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Modified HIV envelope proteins with enhanced binding to neutralizing monoclonal antibodies

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Abstract

The target for neutralizing antibodies against human immunodeficiency virus (HIV) is the trimeric Env protein on the native virion. Conserved neutralizing epitopes of receptor binding sites are located in the recessed core of the Env protein, partially masked by glycosylations and variable loops. In this study, we have investigated the effects of modifications of the HIV Env protein by glycosylation site mutations, deletions of variable loops, or combinations of both types of mutations on their protein functions and reactivities with neutralizing antibodies. Modified Env proteins were expressed in insect or mammalian cells, and their reactivity with epitope-specific broadly neutralizing monoclonal antibodies (Mabs) was determined by flow cytometry. A unique mutant designated 3G with mutations in three glycosylation motifs within the V3/C3 domains surrounding the CD4 binding site showed higher levels of binding to most broadly neutralizing Mabs (b12 and 2F5) in both insect and mammalian expression systems. Mutants with a deletion of both V1 and V2 loop domains or with a unique combination of both types of mutations also bound to most neutralizing Mabs at higher levels compared to the wild-type control. Most mutants maintained the ability to bind CD4 and to induce syncytium formation at similar or higher levels as compared to that of the wild-type Env protein, except for a mutant with a combination of variable loop deletions and deglycosylation mutations. Our study suggests that modified HIV Env proteins with reduced glycosylation in domains surrounding the CD4 binding site or variable loop-deleted mutants expose important neutralizing epitopes at higher levels than wild type and may provide novel vaccine immunogens.
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Keywords: Human immunodeficiency virus (HIV); Modified envelope proteins; Enhanced antigenicity

Introduction

Although human immunodeficiency virus type 1 (HIV-1) was identified as the etiologic agent of AIDS in humans nearly two decades ago, an effective vaccine against the virus has not been developed yet. Considering the high cost and serious side effects of anti-retroviral therapy and continued increase in new infections with the virus in developing countries, there is a great demand for developing a vaccine against the virus. The envelope glycoprotein (Env) of HIV-1 is the only viral target for neutralizing antibodies. The gp120 and gp41 glycoproteins are bound to each other noncovalently and form oligomers on the cell surface and on virions (Chan et al., 1997; Lu et al., 1995). Infection of CD4+ lymphocytes is initiated by binding of gp120 to the CD4 molecule on the cell surface, followed by binding of the gp120–CD4 complex to CXCR4 or CCR5 chemokine receptors (Wu et al., 1996).

It has been difficult to elicit neutralizing antibodies by vaccination that are broadly reactive against many HIV-1 isolates. However, a small number of human monoclonal antibodies (Mabs) with broad neutralizing activities against primary strains have been isolated and characterized from infected individuals, and have revealed several neutralization epitopes on HIV-1 Env gp120–gp41. These Mabs include b12 that recognizes an epitope overlapping the CD4

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binding site (CD4bs) (Roben et al., 1994; Saphire et al., 2001) and 2F5 that binds to an epitope near the transmembrane (TM) domain of the gp41 glycoprotein (Parker et al., 2001), indicating that gp41 is also a target for neutralizing antibodies. Mab 2G12 recognizes an epitope around the V3/C3/V4 domain of gp120 that is dependent on the presence of N-linked glycans in this region (Scanlan et al., 2002; Trkola et al., 1996). Another Mab 17b recognizes a region close to the coreceptor binding site, which is better exposed following CD4 binding (CD4i epitope) (Kwong et al., 1998; Sullivan et al., 1998). Antibodies to V2 and V3 loops display mainly isolate or type-specific neutralizing activities, which can be detected early in infection (Etemad-Moghadam et al., 1998). The CD4bs antibodies are relatively prevalent in patient sera (Moore and Ho, 1993), whereas antibodies to CD4i or 2G12 epitopes are rare (Poignard et al., 2001; Thali et al., 1993).

The elimination of potential N-linked glycosylation sites located in the V1V2 loops or the deletion of the V1 or V2 loops from the HIV glycoprotein resulted in an increased susceptibility of these mutant viruses to neutralization by certain Mabs or by sera collected from infected individuals (Cao et al., 1997; Ly and Stamatatos, 2000; Srivastava et al., 2003; Stamatatos et al., 1998a). This change in viral neutralization indicates that such Env modifications increase the number or exposure of available neutralization epitopes within the oligomeric viral Env protein. Thus, to elicit neutralizing antibodies that have broad cross-reactivity against a large number of genetically diverse HIV-1 isolates, it is of interest to design vaccine candidates in which the conserved regions of gp120 are exposed.

Not much is known about antigenic properties after modifications in glycosylation or variable loop deletions of the HIV trimeric Env glycoprotein. Also, the effects of combined mutation of variable loop deletions and N-linked glycan motifs on exposing important neutralizing epitopes and on their functions have not been studied yet. To address these issues, we genetically engineered the HIV-1 primary isolate 89.6 Env protein and generated a series of Env modifications including deletions in the V1/V2 loop region and mutations at sites of N-linked glycans within the V3/C3 and gp41 domains. We have investigated the reactivities of modified HIV-1 Env proteins with potent neutralizing Mabs as well as their CD4 binding and fusion functions.

Results

Construction of genetically modified HIV 89.6 Env proteins and their expression

To study the effects of multiple mutations on Env protein functions and exposure of neutralizing epitopes, a series of glycosylation site mutations in gp120 and gp41 and variable loop deletions was generated as shown in Fig. 1. Glycosylation site mutant 3G contained mutations

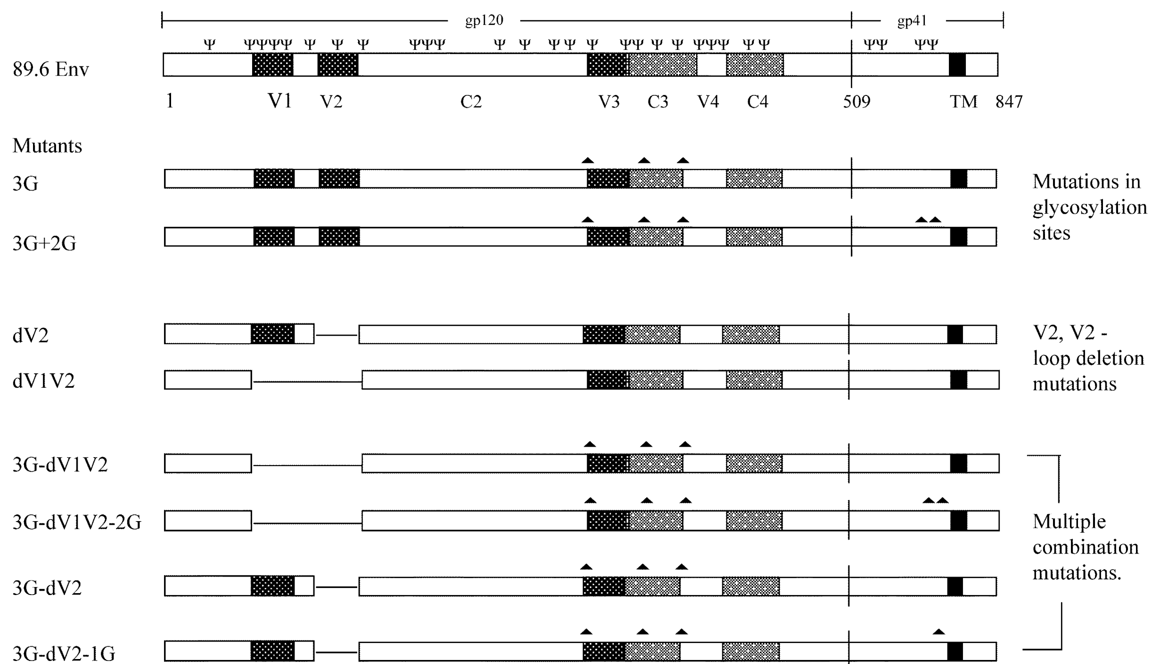


Fig. 1. The construction of genetically modified HIV 89.6 Env proteins. The symbol Ψ indicates the N-glycosylation motifs. The V1V2 (aa 128–194) and V2 (aa 164–194) domains shown as lines indicate deletions and replacement with a 3-amino acid linker (Gly–Ala–Gly). The glycosylation motifs mutated are denoted by triangles. 3G denotes three glycosylation motif mutations of Asn to Gln at aa 304, 341, and 363 in the V3/C3 domains. 2G denotes glycosylation motif mutations of Asn to Gln at aa 623 and 635, and 1G at aa 635 in gp41 near the TM domain. Numbers indicate amino acid (aa) residues based on HIV 89.6 strain.

of Asn to Gln at aa 304, 341, and 363 in the V3/C3 domain of gp120. The 3G–2G mutant was made by combining mutations of 3G and mutations of Asn to Gln at aa 623 and 635 near the gp41 TM domain (designated 2G). These gp41 mutations were designed with the expectation that they might expose the gp41 epitopes. The variable loop mutants dV1V2 and dV2 contained deletions of aa from 128 to 194 and from 164 to 194, respectively, which were replaced with the 3 aa Gly–Ala–Gly. Mutant 3G-dV1V2-1G was generated by combining mutations 3G, dV2, and a mutation of Asn to Gln at an aa 635 near the TM domain of gp41.

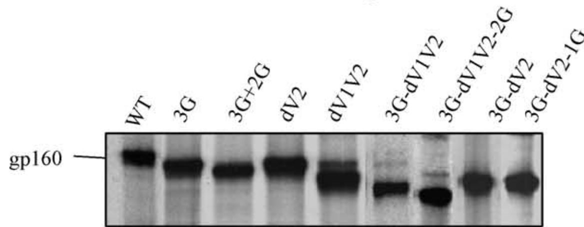
The recombinant baculovirus (rBV) expression system has been used extensively to produce virus-like particles (VLPs) including HIV (Deml et al., 1997; Kang et al., 1999; Wagner et al., 1996, 1998), simian immunodeficiency virus (Kang and Compans, 2003), chimeric simian HIV (SHIV) (Guo et al., 2003), papillomavirus (Shi et al., 2001), and rotavirus (Coste et al., 2000), and these studies demonstrated that VLPs could elicit strong immune responses in animal models. Thus, noninfectious VLPs containing high quantities of oligomeric HIV Env proteins may represent potential candidate immunogens for a vaccine and it is important to study neutralizing epitope exposure of HIV Env expressed by the rBV expression system. Nevertheless, considering that there is a difference in posttranslational modification of N-glycans between insect cells (high-mannose-type oligosaccharides) and mammalian cells (complex type oligosaccharides) (Lanford et al., 1989) and that some neutralizing antibodies, for example, Mab 2G12, depend on carbohydrates of gp120, we compared the wild type (WT) and mutant HIV-1 89.6 Env glycoproteins expressed by both rBV and recombinant vaccinia virus (rVV) expression systems. Sf9 insect cells were infected with a rBV expressing WT or mutant Env protein, radiolabeled, and used for immunoprecipitation by monkey anti-SHIV 89.6 sera (Fig. 2A). Env mutant proteins were expressed mostly as uncleaved precursor forms and were found at similar levels as the WT HIV Env protein in cell lysates. Mutant proteins migrated faster than the WT protein as expected from the mutations introduced. These results indicate that the mutated glycosylation sites were actually used in the WT protein and that the variable loops were deleted as designed. For the analysis of expression in mammalian cells, CV-1 cells were infected with a rVV expressing the wild type or mutant Env protein (Fig. 2B). Most mutants showed similar levels of whole-cell protein expression compared to the WT protein except for the 3G-dV1V2 mutant that showed slightly lower expression. The release of surface HIV Env into the culture supernatant was observed at higher levels in cells infected with the 3G, dV2, and dV1V2 mutants, but was lower in 3G-2G, 3G-dV1V2, and 3G-dV1V2-2G than in WT.

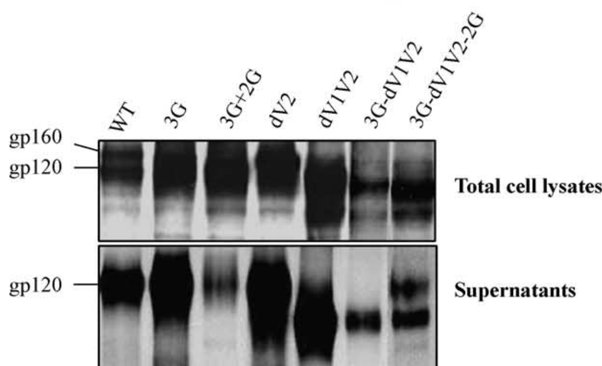
To determine the cell surface expression of WT and mutant Env proteins, we applied a similar approach using fluorescence-activated cell sorting (FACS) analysis as in previous studies (Koch et al., 2003; Si et al., 2001; Wyatt et al., 1995). Infected cells were stained with a human polyclonal anti-serum and flow cytometry analysis was performed. Positive cells were gated and florescence intensity was compared (Fig. 2C). Even considering the possible different reactivities with polyclonal antibodies between WT and mutants, levels of HIV Env on cell surface were found to be similar between WT and mutants without significant differences in both rBV expression (Fig. 2D) and rVV expression (Fig. 2E).

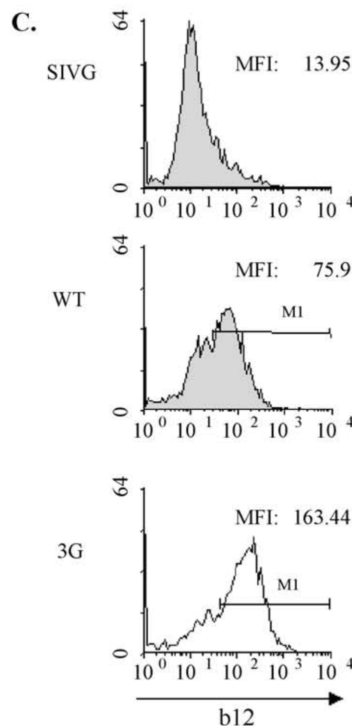
Syncytium formation and CD4 binding

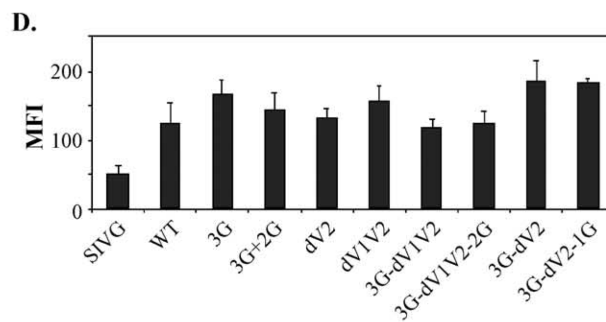
The HIV 89.6 *env* gene is derived from a dual-tropic R5X4 virus, and its expression on cells expressing CD4 and either CXCR4 or CCR5 results in syncytium formation (Doranz et al., 1996). To determine if the mutant HIV Env proteins maintain fusion activity leading to syncytium formation, HeLa-CD4 cells infected with rVVs expressing WT or mutant Env protein were observed. Formation of multinucleated cells (syncytia) was readily visible under phase-contrast microscopy (Fig. 3A). A summary of these results is shown in Fig. 3B. Mutant 3G showed higher syncytium formation ability resulting in an increase in size and number of syncytia compared to WT, whereas mutant 3G-dV1V2 did not show any visible syncytia formation. All other mutants retained the ability to form syncytia at similar levels as WT. When the proteins were expressed in HeLa cells not expressing CD4, neither the mutants nor the WT Env showed syncytium formation (data not shown).

To determine whether the observed difference in syncytium formation ability is due to differences in ability to bind CD4 molecules, CV-1 cells were infected with rVVs expressing WT or mutant Env proteins, incubated with various concentrations of soluble CD4 molecules, and bound CD4 determined using flow cytometry analysis of surface-stained cells with anti-CD4 antibody (Fig. 4). All mutants retained the ability to bind CD4 at levels similar to WT Env except a V2-loop-deleted mutant that showed slightly higher levels of CD4 binding. When the relative affinities of mutants to the CD4 molecule were compared in terms of logarithmic slope from the binding curve, dV2 showed the highest affinity value, which is 25% higher than WT (9.57 versus 7.67). All other mutants showed similar affinity values. The WT and mutant HIV Env proteins expressed in insect cells could also bind CD4 as determined by flow cytometry analysis although lower levels of CD4 binding were found compared to expression in CV-1 cells (data not shown). The results obtained here are consistent with previous studies that demonstrated that similar levels of CD4 binding of variable loop deleted (dV1, dV1V2, dV1V2V3) and various glycosylation site mutants of HIV Env were observed (McCaffrey et al., 2004; Stamatatos and Cheng-Mayer, 1998b; Wyatt et al., 1995).









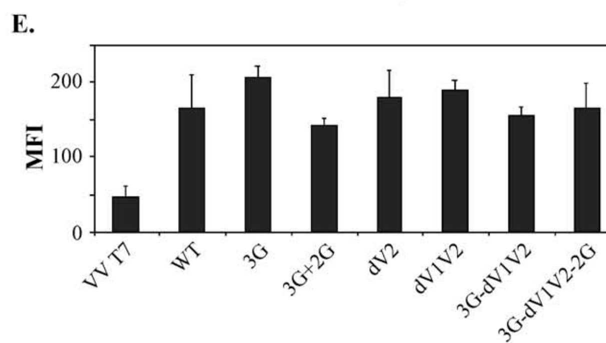


Fig. 2. Immunoprecipitation of wild type and mutant HIV Env proteins. (A) Recombinant baculovirus (rBV) expression system. Sf 9 insect cells were infected with a rBV expressing the wild type or mutant HIV Env protein at a MOI of 10. At day 1 postinfection, cells were metabolically labeled and cell lysates immunoprecipitated using antisera from a monkey infected with SHIV 89.6. Proteins were analyzed by SDS-PAGE. WT (wild-type HIV 89.6 Env protein), 3G, 3G + 2G, dV2, dV1V2, 3G-dV1V2, and 3G-dV1V2-2G have been explained in Fig. 1. (B) Recombinant vaccinia virus (rVV) expression system. HeLa cells were infected with rVVs at a MOI of 2. Cells were metabolically labeled and culture supernatants harvested to determine the HIV Env proteins secreted into the medium. Culture supernatants and cell lysates were immunoprecipitated using antisera from a monkey infected with SHIV 89.6. (C) Flow cytometry profile of cell surface expression of WT and mutant Env stained with Mab b12. SIVG: Sf 9 cells infected with a rBV expressing SIV Gag. WT: Sf 9 cells infected with a rBV expressing HIV 89.6 Env. 3G: Sf 9 cells infected with a rBV expressing mutant 3G Env. Positive cells were gated and mean fluorescent intensity (MFI) was compared. (D) Binding of a patient polyclonal anti-serum to the WT and mutant HIV Env proteins expressed in Sf 9 insect cells. Sf 9 insect cells were infected with rBV at a MOI of 10 and were harvested after 1 day postinfection. Cells (1 × 105) were stained with 80× diluted neutralizing sera from an HIV-1-positive patient and levels of antibody binding were determined by flow cytometry analysis. (E) Binding of a patient polyclonal anti-serum to the WT and mutant HIV Env proteins expressed in mammalian cells using a rVV expression system. CV-1 cells were infected with a rVV at a MOI of 2, harvested after 1 day postinfection, stained with 80× diluted neutralizing sera from an HIV-1-positive patient, and levels of antibody binding were determined by flow cytometry analysis.

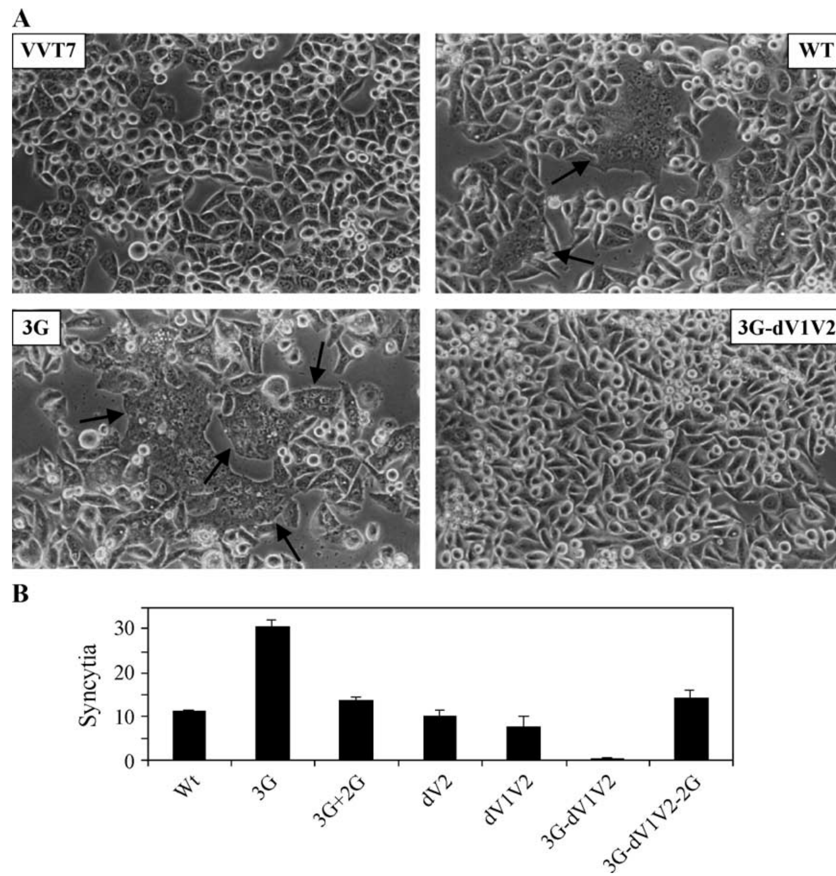


Fig. 3. Syncytium formation induced by HIV Env WT or mutant proteins. (A) Examples of syncytium formation. HeLa-CD4 cells infected with a rVV expressing WT or mutant Env protein at a MOI of 0.5 were monitored for the formation of syncytia under a light microscope and photographed with a 20× objective. Arrows indicate syncytia. (B) The relative syncytium formation activity was evaluated by counting syncytia of multinuclear cells under a microscope with a 10× objective. The numbers indicate an average from counts at five different fields (an area of 1 mm^2) in a well. VV T7, cells infected with rVV expressing bacteriophage T7 polymerase. WT, 3G, and 3G-dV1V2 are described in Fig. 1.

Binding patterns of Mabs to cell surface envelope glycoproteins

Previous studies indicated that binding of antibodies to cell surface-expressed HIV-1 Env was more relevant to the neutralizing potency than binding to the monomeric gp120 protein (Doranz et al., 1996; Roben et al., 1994; Si et al., 2001; Stamatatos et al., 2000). To investigate whether mutations in glycosylation sites or variable loop deletions or combined mutations affect interactions of the HIV-1 Env glycoproteins with antibodies, we applied FACS analysis in comparing the binding efficiency of a panel of Mabs with broad neutralizing activities. Since the levels of cell surface expression were similar but not identical among different mutants, only the positive populations compared to negative control cells were gated to compensate for these differences (Fig. 2C) in evaluating the geometric mean fluorescence intensity (MFI).

For anti-CD4bs Mab b12 that is known to be the most potent and broadly neutralizing monoclonal antibody, mutants 3G, dV1V2, and 3G-dV2-1G expressed in insect cells showed higher binding than the WT. This higher binding level was more prominent using low concentrations of b12 antibody (Fig. 5A). Binding of anti-V3 loop Mab 447-52D to mutants 3G, dV1V2, 3G-2G, and 3G-dV2 was higher than that of WT (Fig. 5B). It is interesting to note that a combination mutant 3G-dV2-1G showed the highest binding to this 447-52D antibody, indicating a possibility that glycosylation of the gp41 domain may affect exposure of the V3 loop epitope in the gp120 domain. These results indicate that mutants 3G, dV1V2, and 3G-dV2-1G expressed on insect cells present V3 loop and CD4 binding site epitopes better than the WT Env protein.

Mab 2G12, which recognizes a carbohydrate-dependent epitope in the V3/C3/V4 regions of gp120, showed the highest binding to the mutant dV1V2 and secondly to the mutant dV2 Env expressed in insect cells (Fig. 5C). All other mutants containing deglycosylations showed lower or similar binding to Mab 2G12 as compared to WT. Mab 17b, which binds to an epitope with increased exposure following CD4 binding (CD4i epitope), showed the highest binding to mutant dV1V2 and then secondly to the mutants 3G-dV2-1G and 3G-dV2 (Fig. 5D). Mab 670-30D, which is specific for the C5 region of gp120 (Nyambi et al., 2000; Zolla-Pazner et al., 1995), did not show differences in binding among mutants and WT.

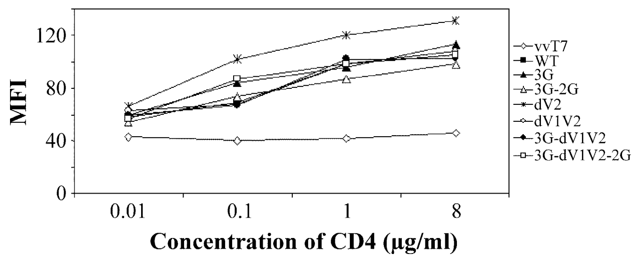


Fig. 4. Binding of soluble CD4 to wild type and mutant HIV Env proteins expressed on cells. WT, 3G, 3G + 2G, dV2, dV1V2, 3G-dV1V2, and 3G-dV1V2-2G are described in Fig. 1. CV-1 cells were harvested at 1 day postinfection, incubated with soluble CD4 (0.01–8 μg/ml), and stained with rabbit antihuman CD4 serum and with FITC-conjugated anti-rabbit IgG serum subsequently. A representative was shown from three independent experiments.

Overall, the variable loop-deleted mutant dV1V2 seems to expose epitopes against 2G12 and 17b better than other mutants.

The 2F5 epitope is relatively well conserved among HIV strains in terms of its linear sequence (Coeffier et al., 2000; Zwick et al., 2001). Most of the mutants containing a 3G mutation (3G, 3G-dV2-1G, 3G-2G, 3G-dV2) showed higher levels of binding to Mab 2F5 than WT (Fig. 5E). The presence of a glycosylation mutation close to the 2F5 epitope (3G-dV2-1G) increased the binding of 2F5 antibody at low concentrations compared to the Env without that mutation (3G-dV2). The variable loop-deleted mutant dV1V2 again showed the highest binding to 2F5 among the other mutants including WT. Overall, among the mutants expressed in insect cells, 3G and some other mutants containing 3G showed higher binding affinities to most antibodies tested except for 2G12, whereas dV1V2 showed higher binding to all antibodies tested including 2G12.

Since it is known that the glycosylation patterns of proteins are different between insect and mammalian cells, we also wanted to determine the binding of mutants expressed on CV-1 cells using rVV expression. Anti-CD4bs Mab b12 with potent neutralizing activity to many HIV clinical isolates showed higher binding to the 3G mutant but lower binding to most other mutants (3G-2G, dV1V2, 3G-dV1V2, 3G-dV1V2-2G) at low concentrations (Fig. 6A). The 3G mutant also showed the highest binding to F105 anti-CD4bs antibody (data not shown). At relatively high concentrations of antibody, mutants dV2 and 3G-dV1V2 showed an increased binding to anti-V3 loop-specific Mab 447-52D (Fig. 6B). Enhanced binding of mutants 3G and dV2 to Mab 17b was also observed only at a high concentration of antibody (Fig. 6D).

For HIV-1 gp120 carbohydrate-dependent Mab 2G12, a variable loop-deleted mutant dV2 resulted in higher binding while all other mutants containing deglycosylation

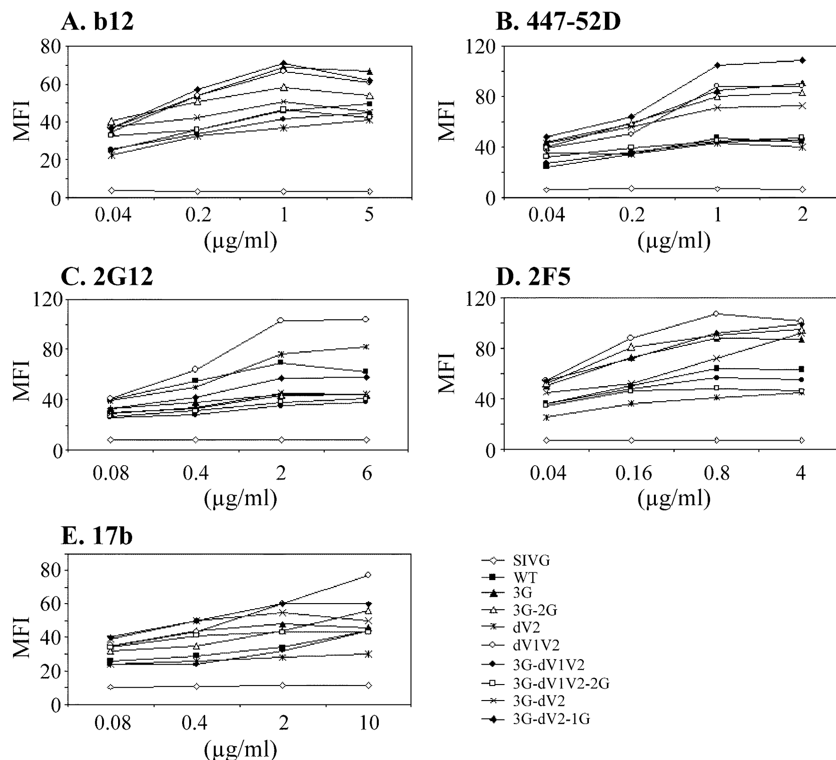


Fig. 5. Binding of neutralizing antibodies to the WT and mutant HIV Env proteins expressed in Sf9 insect cells. Cells were infected with rBV at a MOI of 10 and were harvested 1 day postinfection. Cells (1×10^5 cells/40 μl) were stained with an antibody and levels of antibody binding were determined by flow cytometry analysis. SIVG, cells infected with rBV expressing SIV Gag protein. WT, 3G, 3G + 2G, dV2, dV1V2, 3G-dV1V2, and 3G-dV1V2-2G are described in Fig. 1. (A) Anti-CD4 binding site (CD4bs) Mab, b12. (B) Anti-V3 loop monoclonal antibody (Mab) 447-52D. (C) Carbohydrate and conformation-dependent Mab, 2G12. (D) Mab 2F5 recognizing a gp41 epitope near the TM domain. (E) CD4 induced epitope-specific Mab, 17b. Compared to a negative control, positive cells were gated to evaluate the geometric MFI as indicated. A representative was shown from three independent experiments.

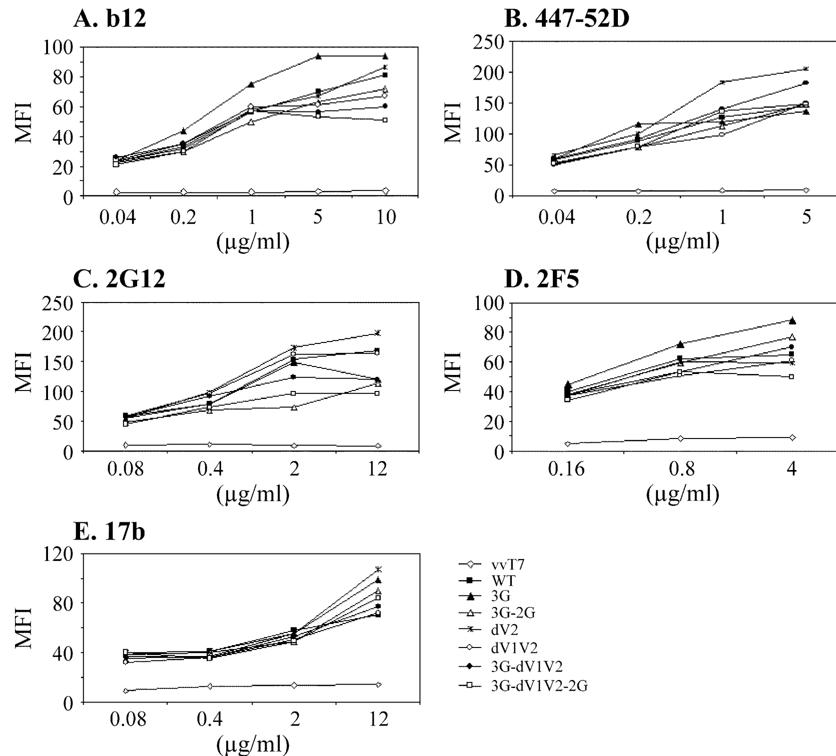


Fig. 6. Binding of neutralizing antibodies to WT and mutant HIV Env proteins expressed in mammalian cells using a rVV expression system. CV-1 cells were infected with a rVV at a MOI of 2 and were harvested after 1 day postinfection. WT, 3G, 3G + 2G, dV2, dV1V2, 3G-dV1V2, and 3G-dV1V2-2G are the same as described earlier. VV T7: rVV expressing T7 polymerase. Cells (1×10^5) were stained with the same antibodies as described in Fig. 5. A representative from three independent experiments is shown.

mutations showed lower binding compared to the WT Env (Fig. 6C). In contrast, a deglycosylation mutant 3G displayed increased binding to anti-gp41 Mab 2F5 compared to other mutants. Overall, a variable loop-deleted mutant dV2 (or dV1V2 in insect cell expression) and the deglycosylation mutant 3G or some mutants containing a 3G mutation showed higher binding to most Mabs, which was similar although not identical to the pattern observed in mutants expressed in insect cells. However, several mutations resulted in decreased binding to most Mabs tested, indicating complex interactions between variable loops and glycosylations in maintaining the conformation of HIV Env.

Discussion

Immunogens presenting the epitopes for neutralizing antibodies in a way that mimics their structure on the native HIV-1 Env glycoprotein may be able to induce a polyclonal response that includes broadly reactive neutralizing antibodies. As an approach to identify such an immunogen, we generated a series of genetically modified HIV Env proteins, expressed them in both insect and animal cells, characterized their functional properties, and monitored their antigenic characteristics using broadly reactive neutralizing Mabs.

The most interesting mutant Env protein that we identified is 3G, which has three deglycosylations at aa 304, 341, and 363. To locate these three N-glycan motifs in a three-dimensional structure model of gp120, the structure was plotted as shown in Fig. 7 by following the coordinates of the HXBc2 strain gp120 crystal structure reported previously (Kwong et al., 1998; Wyatt et al., 1998). Since there are few amino acid deletions or insertions in variable loops between HXBc2 and 89.6 strains, aa 304, 341, and 363 in 89.6 strain Env are equivalent to aa 301, 339, and 362 in the HXBc2 strain, respectively. Although there are dramatic antigenic differences between primary and laboratory-adapted HIV-1, the structure of gp120 cores from these isolates was demonstrated to be remarkably similar and sequence changes were mostly positioned on the surface (Kwong et al., 2001). Glycan Asn304 located near the coreceptor binding domain is very well conserved in primary isolates (95%). Also, this site is known to play a role in modulating coreceptor usage and in resisting neutralization by antibodies (Malenbaum et al., 2000, 2001; Polzer et al., 2002). Asn341, which is located in the helix of the outer domain following the V3 loop, is relatively well preserved in HIV-1 isolates (approximately 75%), and mutation of Asn341 to Gln resulted in an increase for binding to the neutralizing b12 Mab (Scanlan et al., 2002). As shown in Fig. 7, Asn363 is located in the middle of the C3 domain near the critical residues for CD4 binding

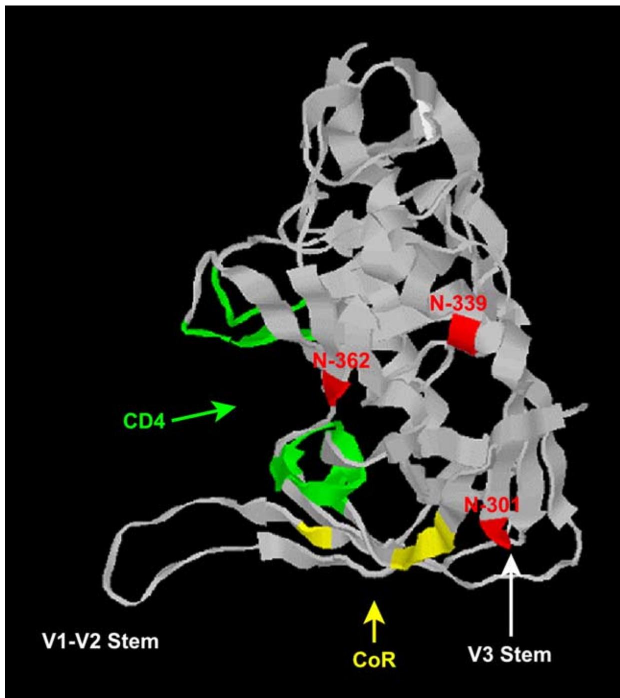


Fig. 7. Location of three Asn aa (mutant 3G) in the three-dimensional structure by plotting in the structure of the HIV-1 HXBc2 gp120 core (Kwong et al., 1998). The structure was visualized using the RasMol view program (www.umass.edu/microbio/rasmol/getras.htm). Three Asn residues at 301, 339, and 362 equivalent to aa 304, 341, and 363 in 89.6 strain Env protein are shown in red in the structure. The critical amino acids involved in binding CD4 and coreceptor are shown in green and yellow, respectively. The stems of the missing variable loops V1/V2 and V3 are indicated.

(Asp369 to Glu371) (Kwong et al., 1998) and the HIV 89.6 Env has an N-glycan motif at this position. The 3G mutant retained the normal level of precursor processing, cell surface expression and CD4 binding, and exhibited significantly enhanced syncytia forming activity. Importantly, this mutant showed higher binding to the most broadly neutralizing Mabs b12, 17b, and 2F5, indicating that neutralizing epitopes may be more highly exposed in this Env structure. Consistent with our results, Koch et al. (2003) recently reported that primary isolate mutants lacking the N-glycan at aa 301 in the V3 loop became to be more sensitive to neutralization by CD4bs and V3 loop-directed antibodies, and that additional mutations of N-glycan motifs proximal to receptor binding regions rendered these mutants sensitive to CD4i antibodies. These antigenic changes might be beneficial in inducing broadly neutralizing antibodies since cross-neutralization by sera from infected individuals is principally attributed to antibodies against the conserved epitopes (Moore and Ho, 1993; VanCott et al., 1995; Zhang et al., 1999).

Unexpectedly, combination mutations of variable loop deletions and deglycosylations resulted in less efficient binding to most neutralizing Mabs. An interesting result was observed in syncytia-forming functions of these mutant proteins. The deglycosylation mutant 3G showed the highest syncytium formation activity while variable loop deletion mutant dV1V2 retained a similar level of syncytium formation as WT. However, the combined mutant 3G-dV1V2 did not show any detectable syncytium formation. The defect of the 3G-dV1V2 mutant in inducing syncytium formation was not due to the inability to bind to CD4, since this mutant showed a similar level of CD4 binding as the WT Env. Interestingly, further deglycosylation of gp41 in mutant 3G-dV1V2-2G restored the syncytium formation to the level of WT. Thus, variable loops and carbohydrates may interact interdependently in maintaining protein function and antigenic properties. Deglycosylations in the gp41 domain (mutants containing 1G or 2G) did not affect CD4 binding or syncytia-forming functions, which is expected from a previous report that mutations of these glycosylation sites were dispensable for viral replication in various cells (Johnson et al., 2001). A unique combination mutant 3G-dV2-1G highly increased the binding of Mabs b12, 447-52D, and 2F5 compared to the counterpart mutant 3G-dV2. These results indicate that glycans in gp41 play a role in gp120 and gp41 interactions or modulating the antigenicity of both gp41 and gp120.

The effect of variable-loop deletions on exposing important neutralizing epitopes is not well understood yet. Variable loop-deleted Env mutants expressed in mammalian cells did not show a significant increase in binding to most Mabs compared to the wild-type Env although the dV2 mutant showed a slight increase in binding to the V3 loop-specific 447-52D, carbohydrate-dependent 2G12, and CD4i-epitope recognizing 17b Mabs at relatively high concentrations of antibody. Our results are consistent with studies of a V2 loop-deleted mutant HIV SF162 Env expressed in oligomeric gp140 form that showed higher exposure of the V3 loop epitope but not CD4bs epitope (Stamatatos et al., 2000). HIV-1 Env mutants lacking the V1 and V2 loops exhibited increased sensitivity to neutralization by antibodies directed against the V3 loop and a CD4-induced epitope on gp120, and by sera collected from patients infected with different clades of HIV-1 primary isolates (Cao et al., 1997; Stamatatos and Cheng-Mayer, 1998b). Serum from a monkey immunized with a V2 loop-deleted immunogen was shown to have higher levels of antibodies capable of neutralizing heterologous HIV-1 isolates than the parental strain SF162 (Barnett et al., 2001). However, only selected heterologous isolates were susceptible to neutralization by this serum and titers were lower than those against the homologous SF162 strain. The same group also reported that the V2 loop deletion did not increase the immunogenicity of the CD4bs even though it altered the immunogenicity of the V3 loop and C5 domain (Srivastava et al., 2003). Also, by immunizing with rVV expressing a V1–V2 loop-deleted HIV-1 gp160 and boosting with the same mutant protein, higher neutralizing activity was not achieved (Kim et al., 2003). Our study and others therefore suggest that simply deleting the variable loops may not expose conserved neutralizing

epitopes like the CD4 binding site better than the wild-type Env protein.

The antigenicity of HIV Env proteins expressed in the rBV expression system has not been studied well although it may be an attractive system to produce a vaccine candidate (Deml et al., 1997; Guo et al., 2003; Kang and Compans, 2003; Kang et al., 1999; Wagner et al., 1996, 1998). In contrast to mammalian cells, significantly enhanced binding to all neutralizing Mabs tested was observed in the V1–V2 variable loop-deleted mutant (dV1V2) expressed in insect cells. This may be due to differences in posttranslational modification between insect and animal cells. It has been shown that the proteolytic processing of HIV Env is not efficient in the baculovirus virus expression system (Hu et al., 1987). Our results show that, in insect cells, mutant and WT HIV Env proteins were expressed predominantly in the unprocessed gp160 form. However, inefficient processing does not imply that such Env proteins cannot be good vaccine candidates. SF162 Env with V2 loop deletion when expressed in a cleavage-defective oligomeric gp140 showed higher binding to antibodies against the CD4bs (b12) or V3 epitopes (391-95D) (Stamatatos et al., 2000). It was also shown that elimination of the cleavage site did not affect the ability of a mutant protein to elicit neutralizing antibodies (Srivastava et al., 2003). Another difference is that N-glycans in insect cells are high-mannose-type oligosaccharides compared to the complex-type oligosaccharides usually produced in mammalian cells (Lanford et al., 1989). Reflecting this difference, greater differences in binding to carbohydrate-dependent Mab 2G12 were observed in Env proteins expressed in insect cells.

To our knowledge, this is the first report investigating the antigenicity of HIV Env mutants expressed in insect cells and the effects of combined Env mutations. Based on the results, several mutants (3G, dV1V2, 3G-dV1V2-1G) produced in the baculovirus expression system may be good candidates to evaluate the effect of deglycosylation or variable loop deletions on inducing broadly neutralizing antibodies. We are currently comparing the immunogenicity of WT and mutant Envs to address some of these questions. These ongoing studies will provide valuable information on designing AIDS vaccine candidate antigens inducing neutralizing antibodies.

Materials and methods

Cells, viruses, and antibodies

Spodoptera frugiperda Sf 9 cells were maintained in serum-free SF900 II medium (GIBCO-BRL) in spinner flasks at a speed of 70–80 rpm. Baculovirus titers were determined by a plaque assay following the manufacturer's protocol (BD PharMingen). A baculovirus-expressing SIV Gag was previously described (Yamshchikov et al., 1995). CV-1, HeLa, and HeLa T4 cells were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum (HyClone Laboratories). The recombinant vaccinia virus (rVV) vTF7-3 and monkey anti-SHIV 89.6 sera were kindly provided by Drs. Bernard Moss (NIH) and Patricia Fultz (University of Alabama at Birmingham), respectively. All antibodies used in this study for flow cytometry analysis, soluble human CD4 (cat #7356), and rabbit antihuman CD4 serum (cat #806) were obtained from the NIH AIDS Research and Reference Reagent Program. HIV-1 neutralizing serum (cat #1984) neutralizes several HIV-1 strains, including IIIB/LAV, RF, MN, and SF2. Anti-V3 loop monoclonal antibody (Mab) 447-52D (cat #4030) neutralizes laboratory strains that contain the GPGR sequence at the apex of the V3 loop (Gorny et al., 1992). Anti-CD4 binding site (CD4bs) Mab b12 (cat #2640) is a potent antibody that neutralizes diverse primary HIV-1 isolates (Burton et al., 1991). Anti-CD4bs Mab F105 (cat #857) reacts with a discontinuous gp120 epitope and neutralizes SF2, IIIB, and MN strains of HIV-1 (Posner et al., 1993). Carbohydrate and conformation-dependent Mab 2G12 (cat #1476) neutralizes laboratory HIV-1 strains IIIB, RF, and a broad variety of primary isolates (Buchacher et al., 1994). CD4-induced epitope-specific Mab 17b (cat #4091) has neutralizing activity against several laboratory strains of HIV-1 and some primary isolates (Sullivan et al., 1998). A gp41 epitope-specific Mab 2F5 (cat #1475) neutralizes a broad variety of laboratory HIV-1 strains and primary isolates (Purtscher et al., 1996).

Plasmid construction

We used a rBV transfer vector pc/pS1-89.6 encoding the HIV 89.6 Env protein with the cytoplasmic tail truncated to 17 amino acids (aa), allowing better cell surface expression compared with the full-length Env glycoprotein (Yao et al., 2000). For site-directed mutagenesis, the Env encoding gene from pc/pS1-89.6 was subcloned to a small shuttle vector pGEM (Promega) that is easy to manipulate and the cloned plasmid designated pGEM-89.6. For mutations eliminating glycosylation sites, we applied a QuickChange site-directed mutagenesis kit (Stratagene) using the high fidelity polymerase with a proofreading activity. The paired mutagenic PCR primers (Sigma Genosys) containing the Gln codon CAG at the Asn position to be mutated were designed to be complementary to each other. The primer pairs are as follows and the numbers indicate the position being mutated to Gln based on HIV 89.6 Env. F-304, 5′ ACA AGA CCC AAC CAG AAT ACA AGA AGA AGG 3′; R-304, 5′ CCT TCT TCT TGT ATT CTG GTT GGG TCT TGT 3′; F-341, 5′-AGT AGA GCA AAA TGG CAG AAC ACT TTA CAA CAG-3′; R-341, 5′-CTG TTG TAA AGT GTT CTG CCA TTT TGC TCT ACT-3′; F-363, 5′AAA ACA ATA GCC TTT CAG CAA TCC TCA GGA GG-3′; R-363, 5′-CC TCC TGA GGA TTG CTG AAA GGC TAT TGT TTT-3′; F-623, 5′ GATGAT ATT TGG AAT CAG ATG ACC TGG ATG

GAG 3′; R-623, 5′ CTC CAT CCA GGT CAT CTG ATT CCA AAT ATC ATC 3′; F-635, 5′ GG GAA AGA GAA ATT GAC CAG TAC ACA GAC TAT A 3′; R-635, 5′-T ATA GTC TGT GTA CTG GTC AAT TTC TCT TTC CC 3′. The thermocycle was 95 °C for 50 s, 55 °C for 1 min 20 s, and 68 °C for 10 min. The PCR product was treated with *Dpn*I (Stratagene) to digest a methylated template DNA. The *Dpn*I-treated PCR product was transformed into competent *Escherichia coli* bacteria DH5α and the intended mutated region of pGEM plasmids was confirmed by DNA sequencing (Emory University Sequencing Facility). For multiple mutations (3G or 3G-2G in Fig. 1), additional mutations were introduced sequentially using the mutated pGEM-89.6 as a template.

The technique of overlap PCR extension was applied for generating variable loop deletion mutations (Ho et al., 1989). For V1–V2 loop deletion of aa 128–194, two PCRs were performed to amplify two coding sequences covering aa 1–128 and the remaining part of the gene covering aa 194 to the C-terminal end. The deleted domain was replaced with a linker of three amino acids, Gly–Ala–Gly between aa 128 and 194. N-terminus PCR primer set; F1: GGTACCCGGGCAGAAGACAG (underline denotes *Sma*I recognition site) and R-V1V2: **CCT TCC TGC** TCC AAC ACA GAG TGG GGT TAA TTT T (bold type denotes codons for the 3-amino acid linker and contains the overlapping nucleotides with the C-terminus product). C-terminus PCR primer set: R1: TCGACTCTAGACCCCTC-GATCA (underline denotes *Xba*I recognition site) and F2-V1V2, **GGAGCAGGA**AGGTTAATAAGTTGTAACAC (bold type denotes codons of the 3-amino acid linker and contains the overlapping nucleotides with the N-terminus product). After PCR reactions, two PCR products were isolated separately, purified, and linked together via the 2nd round PCR with F1 and R1 primers. This PCR product was cloned into the pGEM plasmid after digestion with *Sma*I and *Xba*I restriction enzymes. For V2 loop deletion of aa 164–194, a similar protocol was applied using the following primers; F-dV2: 5′ GGA GCC GGG AGG TTA ATA AGT TGT AAC ACC TCA GTC-3′, R-dV2: 5′ GAT ATA GAA AGA GCA ATT TTT TAT TTC 3′. All mutant pGEM plasmids were sequenced to confirm that the desired mutations were obtained. For combined mutations containing variable loop deletions and deglycosylations, site-directed mutagenesis of glycosylation sites was performed using the variable loop-deleted pGEM mutant plasmid (dV1V2 or dV2 in Fig. 1) as a template DNA.

Generation of recombinant baculoviruses (rBV) and vaccinia viruses (rVV)

The confirmed mutant HIV *env* genes (in pGEM vector) as described above were cloned into the baculovirus transfer vector pc/pS1 for the construction of rBVs. To obtain rBVs expressing mutant HIV Env proteins, Sf9 insect cells were cotransfected with linearized AcNPV Baculogold DNA (BD PharMingen) and a pc/pS1 transfer vector containing the mutated HIV *env* gene following the manufacturer's procedure. Five days after co-transfection, the media containing recombinant viruses were collected by centrifugation at 1500 × *g* for 20 min. Since Baculogold DNA generates greater than 99% pure recombinant virus population (BD PharMingen), the recombinant virus in the culture supernatant was further propagated and a plaque assay performed and used for protein expression.

For construction of rVVs expressing mutant HIV Env protein, the confirmed mutant HIV *env* genes from pGEM DNAs were cloned into the plasmid pRB21 with a strong synthetic VV early/late promoter (Blasco and Moss, 1995) using *Eco*RI and *Hin*dIII restriction enzymes. CV-1 cells infected with a mutant nonplaque-forming VV vRB12 were transfected with a pRB21 plasmid containing a mutant or wild-type HIV *env* gene using Lipofectin transfection reagent (Invitrogen) following the manufacturer's procedure. Recombinant VVs were identified by their ability to form plaques and single plaques were purified for three rounds following a standard protocol (Blasco and Moss, 1995). The rVV stocks were propagated on CV-1 cells and their titers determined and used for protein expression.

Protein expression

Expression of modified HIV Env proteins was confirmed by metabolic labeling of infected Sf9 insect cells or HeLa cells and immunoprecipitation with a monkey anti-SHIV 89.6 sera. Sf9 cells were infected with an rBV containing wild type or mutant HIV *env* gene at a multiplicity of infection (MOI) of 10. The cells were starved with SF900 II methionine and cysteine-deficient medium (GIBCO-BRL) at 24 h postinfection for 30 min, and labeled with 100 μC of [^{35}S]methionine and [^{35}S]cysteine mix/ml for 1 h at 27 °C. Cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.5], 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, protease inhibitor cocktail tablet (Roche Molecular Biochemicals) for 10 min on ice. The cell lysate was immunoprecipitated with monkey anti-SHIV sera at 4 °C for 2 h and protein A overnight. The precipitate was washed three times in RIPA buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and subsequently by autoradiography. For rVV expression, HeLa cells (1 × 10^6 cells) were infected with rVV at MOI of 1. Cells were starved with DMEM with Met and Cys for 30 min at 16 h postinfection. Cells were labeled as above and lysed in 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 1% Triton X-100, 1% Na-deoxycholate, 150 mM NaCl. The cell lysates were immunoprecipitated and analyzed as above.

Antibody binding analysis by flow cytometry

Measurement of antibody binding to Sf9 or CV-1 cells expressing modified HIV-1 Env proteins was performed by

fluorescence-activated cell sorting (FACS) analysis following a similar procedure as described (Koch et al., 2003; Si et al., 2001, 2003; Wyatt et al., 1995; Zolla-Pazner et al., 1995). Sf9 insect cells were infected with a rBV (MOI = 10) expressing SIV Gag (SIVG) as a negative control, wild type (WT), or mutant Env protein and cultured in suspension. After 1 day postinfection, cells were harvested. Sf9 cells (1×10^5) were stained in 40 μl of staining buffer (phosphate-buffered saline (PBS), 2% bovine serum albumin). The concentrations of Mabs used for staining 1×10^5 cells were 0.04–2 μg/ml of 447-52D, 0.04–10 μg/ml of b12, 0.2–5 μg/ml of F105, 0.08–12 μg/ml of 2G12, 0.08–12 μg/ml of 17b, and 0.16–4 μg/ml of 2F5. Neutralizing serum was diluted 40- to 80-fold. After 1 h of incubation at room temperature (RT), cells were washed twice with 200 μl of staining buffer and resuspended in 40 μl of staining buffer containing a 1:200 dilution of phycoerythrin-conjugated goat antihuman antibody (Southern Biotech). The binding reaction mixtures were incubated at RT for another 1 h. Cells were washed two times with staining buffer, suspended in 200 μl of 0.5% paraformaldehyde in PBS buffer, and analyzed with a FACS Calibur instrument (Becton Dickinson) and WINMDI 2.8 software (Scripps Research Institute Cytometry Software). Comparing to a negative control, only positive cells were gated to evaluate the geometric mean fluorescence intensity (MFI) (see Fig. 2C).

CV-1 cells were infected with rVVs (MOI = 2) expressing wild type (WT) or mutant HIV Env protein or vTF7-3 expressing T7 polymerase (VV T7) as a negative control. At 18–20 h postinfection, CV-1 cells were detached with 5 mM EDTA in PBS, washed in PBS, and suspended in staining buffer. CV-1 cells (1×10^5) were stained with antibodies and analyzed by FACS in the same way as described above.

CD4 binding and syncytium formation assays

For CD4 binding assay, CV-1 cells grown on 96-well plate (4×10^4 cells/well) were infected with a rVV at a MOI of 2. At 18–20 h postinfection, cells were washed with PBS and fixed with 0.05% glutaraldehyde in PBS at 4 °C for 1 h. After washing with staining buffer, cells were incubated with soluble human CD4 at concentrations of 0.01–8 μg/ml in staining buffer at RT for 1 h. The amounts of bound CD4 were determined using rabbit antihuman CD4 serum (1:10 000) followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG polyclonal antibody (1:2000). HRP substrate ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-sulfonic acid)] containing H_2O_2 (Sigma) was used to develop color and OD_{405} was read by ELISA reader (MTX Lab Systems). Alternatively, the CD4 binding was also determined by FACS analysis. The infected CV-1 or Sf9 insect cells were harvested at 20 h postinfection, and incubated serially with CD4, rabbit antihuman CD4 serum, and FITC-conjugated goat anti-rabbit IgG (1:200) as described above.

In previous studies, the fusion activity of HIV Env has been determined by analyzing the formation of syncytia (Doranz et al., 1996; Raja et al., 2003; Wyatt et al., 1995). HeLa-CD4 cells were infected with rVVs expressing wild type or mutant Env protein at a MOI of 0.5. Between 16 and 20 h postinfection, the formation of syncytia was monitored under a light microscope and photographed. Syncytia of multinuclear cells were counted at five different fields in a well and averaged to evaluate the syncytium forming ability.

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