Highly conserved HIV-1 gp120 glycans proximal to CD4-binding region affect viral infectivity and neutralizing antibody induction

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Glycosylation plays important roles in gp120 structure and HIV-1 immune evasion. In the current study, we introduced deglycosylations into the 24 N-linked glycosylation sites of a R5 env MWS2 cloned from semen and systematically analyzed the impact on infectivity, antigenicity, immunogenicity and sensitivity to entry inhibitors. We found that mutants N156-T158A, N197-S199A, N262-S264A and N410-T412A conferred decreased infectivity and enhanced sensitivity to a series of antibodies and entry inhibitors. When mice were immunized with the DNA of wild-type or mutated gp160, gp140 or gp120; N156-T158A, N262-S264A and N410-T412A were more effective in inducing neutralizing activity against wild-type MWS2 as well as heterologous IIIB and CH811 Env.s. In general, gp160 and gp140 induced higher neutralizing activity compared with gp120. Our study demonstrates for the first time that removal of individual glycan N156, N262 or N410 proximal to CD4-binding region impairs viral infectivity and results in enhanced capability to induce neutralizing activity.

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Introduction

The ideal strategy for HIV-1 prevention is an effective prophylactic vaccine. Seeking an antigen capable of inducing broad and potent neutralizing antibody is the primary goal of prophylactic vaccine development. HIV-1 gp120 is considered the principal target for preventative vaccines (Graham and Wright, 1995; Pantophlet and Burton, 2006). However, HIV-1 has evolved effective mechanisms to escape antibody neutralization. For instance, gp120 is highly glycosylated, with carbohydrates accounting for as much as 50% of its mass. Such carbohydrate moieties are proposed to act as shields occluding highly immunogenic epitopes on gp120. Early vaccines based on unmodified gp120 monomers induced limited breadth of neutralization, only effective against T cell line adapted isolates (Berman et al., 1990; Cooney et al., 1993; Gilbert et al., 2005; Schultz and Bradac, 2001) rather than primary strains (Bures et al., 2005; Connor et al., 1998; Flynn et al., 2005; Hanson, 1994; Mascola et al., 1996; Matthews, 1994; Pittsuttithum et al., 2006). Although there is growing evidence that trimeric gp140 may represent a better vaccine candidate (Beddows et al., 2007; Earl et al., 2001; Jeffs et al., 2004; Kim et al., 2005), development of an effective preventive vaccine remains elusive.

The carbohydrate moieties on gp120 are synthesized by the infected host cells and act as “shields” to mask the conserved CD4 binding site. gp120 promotes virus entry into host cells by sequential binding to receptor CD4 and co-receptor CCR5 or CXCR4. Deletion of these glycans may modulate the recognition of receptor or co-receptor binding sites masked by carbohydrates. Several studies have described that modification of gp120 glycosylation sites affects viral entry by interfering with receptor binding (Dunfee et al., 2007; Malenbaum et al., 2000). For instance, removal of several individual N-linked glycosylation sites of HXB2 gp120 in the canonical Asn-x-Ser/Thr sequence impairs viral infection (Lee et al., 1992). Deglycosylation at a single N-linked glycan site N197 alters the steric configuration of CD4 binding sites and promotes CD4-independent infection of HIV-1 (Kolchinsky et al., 2001). In addition, N-linked glycosylation sites may be involved in regulating the interaction between CD4 and co-receptors CCR5 (Ly and Stamatatos, 2000; Ogert et al., 2001; Pollakis et al., 2001) or CXCR4 (Dumonceaux et al., 2001; Polzer et al., 2001).

The current challenge for design of a protective HIV-1 vaccine based on gp120 is to develop suitable strategies to enhance its antigenicity and immunogenicity. Although intense investigation has revealed that the antigenicity of HIV-1 Env can be changed by removing certain glycans (Cole et al., 2004; Duenas-Decamp et al., 2007; Earl et al., 2001; Jeffs et al., 2004; Kim et al., 2005), development of an effective preventive vaccine remains elusive.

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whether and how deglycosylations affect the immunogenicity of Env has not been characterized systematically. Certain site specific deglycosylations or their combinations on HIV-1 Env have been suggested to enhance the induction of neutralizing activity (Bolmstedt et al., 1996). Similar results have also been observed with SIV Env (Mori et al., 2005; Reitter et al., 1998). In particular, multiple deglycosylation combinations in the V1V2 region of HIV-1 89.6 Env induce neutralizing activity more effectively than the wild-type (WT) Env (Quinones-Kochs et al., 2002). Moreover, deletion of a single glycosylation site at N197 of HIV-1 89.6 gp140 trimer enhances the ability to induce neutralizing responses (Li et al., 2008). However, the effect of deglycosylations on immunogenicity needs to be clarified as most previous studies deleted glycans by introducing a conservative first-site amino acid substitution from Asn-x-Thr/Ser to Gln-x-Thr/Ser and were mainly based on laboratory-adapted or clade B gp160, gp140 or gp120 (Lee et al., 2002; Quinones-Kochs et al., 2002). It is not clear whether such modification of structure and function of Env are due to amino acid substitutions or the loss of glycans. Moreover, the impact of removal of glycans proximal to CD4 binding region remains to be addressed, while a systematic comparison of the immunogenicity of gp160, gp140 and gp120 delivered via DNA immunization has not been fully explored.

Given that sexual transmission is the leading mode of HIV-1 infection worldwide, it will be important to design vaccines against sexual transmitted isolates, in particular those clades spreading most rapidly in the world. In the current study, we introduced a series of conservative third-site substitutions from serine/threonine to alanine or threonine/serine in the 24 N-linked glycosylation sites of gp120 based on a clade C MWS2 env cloned from semen of a subject known to have infected women by vaginal intercourse. The strategy of using conserved third-site substitutions in the canonical Asn-x-Thr/Ser/Thr sequences from Ser/Thr to Ala or Thr/Ser allows parallel assessments of glycan mutants and amino acid mutants. We examined the effect of removal of individual gp120 glycans on viral infectivity as well as sensitivity to antibodies, CCR5 antagonists and carbohydrate-binding agents (CBAs). Additionally, we compared the immunogenicity of gp160, gp140 and gp120 by vaccination of mice with corresponding DNA.

Results

Infectivity of Env glycan mutants

To better understand the roles of Env glycans, we constructed deglycosylated mutants based on a primary clade C R5 MWS2 Env cloned from semen. Subsequent experiments were carried out in an infection assay using Env pseudotyped viruses to infect TZM-bl cells. The infectivity of WT, respectively, while other glycan mutants had infectivity ranging from 61% to 113% of the WT (Table 1 and Fig. 1A). A similar decrease).

![Table 1 Infectivity and sensitivity to HIV-IG of N-linked glycan mutants of MWS2 Env.](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>N-glycan site</th>
<th>Env mutant</th>
<th>Amino acid change</th>
<th>Viral infectivity</th>
<th>HIV-IG neutralization a</th>
</tr>
</thead>
<tbody>
<tr>
<td>88</td>
<td>N88-T90A</td>
<td>NVT → NVA</td>
<td>100%</td>
<td>5.20</td>
</tr>
<tr>
<td>131</td>
<td>N88-T90S</td>
<td>NVT → NVS</td>
<td>68%</td>
<td>5.81</td>
</tr>
<tr>
<td>134</td>
<td>N131-T133A</td>
<td>NTS → NSA</td>
<td>74%</td>
<td>0.82</td>
</tr>
<tr>
<td>140</td>
<td>N131-T133S</td>
<td>NTS → NNS</td>
<td>86%</td>
<td>ND</td>
</tr>
<tr>
<td>156</td>
<td>N156-T158A</td>
<td>NTS → NTA</td>
<td>76%</td>
<td>1.25</td>
</tr>
<tr>
<td>156</td>
<td>N156-T158S</td>
<td>NTS → NTA</td>
<td>76%</td>
<td>1.25</td>
</tr>
<tr>
<td>160</td>
<td>N160-T162A</td>
<td>NTS → NVS</td>
<td>76%</td>
<td>1.25</td>
</tr>
<tr>
<td>160</td>
<td>N160-T162S</td>
<td>NTS → NVA</td>
<td>78%</td>
<td>1.25</td>
</tr>
<tr>
<td>186</td>
<td>N186-S188A</td>
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<td>102%</td>
<td>6.13</td>
</tr>
<tr>
<td>197</td>
<td>N197-S199A</td>
<td>NTS → NTA</td>
<td>102%</td>
<td>6.13</td>
</tr>
<tr>
<td>230</td>
<td>N230-T232A</td>
<td>NTK → NKA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>234</td>
<td>N234-T236A</td>
<td>NKT → NKA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>234</td>
<td>N234-T236S</td>
<td>NKT → NKS</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>234</td>
<td>N234-T236S</td>
<td>NKT → NKS</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>262</td>
<td>N262-S264A</td>
<td>NKS → NVA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>262</td>
<td>N262-S264A</td>
<td>NKS → NVA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>276</td>
<td>N276-T278A</td>
<td>NIT → NIA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>276</td>
<td>N276-T278A</td>
<td>NIT → NIA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>295</td>
<td>N295-T297A</td>
<td>NTA → NCA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>295</td>
<td>N295-T297S</td>
<td>NTA → NCA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>301</td>
<td>N301-T303A</td>
<td>NTA → NNA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>332</td>
<td>N332-S334A</td>
<td>NIS → NIS</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>339</td>
<td>N339-T341A</td>
<td>NTK → NKA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>335</td>
<td>N335-S337A</td>
<td>NIS → NIS</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>368</td>
<td>N368-S388A</td>
<td>NTS → NTA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>392</td>
<td>N392-T394A</td>
<td>NTA → NTA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>432</td>
<td>N432-T434A</td>
<td>NTS → NTS</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>432</td>
<td>N432-T434A</td>
<td>NTS → NTS</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>448</td>
<td>N448-T450A</td>
<td>NIT → NIA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>448</td>
<td>N448-T450S</td>
<td>NIT → NIS</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>462</td>
<td>N462-T464A</td>
<td>NRT → NRA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>462</td>
<td>N462-T464S</td>
<td>NRT → NRS</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>465</td>
<td>N465-T467A</td>
<td>NTE → NEA</td>
<td>73%</td>
<td>3.32</td>
</tr>
<tr>
<td>465</td>
<td>N465-T467S</td>
<td>NTE → NES</td>
<td>73%</td>
<td>3.32</td>
</tr>
</tbody>
</table>

Bold data imply that mutants had significantly decreased infectivity, or increased/decreased sensitivity to antibodies or entry inhibitors. Data are representative of at least three independent experiments (± decrease). a IC50 (µg/ml).

To assess whether the reduction of pseudotype-virus infectivity was due to decreased protein expression, we detected the expression of Env in HeLa cells by Western blot analysis. We analyzed the gray-scale values using Bio-Rad Discovery software. The relative gp160 expression levels of WT, N156-T158A, N262-S264A and N410-T412A were 100, 115, 87 and 129, while the relative infectivity levels of WT, N156-T158A, N262-S264A and N410-T412A were 100, 15, 25 and 77, respectively (Fig. 1B and Table 1), indicating that there was no correlation between the level of protein expression and viral infectivity. We further examined cell surface expression of Env by flow cytometry. The expression levels of WT, N156-T158A, N262-S264A and N410-T412A Env were similar as detected by HIV-IG (Fig. 1C). We also assessed expression using the PG9 MAb shown to recognize overlapping epitopes preferentially expressed on the membrane-anchored trimeric Env with its neutralization activity dependent on glycan N160 and to a lesser extent glycan N156 (Davenport et al., 2011; Doore and Burton, 2010; Walker et al., 2009). At the antibody concentration used, we did not observe significant difference in terms of cell surface expression when PG9 (Fig. 1C) was used to detect the expression of WT and glycan mutants. Our data together indicate that the decreased infectivity of Env glycan mutants is unlikely due to the reduction of Env expression.

To confirm that our observations were glycan specific rather than due to amino acid change, we constructed a panel of Env mutants by third-site substitution from Ser to Thr or Thr to Ser at the N-linked
Sensitivity of Env glycan mutants

The N-linked glycan sites on gp120 are highly conserved, having been dubbed as being on the “silent face” of gp120 (Kwong et al., 1998; Wyatt and Sodroski, 1998). Such carbohydrates can render the underlying protein surface invisible to humoral immunity (Pantophlet and Burton, 2006; Wyatt and Sodroski, 1998); depletion of glycans could make gp120 more accessible to antibodies. To test this hypothesis, we examined the sensitivity of the 24 MWS2 Env glycan mutants to HIV-IG. The IC50 of HIV-IG against WT was 5.20 μg/ml, whereas N131-T133A, N134-T136A, N156-T158A, N197-S199A, N262-S264A, N332-S334A, N339-T341A, N410-T412A and N448-T450A mutants demonstrated enhanced sensitivity to HIV-IG, with IC50 values of 0.82, 1.25, 0.42, 0.69, 0.77, 1.66, 1.47, 1.30 and 1.20 μg/ml, respectively (Fig. 2A and Table 1). Other glycan mutants had similar sensitivity to HIV-IG as that of WT. Our data suggest that deglycosylation at these sites may increase the number or exposure of available neutralization epitopes on gp120 to antibodies which already exist in human immunoglobulins from HIV+ individuals.

To further investigate the significance of these glycosylation sites, we retrieved gp160 sequences from the HIV database (http://hiv-web.lanl.gov) and analyzed the conservation level of glycosylation sites. Compared with that of the glycan mutants N156-T158A, N262-S264A and N448-T450A, the infectivity of N156-T158S, N262-S264T and N448-T450S was largely maintained (Table 1). As infectivity is closely related with the steric configuration of Env, these data suggest that removal of glycans, in particular at N156, N262 or N448, is likely to have modified the structure and function of the HIV-1 Env.
glycosylation sites in the gp120 region of clade A, B, C and D Envs. More than half of the glycosylation sites have a conservation ratio over 80% (Supplementary Fig. 1). In particular, N156, N197 and N262 are highly conserved, with an identity greater than 88%. In comparison, N410 is significantly less conserved. Subsequently, experiments were focused on N156-T158A, N197-S199A, N262-S264A and N410-T412A Env mutants because the role of these glycans in infection and antigenicity has yet to be defined.

To elucidate the potential exposure of neutralizing epitopes induced by removal of specific glycans, we carried out infection experiments using a range of well-characterized MAbs and CD4-IgG2 (Pro-542). The neutralization sensitivity of N156-T158A and N197-S199A to CD4-IgG2, which recognizes conserved CD4 binding sites, was significantly augmented, while N410-T412A did not alter susceptibility to CD4-IgG2 (Fig. 2B and Table 2). However, N262-S264A decreased the sensitivity to CD4-IgG2 and showed slightly decreased sensitivity to 4E10, a monoclonal antibody that recognizes the gp41 sequence NWFDIT, conserved among many HIV-1 strains, whereas N156-T158A, N197-S199A and N410-T412A showed slightly enhanced sensitivity to 4E10. Compared to the WT, N262-S264A and N410-T412A mutants showed enhanced sensitivity to a broadly neutralizing MAb 2G12 (~15–13-fold, respectively), the binding epitope of which involves high-mannose type oligosaccharides at N295, N332, N339, N386, N392 and N448, with the sugar chains at positions N262, N332 and N392 being most critical (Calarese et al., 2003; N332, N339, N386, N392 and N448, with the sugar chains at positions N262, N332 and N392 being most critical (Calarese et al., 2003; Sanders et al., 2002; Scanlan et al., 2002). N156-T158A also demonstrated a modest increased sensitivity to 2G12 (~4-fold) while the sensitivity of N197-S199A to 2G12 remained unaffected. N156-T158A was significantly sensitive to 17b in the absence of CD4. Other mutants showed marginally enhanced sensitivity to 17b. Together our results indicate that removal of these glycans may be beneficial to the exposure of CD4 binding sites.

Coreceptor use and sensitivity to entry inhibitors of Env glycan mutants

Previous studies by others showed that modification of certain glycans on gp120 affected coreceptor binding/switch or facilitated CD4-independent entry (Kolchinsky et al., 2001; Pollakis et al., 2001; Polzer et al., 2002). We therefore examined the infectivity of Env mutants versus WT in CHO-CCR5 and U87.CD4.CXCR4 cells, respectively. All Envs including WT were noninfectious in these cells (data not shown), indicating that removal of N156, N197, N262 or N410 glycan does not result in CD4-independent infection or coreceptor switch.

Nevertheless, in the current study, several Env mutants, in particular N156-T158A, showed enhanced neutralization sensitivity to 17b in the absence of soluble CD4 (Table 2 and Fig. 2B), suggesting that deglycosylation in these sites on gp120 may be beneficial for the exposure of a CD4 induced epitope which only exists in the CD4-ligated form of gp120. We subsequently investigated the sensitivity of glycan mutants to a range of CCR5 antagonists to assess whether deglycosylations also affect Env-CCR5 interaction. Env glycan mutants, particularly N262-S264A, demonstrated enhanced sensitivity to the tested CCR5 inhibitors maraviroc, SCH-412147, CMPD-167 and TAK-779 (Table 2). These findings indicate that removal of gp120 glycans may also affect gp120-CCR5 interaction.

Previous studies have shown that under the selection pressure of CBAs, HIV-1 selectively lost multiple high-mannose type glycans on gp120 to escape CBA inhibition (Balzarini et al., 2006; Hu et al., 2007). Therefore we tested the sensitivity of MWS2 glycan mutants to CBAs cyanovirin (CV-N) and Galanthus nivalis agglutinin (GNA). Of interest, glycan mutants N156-T158A, N197-S199A, N262-S264A and N410-T412A had increased sensitivity to CV-N and GNA (Table 2). Of note, removal of high-mannose glycan N262 demonstrated significantly enhanced sensitivity to CV-N. The binding sites on gp120 for CV-N and GNA have been suggested to be high-mannose specific (Balzarini, 2007), whereas N156, N197 and N410 are complex type glycans and there has been no evidence indicating the involvement of N262 in the binding of tested CBAs (Balzarini, 2007; Balzarini et al., 2006). Our data suggest that removal of CBA-binding unrelated glycans on gp120 may facilitate the binding of CBAs to their specific glycan residues.

Induction of enhanced neutralizing activity in mice by Env glycan mutants

It is well documented that HIV-1 uses its glycan shields in virus-infected individuals to avoid neutralization (Wei et al., 2003). Indeed, removal of N197 glycan alone or multiple glycans including N156 in the V1V2 region of 89.6 Env results in enhanced ability to induce neutralizing activity (Li et al., 2008; Quinones-Kochs et al., 2002). In the current study, we investigated whether removal of individual N156, N262 or N410 promote neutralizing activity in a mouse model. N197 was not included because its immunogenicity was characterized in a previous study (Li et al., 2008). We immunized mice (n = 9/group) with 30 μg DNA of gp160, gp140 or gp120 glycan mutants three times by electroporation at 3 week intervals. We tested the neutralizing activity of sera from immunized mice against homologous and heterologous Envs using a well-established pseudo-typed virus neutralization system.

Ten days after the final immunization, the gp160 and gp140 forms of N156-T158A, N262-S264A or N410-T412A elicited significantly higher levels of neutralizing activity against the homologous WT MWS2 Env as well as heterologous X4 IIIB and primary R5 CH811 Envs (Fig. 3A). In contrast, the ability of gp120 monomer glycan mutants in inducing neutralizing activity was much more complex. When tested against IIIB or CH811 Env, the sera from N156-T158A, N262-S264A or N410-T412A gp120 vaccinated mice achieved higher neutralizing activity than those from WT gp120. However, when tested against MWS2 Env, the neutralizing activity elicited by gp120 mutant N156-T158A, N262-S264A or N410-T412A was very similar to that of WT gp120. In general, gp160 and gp140

Table 2
Neutralization activity of antibodies and entry inhibitors to HIV-1 MWS2 Env pseudotyped virus

<table>
<thead>
<tr>
<th>IC50 (fold change)</th>
<th>N156-T158A</th>
<th>N197-S199A</th>
<th>N262-S264A</th>
<th>N410-T412A</th>
<th>WT</th>
</tr>
</thead>
</table>

Antibody* |

| HIV-IgG* | 0.42 | 0.69 | 0.77 | 1.30 | 5.20 |
| CD4-IgG2* | 0.05 (36) | 0.05 (36) | 12.72 (71) | 1.53 | 1.82 |

17ba |

| 17ba | 2.82 | >15.00 | >15.00 | >15.00 | >15.00 |
| 2G12a | 14.97 | 68.73 | 3.97 (15) | 4.53 (13) | 58.62 |
| 4E10a | 0.63 | 0.62 | 2.76 | 1.05 | 2.51 |

CCR5 antagonist |

| Maraviroc | 15.21 | 4.73 | 1.61 (17) | 20.97 | 27.75 |
| SCH-412147b | 0.54 | 0.54 | 0.12 (26) | 1.10 | 3.06 |
| CMPD-167b | 0.82 | 1.08 | 0.24 (28) | 2.35 | 6.82 |
| TAK-779b | 204.47 | 102.81 (17) | 113.91 (15) | 679.80 | 1736.16 |

Carbohydrate binding agents |

| CV-N | 0.30 | 0.25 | 0.07 (24) | 0.34 | 1.70 |
| GNA | 1.76 | 1.67 | 2.64 | 6.61 | 12.54 |

Bold data imply that mutants had significantly decreased infectivity, or increased/ decreased sensitivity to antibodies or entry inhibitors. *MWS2 showed resistance to MAbs IgG1b2, 447-52D and 2FS: 2FS recognizes gp41 sequence ELDKWA (MWS2: ALDRWQ); IgG1 b2 recognizes epitopes on gp120 overlapping the CD4 binding region (key residues D185 and P369 changes to N185 and L369, respectively, in MWS2); 447-52D recognizes the highly conserved GPCR sequence of the V3 loop (MWS2: GPCQ). Data are representative of at least three independent experiments (↑ increase; ↓ decrease).

* MWS2 showed resistance to MAbs IgG1b2, 447-52D and 2FS: 2FS recognizes gp41 sequence ELDKWA (MWS2: ALDRWQ); IgG1 b2 recognizes epitopes on gp120 overlapping the CD4 binding region (key residues D185 and P369 changes to N185 and L369, respectively, in MWS2); 447-52D recognizes the highly conserved GPCR sequence of the V3 loop (MWS2: GPCQ).

Data are representative of at least three independent experiments (↑ increase; ↓ decrease).
DNAs induced higher neutralizing activity compared with monomeric gp120 (Fig. 3B).

Characterization of the neutralizing activity of mouse sera

We initially performed neutralization activity assay of sera at 3-fold dilution of 1:20 to 1:540 against MWS2 Env pseudotyped virus. We noticed that mouse sera at lower concentration caused slight enhancement of virus infection in TZM-bl cells (Fig. 4A). We therefore chose 1:20 dilution for our neutralizing experiments in accordance with standard protocols previously established by others (Devito et al., 2004; Montefiori, 2009; Radaelli et al., 2007). It is well known that neutralization assays using mouse sera can have background problems. To further confirm the specificity of mouse sera, we tested the neutralization activity of immunized mouse serum against VSV-G pseudotyped virus and did not observe significant neutralization when mouse sera from different groups were used (Fig. 4B). 20 days after the final immunization, neutralizing activity in different groups against MWS2 Env pseudotyped virus decreased to an equivalent level (Fig. 4C). Due to the lack of homologous MWS2 Env protein, we also examined the levels of Env-specific IgG in mouse sera by ELISA using heterologous CN54 gp140 protein as capture antigen. We confirmed induction of gp140-specific IgG in mice vaccinated with MWS2 gp140 DNA (Fig. 4D).

Collectively, our results illustrate that gp160 and gp140 DNAs of N156-T158A, N262-S264A or N410-T412A are capable of inducing neutralizing activity against homologous and, to a lesser extent, some heterologous primary as well as laboratory-adapted isolates.

Discussion

An ideal strategy to block the acquisition of HIV-1 infection is the development of effective preventive vaccines which can generate neutralizing antibodies against primary HIV-1 isolates, in particular those associated with sexual transmission. Because glycans on HIV-1 play important roles in viral infection and immune escape from the host (Reitter et al., 1998; Wei et al., 2003), glycan modification may represent a rational approach to elicit protective neutralizing antibody responses.

Deglycosylations of HIV-1 Env was previously reported to influence HXB2 infectivity (Lee et al., 1992). Here we found that replacement of serine or threonine with alanine in the Asn-x-Ser/Thr glycosylation sites of MWS2 env cloned from semen, including N262, proximal to the highly conserved core structure of CD4 binding region on the inside, resulted in sharp reduction of HIV-1 infection. Similar results were observed when N262 on HIV-1 Bal and IIIB Envs was deglycosylated (Supplementary Fig. 2). The rigidity of the highly conserved amino acid sequences of gp120 usually contribute to the maintenance of steric conformation. Because the amino acid sequence of glycosylation site at N262 is very close to the core structure of CD4 binding sites (CD4BS) on the inside (Fig. 5), depletion of glycosylation site at N262 may lower the entropy for interaction of adjacent secondary-structure elements in C4 and C5 region involved in CD4BS. The glycosylation is also known to affect the correct protein folding. Deglycosylation at N262 may induce the modification of β-strand-turn-β-strand structure in the C2 region and impair the highly conserved core structure of Env. Taken together, deglycosylation at N262 may result in rearrangement of CD4BS or conformational change of Env and further affect receptor and/or coreceptor binding.

Fig. 3. Generation of neutralizing activity to site-specific deglycosylated gp160, gp140 or gp120 in mice. (A) Mice were immunized i.m. with MWS2 gp160, gp140 or gp120 DNA by electroporation and the immunization was repeated three times in 3 week intervals. Sera were collected from mice (n = 9/group) 10 days after the final immunization and tested at a single dilution (1:20) for neutralization activity against a panel of wild-type (WT) HIV-1 MWS2, IIIB, CH811 Env pseudotyped virus in TZM-bl cells. The luciferase activity of cell lysates was determined following infection for 48 h. Neutralization sensitivity was shown as the percentage inhibition of viral infectivity. The infectivity without sera was set to 100%. Results represent mean of three duplications (Mean ± SD; *, median; ..., mean); (B) Summary of mouse serum neutralizing activity against HIV-1 MWS2, IIIB and CH811 Env pseudotyped virus at day 10 after the final immunization. Statistical analysis was assessed by unpaired Student’s t-test. (Mean ± SEM; *, p < 0.05; **, p < 0.01).
This was further strengthened by the result that N262-S264A had decreased sensitivity to CD4-IgG2.

We observed that several of the 24 deglycan mutants showed enhanced sensitivity to HIV-IG (human immunoglobulins pooled from HIV+ patients). It is reasonable to conclude that such deglycosylations may increase the number or exposure of available neutralization epitopes on gp120 to antibodies which already exist in HIV-IG. MWS2 WT Env was resistant to b12, 447-52D and 2F5, because it lacks the key amino acid residues in the antibody epitopes. MWS2 env gene was cloned from semen; whether there is any biological significance remains to be further addressed. In agreement, a previous study revealed that transmitted or early founder HIV-1 Envs demonstrated equivalent or modestly enhanced resistance to broadly neutralizing antibodies (Keele et al., 2008). Although N262 is a high-mannose type glycan, it is not the binding site of 2G12 (Sanders et al., 2002; Scanlan et al., 2002). It is worth noting that deglycosylated mutant N262-S264A had significantly decreased sensitivity to CD4-IgG2, which recognizes conserved CD4 binding sites. The modification of canonical glycosylation site at N262 may reduce rigidity of the nearby amino acids or alter the structural arrangement of the neighboring canonical glycosylation sites.
glycans, thereby allowing Env to stabilize in an abnormal conformation that is not exhibited before CD4 engagement, resulting in rearrangement of the key epitopes required for antibody recognition, CD4 binding and/or membrane fusion. This is in agreement with our observation that deglycosylation at N262 resulted in sharp reduction of HIV-1 infection.

Deglycosylations may also affect the interaction between gp120 and its coreceptors based on the results from SF162 (Ly and Stamatatos, 2000) and DH12 (Dumonceaux et al., 2001). We observed in the present study that removal of certain glycans proximal to the CD4-binding region resulted in significantly enhanced sensitivity to small-molecule CCR5 inhibitors, including maraviroc, SCH-412147, CMPD-167 and TAK-779. It is probable that removal of the highly conserved glycosylation sites which likely modify the conformation of CD4BS and/or Env may also affect the interaction between gp120 and CCR5. In addition, the fusion kinetics may also have been affected. Of interest, glycan mutants N156-T158A, N197-S199A and N410-T412A had increased sensitivity to tested CBAs, of which N262-S264A showed enhanced sensitivity to CV-N. N156, N197 and N410 are complex-type glycans (Gallaher et al., 1995; Leonard et al., 1990; Zhu et al., 2000) which are not recognized by high-mannose specific binding CBAs (Balzarini, 2007). Although N262 is a high-mannose type glycan, previous studies revealed that, under the selection pressure of CBAs, CBA-resistant HIV-1 variants still maintained the high-mannose glycan N262 on gp120 (Balzarini, 2007; Balzarini et al., 2006). Therefore, N262 is unlikely to be recognized by CV-N and GNA. Deglycosylation at N262 may rearrange the spatial structure of gp120 and increase the exposure of CBA-targeted carbohydrates including adjacent glycans N295, N332 and N448. We have recently demonstrated that removal of N295 or N448 renders HIV-1 resistant to high-mannose specific binding CBA griffisin, indicating the specificity of N295 and N448 in griffisin-gp120 interaction (Huang et al., 2011). Further understanding the effect of deglycosylations on the sensitivity of Env to entry inhibitors may have implications for therapeutic and prophylactic use of entry-based antiretrovirals.

Deglycosylations can affect Env immunogenicity. For instance, removal of N197 or multiple glycans on HIV-1 89.6 Env induced enhanced neutralizing activity (Li et al., 2008; Quinones-Kochs et al., 2002). Our results indicated that removal of N156, N262 or N410 alone was sufficient to enhance neutralizing activity against homologous and heterologous Env pseudotyped viruses. We also confirmed that removal of N197 had similar effect as reported previously (Li et al., 2008), while removal of N332 or N448 did not influence the immunogenicity compared with WT (data not shown). In general, gp140 DNA of glycan mutant N156, N262 or N410 induced the highest neutralizing activity among the three DNA forms. As suggested by others, the high glycosylation of trimeric molecules (4–35 trimmers per virion) shield immunogenic epitopes on HIV-1 virion from neutralization (Swanson and Malim, 2008). Immunization with gp120 monomer usually elicits non-neutralizing antibodies (non-NtAbs) which target the inner domain of Env trimers or other epitopes not exhibited in native Env. In contrast, glycan-removed gp140 may efficiently reveal immunogenic epitopes that are not well exposed in native trimers. The basis for the augmented NtAb responses induced by deglycosylated Env mutants remains to be further investigated. Nevertheless, our observations add important information to the approach aiming to induce neutralizing antibodies that recognize the functional trimeric Env complex (Dey et al., 2007; Fouts et al., 2002; Morner et al., 2009; Pantophlet and Burton, 2006). Given the relative poor immunogenicity of HIV-1 Env DNA (Robinson, 2009), while beyond the scope of this study, it will be important to determine whether a heterologous DNA prime followed by boosting with matched deglycosylated Env protein could increase the observed immune activity reported here. Previous studies have suggested that such heterogeneous DNA prime-protein boost strategies can potentially increase neutralization titers and breadth of activity (Vaine et al., 2010).

Given that HIV Env is an important target for prophylactics and therapeutics, rational modification of Env to improve its antigenicity and immunogenicity may provide essential information for the design of effective preventative and/or therapeutic vaccines. The findings in this study demonstrate that removal of certain glycans proximal to CD4-binding region not only affects viral infectivity, but also impacts Env antigenicity and immunogenicity, providing valuable information for vaccine development.

Materials and methods

Env clones, cell lines, antibodies, CCR5 antagonists and CBAs

Env clones MWS2, IIIB and BaL in pcDNA3.1 were previously described (Hu et al., 2000b; Hu et al., 2005). CH811 env was cloned from a blood sample isolated from a Chinese patient and the approach for molecular cloning of env gene was previously described (Hu et al., 2000b). 293 T cell line was purchased from the American Type Culture Collection. CHO-CCR5 cell line was previously described (Hu et al., 2000a). TZM-bl, U87.CD4.CCR5, U87.CD4.CXCR4, VSV-G, HIV-IG, IgG1-b2, 2G12, 447-52D, 17b and 4E10 were obtained from the AIDS Research and Reference Reagent Program. CCR5 inhibitors maraviroc, SCH-412147, CMPD-167 and TAK-779 were previously described (Hu et al., 2010; Veazey et al., 2005). CV-N (MW 11 kDa) was previously described (Hu et al., 2007). GNA (MW 12.5 kDa) was purchased from Sigma. VFT1.1 expressing T7 RNA polymerase was previously described (Hu et al., 2000b). PC9 was kindly provided by Professor Dennis Burton.

Genetic engineering of N-linked glycan mutants of gp160, gp140 and gp120

The env mutants were made based on a pcDNA3.1 vector containing the env gene of HIV-1 MWS2 using the QuickChange Site-Directed Mutagenesis Kit (Agilent Tech-Stratagene). A conservative third-site substitution from serine/threonine (Ser/Thr) to alanine (Ala) or Thr/Ser was introduced into the 24 N-linked glycosylation sites (Asn-x-Ser/Thr) in the gp120 region of Env, respectively. The gp140 and gp120 mutants were created by introducing a stop codon into gp160 mutants using high-fidelity PCR. The incision enzyme site between gp120 and gp41 in gp140 was removed by site-directed mutagenesis. Clones containing programmed mutations were identified by sequencing. The sequence of open reading frames was confirmed. Plasmids were prepared by QIAfilter Maxi kit (Qiagen).

 Infectivity assay and sensitivity to antibodies or inhibitors

Stocks of pseudotyped reporter viruses were prepared as described elsewhere. In brief, 293 T cells were co-transfected with 2 μg of HIV-1 Env or VSV-G expression constructs and 3.3 μg of pSG3Δenv or pNL4-3.Luc.R.E’ plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Supernatants containing viruses were harvested 48 h post-transfection, mixed with heat-inactivated fetal bovine serum (FBS) to a final concentration of 20% (V/V), filtered through a 0.45 μm syringe filter and stored at −80°C. Virus titer was determined as previously described (Montefiori, 2009).

TZM-bl or U87.CD4.CCR5 cells were seeded at 6 × 10^3 cells per well in a 96-well plate overnight and infected with 50 μl pseudotyped viruses. The luciferase activity of cell lysates was determined 48 h post-infection. In experiments assessing inhibition, single cycle infection was conducted in TZM-bl cells in the presence of serially diluted antibodies, entry inhibitors or CBAs.
Western blotting

HeLa or 293 T cells were infected at 37°C for 1 h with or without the vTF1.1 vaccinia virus and then transfected with gp160 constructs for 5 h using Lipofectamine 2000 (Invitrogen). 48 h post-transfection, cells were harvested and lysed in lysis buffer (Pierce) in the presence of protease inhibitor cocktail (Roche). Equal amounts of proteins were resolved by 12% SDS-PAGE and transferred onto PVDF membrane (Millipore) using the Bio-Rad microassay system (Bio-Rad). Membrane was blocked in TBS-T (TBS-0.1% Tween20) plus 5% non-fat milk at 4°C overnight and subsequently incubated with human immunoglobulins pooled from HIV + patients (HIV-IgG) for 1 h at room temperature. After washes with PBS-T, the membrane was incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Following 3 washes with PBS-T, immunoreactive bands were revealed by ECL using the Super Signal West Dura Extended Duration Substrate (Pierce) and visualized by a CCD camera (Alpha Innotech). The grayscale values of immunoreactive bands were analyzed using Bio-Rad Discovery software.

Flow cytometry

293 T cells were transfected with gp160 constructs for 5 h using Lipofectamine 2000 (Invitrogen). 48 h post-transfection, cells were harvested and washed. Cells were stained with 1 μg HIV-IG, PG9 or control human IgG (Serotec) in 100 μl PBS containing 3% FCS for 30 min at 4°C, extensively washed and subsequently incubated with RPE-conjugated goat-anti human IgG (Sigma) for 30 min at 4°C before washes and fixation. Stained cells were analyzed on a FACStar device (Becton Dickinson).

Immunization of mice

BALB/c female mice, 6 to 8 weeks old, were purchased from the Experimental Animal Center (Hunan, China). Animals were maintained under specific pathogen free (SPF) conditions, and randomly divided into thirteen groups (9 mice per group). In vivo electroporation was carried out according to the method described previously (Aihara and Miyazaki, 1998). Mice were immunized with plasmid DNA dissolved in 30 μl of PBS at a dosage of 30 μg WT, glycan mutant DNA or vector pCDNA3.1 by injection into the quadriceps muscles. After injection in the right quadriceps muscle, a pair of electrode needles 5 mm apart was inserted into the muscle to cover the DNA injection sites and electric pulses were delivered using the ECM 830 Square Wave Electroporation System (BTX). Three pulses of 100 V each, followed by three pulses of the opposite polarity, were delivered to each injection site at a rate of one pulse per second. Each pulse lasted for 50 ms. All mice were vaccinated three times in 3 week intervals. After the final immunization, mice were bled from the orbital sinus. All animals were handled in strict accordance with good animal practice as defined by the Hubei Laboratory Animal Science Association and all animal work was approved by the Institutional Animal Care and Use Committee (Approval ID: S03409100L and S03409110D).

Measurement of Ab levels by ELISA

To detect the level of Env specific IgG, plates were coated with 50 μl of 5.0 μg/ml trimeric CN54 gp140 overnight as previously described (Arias et al., 2011). After blocking with 300 μl per well of PBS containing 1% BSA for 1 h at room temperature, the plates were washed with PBS-T 3 times. Sera from different groups of the immunized mice were added into the wells in a series of three-fold dilutions. Following incubation at 37 °C for 1 h, the plates were washed 3 times and incubated with a 1/1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Abcam) in PBS-T. After 5 washes, the reaction was developed with TMB (Sigma), stopped with 2 M H2SO4, and absorbance was read at 450 nm by using an ELISA plate reader (Tecan). Sample dilutions were considered positive if the OD recorded for that dilution was at least 2-fold higher than the OD recorded for the control sample at the same dilution. Endpoint titers were analyzed using GraphPad Prism 4 (GraphPad Software).

Neutralization assay

The ability of serum neutralization was determined as previously described (Montefiori, 2009). One day before the neutralization assay, 6 × 10^3 TZM-bl cells per well were plated in 96-well plates. Mice serum samples were heat-inactivated at 56 °C for 1 h and then diluted (1:20 or 3-fold dilution of 1:20 to 1:540) in triplicates. Unless otherwise indicated, 200 TCID50 (50% tissue culture infective dose) pseudotyped viruses were incubated with diluted serum in a total volume of 100 μl at 37 °C for 1 h. Cells incubated with viruses in the absence of serum were used as positive control, while uninfected cells were used as background control. Following 1 h incubation, the mixture of virus and serum was added to TZM-bl cells in 100 μl of DMEM containing DEAE (40 μg/ml). The luciferase activity of cell lysates was determined 48 h post-infection.

Statistical analysis

Unless otherwise indicated, data were presented as means ± standard deviation (SD) or standard error of the mean (SEM). The IC50 was determined using the Ricketts and Head equation by Sigmaplot 11. The significance of difference in the neutralization activity between different groups was assessed by unpaired Student’s t-test. p value <0.05 was considered statistically significant and labeled as *, while p value <0.01 was labeled as **.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.virol.2011.11.023.

References

from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. Nature 345 (6276), 622–625.


