are mechanistic differences between these two phenomena. These data also confirm evidence of the importance of the gp41 CHR regions to envelope function and lend insight into areas within the protein sequence that potentially can be manipulated for labeling purposes while retaining function.

Acknowledgments

The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. (42), U87.CD4.CXCR4 from Dr. HongKui Deng and Dr. Dan R. Littman, (43) pNL4-3.HSA.R-E- and pNL4-3.Luc.R-E- from Dr. Nathaniel Landau (30), and pHXB2-env from Dr. Kathleen Page and Dr. Dan Littman (44), HIV-1 gp41 Hybridoma (Chessie 8) from Dr. George K. Lewis, the HIV-1 p24 hybridoma (183-H12-5C) from Dr. Bruce Chesebro and Dr. Hardy Chen (45), pCMV-rev from Dr. Marie-Louise Hammarström and Dr. David Rekosh (27) and pCEP4-Tat from Dr. Lung-Ji Chang (28). pCAGGS JRFL gp160 WT was a gift from Dr. James Binley.

Funding information: This work was supported by startup funds from the University at Buffalo School of Medicine and Biomedical Sciences. This publication was supported in part by the University of Rochester Developmental Center for AIDS Research (NIH P30AI078498).

References

1. Jacobs A, Simon C, Caffrey M. Thermostability of the HIV gp41 wild-type and loop mutations. *Protein Pept Lett.* 2006; 13:477–480. [PubMed: 16800801]
2. Melikyan GB, Markosyan RM, Hemmati H, Delmedico MK, Lambert DM, Cohen FS. Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. *J Cell Biol.* 2000; 151:413–423. [PubMed: 11038187]
3. Jiang S, Lin K, Strick N, Neurath AR. Inhibition of HIV-1 infection by a fusion domain binding peptide from the HIV-1 envelope glycoprotein GP41. *Biochem Biophys Res Commun.* 1993; 195:533–538. [PubMed: 8373393]

4. Mkrtchyan SR, Markosyan RM, Eadon MT, Moore JP, Melikyan GB, Cohen FS. Ternary complex formation of human immunodeficiency virus type 1 Env, CD4, and chemokine receptor captured as an intermediate of membrane fusion. *J Virol*. 2005; 79:11161–11169. [PubMed: 16103167]
5. Wild C, Oas T, McDanal C, Bolognesi D, Matthews T. A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. *Proc Natl Acad Sci U S A*. 1992; 89:10537–10541. [PubMed: 1438243]
6. Matthews T, Salgo M, Greenberg M, Chung J, DeMasi R, Bolognesi D. Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. *Nat Rev Drug Discov*. 2004; 3:215–225. [PubMed: 15031735]
7. Wild C, Greenwell T, Shugars D, Rimskey-Clarke L, Matthews T. The inhibitory activity of an HIV type 1 peptide correlates with its ability to interact with a leucine zipper structure. *AIDS Res Hum Retroviruses*. 1995; 11:323–325. [PubMed: 7786578]
8. Chan DC, Fass D, Berger JM, Kim PS. Core structure of gp41 from the HIV envelope glycoprotein. *Cell*. 1997; 89:263–273. [PubMed: 9108481]
9. Tan K, Liu J, Wang J, Shen S, Lu M. Atomic structure of a thermostable subdomain of HIV-1 gp41. *Proc Natl Acad Sci U S A*. 1997; 94:12303–12308. [PubMed: 9356444]
10. Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC. Atomic structure of the ectodomain from HIV-1 gp41. *Nature*. 1997; 387:426–430. [PubMed: 9163431]
11. Wang S, Y Joanne, Shu Wei, Stoller Marisa O, Nunberg Jack H, Lu Min. Interhelical Interactions in the gp41 Core: Implications for Activation of HIV-1 Membrane Fusion. *Biochemistry*. 2002; 41:7283–7292. [PubMed: 12044159]
12. Weng Y, Y Zhongning, Weiss Carol D. Structure-Function Studies of the Self-Assembly Domain of the Human Immunodeficiency Virus Type 1 Transmembrane Protein gp41. *Journal of Virology*. 2000; 74:5368–5372. [PubMed: 10799616]
13. Caffrey M, Cai M, Kaufman J, Stahl SJ, Wingfield PT, Covell DG, Gronenborn AM, Clore GM. Three-dimensional solution structure of the 44 kDa ectodomain of SIV gp41. *EMBO J*. 1998; 17:4572–4584. [PubMed: 9707417]
14. Caffrey M. Model for the structure of the HIV gp41 ectodomain: insight into the intermolecular interactions of the gp41 loop. *Biochim Biophys Acta*. 2001; 1536:116–122. [PubMed: 11406346]
15. Neurath AR, Strick N, Jiang S. Synthetic peptides and anti-peptide antibodies as probes to study interdomain interactions involved in virus assembly: the envelope of the human immunodeficiency virus (HIV-1). *Virology*. 1992; 188:1–13. [PubMed: 1373549]
16. Miyauchi K, Marin M, Melikyan GB. Visualization of retrovirus uptake and delivery into acidic endosomes. *Biochem J*. 2010; 434:559–569. [PubMed: 21175427]
17. Page KA, Landau NR, Littman DR. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. *J Virol*. 1990; 64:5270–5276. [PubMed: 2214018]
18. Krell T, Greco F, Engel O, Dubayle J, Kennel A, Charlotiaux B, Brasseur R, Chevalier M, Sodoyer R, El Habib R. HIV-1 gp41 and gp160 are hyperthermostable proteins in a mesophilic environment. Characterization of gp41 mutants. *Eur J Biochem*. 2004; 271:1566–1579. [PubMed: 15066182]
19. Jiang S, Lin K, Strick N, Neurath AR. HIV-1 inhibition by a peptide. *Nature*. 1993; 365:113. [PubMed: 8371754]
20. Lu M, Blacklow SC, Kim PS. A trimeric structural domain of the HIV-1 transmembrane glycoprotein. *Nat Struct Biol*. 1995; 2:1075–1082. [PubMed: 8846219]
21. Buzon V, Natrajan G, Schibli D, Campelo F, Kozlov MM, Weissenhorn W. Crystal structure of HIV-1 gp41 including both fusion peptide and membrane proximal external regions. *PLoS Pathog*. 2010; 6:e1000880. [PubMed: 20463810]
22. Miyauchi K, Kim Y, Latinovic O, Morozov V, Melikyan GB. HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. *Cell*. 2009; 137:433–444. [PubMed: 19410541]
23. Konopka K, Pretzer E, Celada F, Duzgunes N. A monoclonal antibody to the gp120-CD4 complex has differential effect on HIV-induced syncytium formation and viral infectivity. *J Gen Virol*. 1995; 76(Pt 3):669–679. [PubMed: 7897355]

24. Yee M, Konopka K, Balzarini J, Duzgunes N. Inhibition of HIV-1 Env-Mediated Cell-Cell Fusion by Lectins, Peptide T-20, and Neutralizing Antibodies. *The open virology journal*. 2011; 5:44–51. [PubMed: 21660189]
25. Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, Kabat D. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J Virol*. 1998; 72:2855–2864. [PubMed: 9525605]
26. Schulke N, Vesanen MS, Sanders RW, Zhu P, Lu M, Anselma DJ, Villa AR, Parren PW, Binley JM, Roux KH, Maddon PJ, Moore JP, Olson WC. Oligomeric and conformational properties of a proteolytically mature, disulfide-stabilized human immunodeficiency virus type 1 gp140 envelope glycoprotein. *J Virol*. 2002; 76:7760–7776. [PubMed: 12097589]
27. Lewis N, Williams J, Rekosh D, Hammarskjöld ML. Identification of a cis-acting element in human immunodeficiency virus type 2 (HIV-2) that is responsive to the HIV-1 rev and human T-cell leukemia virus types I and II rex proteins. *J Virol*. 1990; 64:1690–1697. [PubMed: 2157051]
28. Chang LJ, Urlacher V, Iwakuma T, Cui Y, Zucali J. Efficacy and safety analyses of a recombinant human immunodeficiency virus type 1 derived vector system. *Gene therapy*. 1999; 6:715–728. [PubMed: 10505094]
29. Sen J, Jacobs A, Caffrey M. Role of the HIV gp120 conserved domain 5 in processing and viral entry. *Biochemistry*. 2008; 47:7788–7795. [PubMed: 18597484]
30. He J, Choe S, Walker R, Di Marzio P, Morgan D, Landau N. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. *J Virol*. 1995; 69:6705–6711. [PubMed: 7474080]
31. Yi HA, Diaz-Aguilar B, Bridon D, Quraishi O, Jacobs A. Permanent Inhibition of Viral Entry by Covalent Entrapment of HIV gp41 on the Virus Surface. *Biochemistry*. 2011; 50:6966–6972. [PubMed: 21736372]
32. Abacioglu YH, Fouts TR, Laman JD, Claassen E, Pincus SH, Moore JP, Roby CA, Kamin-Lewis R, Lewis GK. Epitope mapping and topology of baculovirus-expressed HIV-1 gp160 determined with a panel of murine monoclonal antibodies. *AIDS Res Hum Retroviruses*. 1994; 10:371–381. [PubMed: 8068416]
33. Wang H, Qi Z, Guo A, Mao Q, Lu H, An X, Xia C, Li X, Debnath AK, Wu S, Liu S, Jiang S. ADS-J1 inhibits human immunodeficiency virus type 1 entry by interacting with the gp41 pocket region and blocking fusion-active gp41 core formation. *Antimicrob Agents Chemother*. 2009; 53:4987–4998. [PubMed: 19786602]
34. Yu X, Lu L, Cai L, Tong P, Tan S, Zou P, Meng F, Chen YH, Jiang S. Mutations of Gln64 in the HIV-1 gp41 N-terminal heptad repeat render viruses resistant to peptide HIV fusion inhibitors targeting the gp41 pocket. *J Virol*. 2012; 86:589–593. [PubMed: 22013063]
35. Chan DC, Chutkowski CT, Kim PS. Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target. *Proc Natl Acad Sci U S A*. 1998; 95:15613–15617. [PubMed: 9861018]
36. He Y, Liu S, Li J, Lu H, Qi Z, Liu Z, Debnath AK, Jiang S. Conserved salt bridge between the N- and C-terminal heptad repeat regions of the human immunodeficiency virus type 1 gp41 core structure is critical for virus entry and inhibition. *J Virol*. 2008; 82:11129–11139. [PubMed: 18768964]
37. Daar ES, Li XL, Moudgil T, Ho DD. High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. *Proc Natl Acad Sci U S A*. 1990; 87:6574–6578. [PubMed: 2395859]
38. McKeating JA, Bennett J, Zolla-Pazner S, Schutten M, Ashelford S, Brown AL, Balfe P. Resistance of a human serum-selected human immunodeficiency virus type 1 escape mutant to neutralization by CD4 binding site monoclonal antibodies is conferred by a single amino acid change in gp120. *J Virol*. 1993; 67:5216–5225. [PubMed: 7688820]
39. Sen J, Yan T, Wang J, Rong L, Tao L, Caffrey M. Alanine scanning mutagenesis of HIV-1 gp41 heptad repeat 1: insight into the gp120-gp41 interaction. *Biochemistry*. 2010; 49:5057–5065. [PubMed: 20481578]

40. Crooks ET, Tong T, Osawa K, Binley JM. Enzyme digests eliminate nonfunctional Env from HIV-1 particle surfaces, leaving native Env trimers intact and viral infectivity unaffected. *J Virol.* 2011; 85:5825–5839. [PubMed: 21471242]
41. Cao J, Bergeron L, Helseth E, Thali M, Repke H, Sodroski J. Effects of amino acid changes in the extracellular domain of the human immunodeficiency virus type 1 gp41 envelope glycoprotein. *J Virol.* 1993; 67:2747–2755. [PubMed: 8474172]
42. Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, Kabat D. Effects of CCR5 and CD4 Cell Surface Concentrations on Infections by Macrophagetropic Isolates of Human Immunodeficiency Virus Type 1. *J Virol.* 1998; 72:2855–2864. [PubMed: 9525605]
43. Bjorndal A, Deng H, Jansson M, Fiore J, Colognesi C, Karlsson A, Albert J, Scarlatti G, Littman D, Fenyo E. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J Virol.* 1997; 71:7478–7487. [PubMed: 9311827]
44. Page KA, Landau NR, Littman DR. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. *J Virol.* 1990; 64:5270–5276. [PubMed: 2214018]
45. Chesebro B, Wehrly K, Nishio J, Perryman S. Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell-tropic isolates: definition of critical amino acids involved in cell tropism. *J Virol.* 1992; 66:6547–6554. [PubMed: 1404602]

Fig. 1.

Diagram of the HIV genome with emphasis on the C-heptad repeat (CHR) domain of the envelope transmembrane subunit, gp41. Amino acids that are not conserved between the two strains are shown in bold.

Fig. 2.

Levels of cell-cell fusion and viral entry substitutions in the CHR region of strain HXB2.

The results are presented as a percentage of the luciferase signal normalized to the wild type level. The error bars represent the standard deviation of three separate experiments.

Fig. 3.

Levels of cell-cell fusion and viral entry substitutions in the CHR region of strain JRFL. The results are presented as a percentage of the luciferase signal normalized to the wild type level. The error bars represent the standard deviation of three separate experiments.

Fig. 4.

Western blot quantification of gp160/gp120/gp41 from HXB2 envelope transfection into 293T cells for cell-cell fusion. The results are presented as a percentage of the WT infrared fluorescence intensity.

Fig. 5.

Western blot quantification of gp160/gp120/gp41 from JRFL envelope transfection into 293T cells for cell-cell fusion. The results are presented as a percentage of the WT infrared fluorescence intensity.

Fig. 6. Western blot quantification of gp160/gp120/gp41 from HXB2 envelope transfection into 293T cells for virus production. The results are presented as a percentage of the WT infrared fluorescence intensity.

Fig. 7.

Western blot quantification of gp160/gp120/gp41 from JRFL envelope transfection into 293T cells for virus production. The results are presented as a percentage of the WT infrared fluorescence intensity.

Fig. 8.

Western blot quantification of gp120 and gp41 levels on HXB2 virus. The results are presented as a percentage of the WT infrared fluorescence intensity.

Fig. 9.

Western blot quantification of gp120 and gp41 levels on JRFL virus. The results are presented as a percentage of the WT infrared fluorescence intensity.

Fig. 10.

Molecular models highlighting the functionality of mutations in the HIV gp41 CHR. The inner NHR helical bundle is shown as a ribbon diagram in grey. The top of the structure is that which is oriented toward the loop region. The top panel of models is a view of the right side of the CHR helix. The bottom panel is a view that is flipped about the vertical axis to display the left side of the CHR helix. Only one of the CHR peptides is shown for simplicity. Amino acid residues without highlighting (light grey) are between 80-120% in either cell-cell fusion or viral entry as indicated. Those highlighted in green are increased above 120% of the wild type level. Those highlighted with dark grey are diminished to levels between 20-50% and those in red are diminished to lower than 20% of wild type level. The structure was rendered with Discovery Studios Visualizer based upon coordinates from the theoretical model 1IF3 with HIV HXB2 as the sequence (4).

Table 1

HXB2 envelope protein quantification. The left hand side shows protein levels in the cell lysates used for cell-cell fusion. On the right hand side are protein levels on the virus.

Residue	gp120 Expression	gp41 Expression	Cell-cell Fusion	gp120 Level	gp41 Level	Viral Entry
W623A	++++	++++	97%	++++	+++	90%
N624A	++++	+++++	136%	++++	+++	121%
H625A	++++	++++	108%	++++	+++	108%
T626A	++++	++++	100%	++++	++++	90%
T627A	+	+	10%	+++	+++	49%
W628A	+	+	7%	++	++	47%
M629A	+	+	15%	++	++	48%
E630A	+	+	15%	+++	+++	70%
W631A	+	+	5%	+	+	68%
D632A	+	+	17%	+	+	78%
R633A	++	++	45%	++++	+++	88%
E634A	++	++	20%	+++	+++	70%
I635A	+++	++++	40%	++++	+++	95%
N636A	++	++	29%	+++	+++	72%
N637A	++++	++++	108%	++++	++++	144%
Y638A	++++	++++	113%	++++	+++	146%
T639A	++++	+++++	113%	++++	++++	131%
S640A	+++	++++	63%	++++	+++++	122%
L641A	+++	++++	62%	++++	++++	149%
I642A	+	+	13%	++++	++++	106%
H643A	+++	+++	62%	++++	++++	142%
S644A	+++	+++	12%	++++	++++	118%
L645A	+++	+++	18%	++++	++++	143%
I646A	+++	+++	13%	++++	++++	111%
E647A	++++	++++	99%	++++	++++	144%
E648A	++++	+++++	122%	++++	++++	97%

Residue	gp120 Expression	gp41 Expression	Cell-cell Fusion	gp120 Level	gp41 Level	Viral Entry
S649A	+++	++++	71%	+++	+++	118%
Q650A	+++	++++	63%	+++	+++	116%
N651A	+++	++++	48%	+++	+++	108%
Q652A	+++	++++	56%	+++	+++	120%
Q653A	++++	+++++	159%	+++	+++	116%
E654A	++++	+++++	177%	+++	+++	162%
K655A	++++	++++	102%	+++	+++	117%
N656A	+++	+++	51%	++	++	47%
E657A	++++	++++	151%	+++	+++	141%
Q658A	++++	++++	95%	+++	+++	110%
E659A	++++	++++	119%	+++	+++	109%

Protein levels of expression and incorporation are shown as: (+) 0-20% WT levels, (++) 20-50% WT levels, (++++) 50-80% WT levels, (+++++) >80% WT levels, and (++++++) >120% WT levels.

Table 2

JRFL envelope protein quantification. The left hand side shows protein levels in the cell lysates used for cell-cell fusion. On the right hand side are protein levels on the virus.

Residue	gp120 Expression	gp41 Expression	Cell-cell Fusion	gp120 Level	gp41 Level	Viral Entry
W623A	+	+	2%	+	+	2%
N624A	++++	++++	30%	++	++	35%
N625A	++++	++++	35%	++	++	24%
M626A	++	++	33%	++	++	21%
T627A	+	+	9%	+	+	8%
W628A	+	+	1%	+	+	1%
M629A	++++	++++	45%	++	++	40%
E630A	++++	++++	52%	++	++	39%
W631A	++	++	1%	+	++	1%
E632A	+	+	60%	+	++	31%
R633A	+	+	16%	+	+	16%
E634A	++	+++	69%	+	+	58%
I635A	++	++	1%	+	+	1%
D636A	+	++	45%	+	+	34%
N637A	+++	+++	73%	+	+	34%
Y638A	++++	++++	40%	+	+	47%
T639A	+++	+++	68%	+	+	47%
S640A	+	++	47%	+	+	61%
E641A	++	++	1%	+	+	2%
I642A	+++	++++	19%	+	+	1%
Y643A	+++	++++	12%	+	+	38%
T644A	++++	++++	73%	+	+	11%
L645A	+++	++++	5%	+	+	6%
I646A	++++	++++	29%	+	+++	29%
E647A	++	++	36%	+	+	36%
E648A	+	++	26%	+	+	30%

Residue	gp120 Expression	gp41 Expression	Cell-cell Fusion	gp120 Level	gp41 Level	Viral Entry
S649A	+++	+++	33%	+	+	33%
Q650A	++++	++++	30%	+	+	30%
N651A	++++	++++	115%	++++	++++	90%
Q652A	++++	++++	112%	++++	++++	102%
Q653A	++++	++++	52%	+	+	52%
E654A	++++	++++	105%	+	+	75%
K655A	++++	++++	93%	+	+	3%
N656A	++++	++++	37%	+	+	37%
E657A	++++	++++	24%	++	++	21%
Q658A	++	++	0%	+	+	9%
E659A	+	+	19%	+	+	1%

Protein levels of expression and incorporation are shown as: (+) 0-20% WT levels, (++) 20-50% WT levels, (++++) 50-80% WT levels, and (+++++) >120% WT levels.

Table 3

Published results for alanine substitutions performed in the CHR region of gp41.

Residue	Isolate	Published Results
W628	HXB2	Impaired precursor cleavage, abrogated viral entry compared to WT (12)
W628	HXB2	Expression similar to WT, with lowered cell surface gp120 and lowered syncytium formation compared to WT (11)*
W631	HXB2	Expression similar to WT, with lowered cell surface gp120 and lowered syncytium formation compared to WT(11)*
I635	HXB2	Expression similar to WT, with lowered cell surface gp120 and lowered syncytium compared to WT (11)*
Y638	HXB2	Expression on the cell surface, proteolytic processing, gp120 association and syncytium formation similar to WT despite gp120 shedding being elevated (11)*
T639	HXB2	No expression, no syncytium formation (41)
I642	HXB2	Expression similar to WT, with lowered cell surface gp120 and lowered syncytium formation compared to WT (11)*
L645	H64333	Expression, surface gp120, and syncytium formation similar to WT (11)*
S649	HXB2	Expression, surface gp120, and syncytium formation similar to WT (11)*
Q652	HXB2	Expression, surface gp120, and syncytium formation similar to WT (11)*

* note that in this study wild type levels of syncytium formation were designated to be those between 50% and 120% of wild type.