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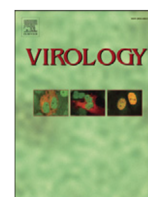

Glycan deletions in the HIV-1 gp120 V1/V2 domain compromise viral infectivity, sensitize the mutant virus strains to carbohydrate-binding agents and represent a specific target for therapeutic intervention

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ABSTRACT

Carbohydrate-binding agents (CBAs), such as the mannose-specific *Hippeastrum* hybrid agglutinin (HHA) and the GlcNAc-specific *Urtica dioica* agglutinin (UDA), frequently select for glycan deletions in all different domains of HIV-1 gp120, except in the V1/V2 domain. To reveal the underlying mechanisms, a broad variety of 31 different virus strains containing one or several *N*-glycan deletions in V1/V2 of the gp120 of the X4-tropic HIV-$1_{NL4.3}$ were constructed by chimeric virus technology. No co-receptor switch to CCR5 was observed for any of the replication-competent mutant virus strains. With a few exceptions, the more glycans were deleted in the gp120 V1/V2 domain, the more the replication capacity of the mutant viruses became compromised. None of the mutant virus strains showed a markedly decreased sensitivity to the inhibitory activity of HHA and UDA. Instead, an up to 2- to 10-fold higher sensitivity to the inhibitory activity of these CBAs was observed. Our data may provide an explanation why glycan deletions in the gp120 V1/V2 domain rarely occur under CBA pressure and confirm the important functional role of the glycans in the HIV-1 gp120 V1/V2 domain. The gp120 V1/V2 loop glycans of HIV-1 should therefore be considered as a hot spot and novel target for specific therapeutic drug intervention.

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Introduction

The gp120 envelope glycoprotein of HIV-1 is a heavily glycosylated protein and contains ~20 to 30 consensus recognition sites for *N*-linked glycosylation (i.e. N-X-S/T where *X*=any amino acid except P). The carbohydrate content of gp120 comprises approximately half of the molecular weight of the entire molecule (Geyer et al., 1988). Studies of recombinant gp120 expression in CHO cells show that all the 24 potential *N*-glycosylation sites in HIV-1_{IIIB} gp120 are indeed occupied by *N*-linked glycans (Leonard et al., 1990). Approximately 11 out of 24 of these glycans were determined to have a high-mannose-type structure. The *N*-glycans play an important role in correct folding, intracellular transport, (co)receptor binding and masking immunodominant domains (i.e. the V3 loop) (Li et al., 1993; Wyatt et al., 1998). Together with the V1/V2 variable domain, the V3 loop forms a pocket surrounding the four-stranded antiparallel β-sheet (i.e. the bridging sheet), which plays an important role in the co-receptor interaction of gp120. The overall conformation of the V3 loop together with internal interactions within the V1/V2 loop is thought to be the primary determinant for the efficient binding of gp120 to CCR5, CXCR4 or both, thus defining the cellular tropism of the virus isolate (Hwang et al., 1991; O'Brien et al., 1990; Ogert et al., 2001; Ross and Cullen, 1998; Shioda et al., 1992). The V2 domain is part of a disulfide-bonded arm of gp120 that also includes the V1 region. Deletion of the V1/V2 loop has been shown to result in non-infectious virions (Wyatt et al., 1993) although other studies have shown that some HIV-1 and SIV strains in which the V1/V2 domain of gp120 had been deleted were still replication-competent (Cao et al., 1997). Point mutations in V2 have also been shown to affect syncytium formation (Sullivan et al., 1993). The lack of the entire V1/V2 loop (Ogert et al., 2001), the removal of 3 to 5 high-mannose residues from 289 to 448 in the C2–C4 region of gp120 (Hu et al., 2007) or even the removal of only the single N195 linked glycan (Li et al., 2008) resulted in an increased sensitivity towards V3 loop-directed antibodies. In addition, deletion of glycosylation sites in the V1 domain of simian immunodeficiency virus (SIV) gp120 resulted in a redirection of B-cell responses to V3 (Cole et al., 2004). Thus the V1/V2 loop in gp120 might partially overlap and/or affect the antigenic epitopes of the principal neutralizing V3 domain.

Lectins are a class of proteins that interact with carbohydrates. The majority of these lectins primarily recognize mannose, glucose,

Abbreviations: HHA, *Hippeastrum* hybrid agglutinin; UDA, *Urtica dioica* agglutinin; PRM-A, pradimicin A; CBA, carbohydrate-binding agent; GlcNAc, *N*-acetylglucosamine; EGFP, enhanced green fluorescent protein; DMEM, Dulbecco minimal essential medium; FCS, foetal calf serum.

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galactose, *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), fucose and/or *N*-acetylneuraminic (sialic) acid. In addition, they preferentially interact with a variety of oligosaccharide glycans composed of one or more of these monosaccharides. It has been shown that especially the mannose-specific plant lectins (e.g. *Hippeastrum* hybrid agglutinin (HHA)), but also GlcNAc-recognizing lectins (e.g. *Urtica dioica* agglutinin (UDA)), show marked potency to inhibit enveloped viruses such as HIV (for a review see Balzarini, 2006a; Botos and Wlodawer, 2005) and hepatitis C virus (HCV) (Bertaux et al., 2007). The inhibition of viral entry by carbohydrate-binding lectins is probably accomplished by crosslinking the membrane glycoproteins of the virus, resulting in blocking the entry (or fusion) of the virus into its target cell (Leikina et al., 2005). We reported previously that mannose-specific lectins [i.e. α(1,3)- and/or α(1,6)-mannose-specific HHA and *Galanthus nivalis* agglutinin (GNA); the α(1,2)-mannose-specific Cyanovirin-N (CV-N), the non-peptidic small-size α(1,2)-mannose-specific antibiotic pradimicin A (PRM-A)] and also the GlcNAc-specific UDA select for a unique drug resistance profile that mainly affects the *N*-glycosylation pattern of the HIV-1 gp120 (Balzarini et al., 2004; 2005a; 2005b; 2006b; 2007). The resistance mutations predominantly appeared at asparagine or serine/threonine residues that are part of the N-X-S/T *N*-glycosylation motif on gp120. Intriguingly, in sharp contrast with the frequently observed glycan deletions at domains of gp120 other than V1/V2, glycan deletions in the V1/V2 domain of HIV-1 gp120 rarely occur under CBA pressure. The gp120 V1 and V2 region of the T-cell laboratory-adapted HIV-1 strain NL4.3 contains six recognition sites for *N*-linked glycosylations (i.e. N136, N141, N156, N160, N186, N195) which represents approximately 25% of the total amount of glycans present on gp120. Among more than 50 virus isolates independently selected under CBA pressure (harbouring an average number of ~4–5 glycan deletions in gp120), only two virus strains contained a glycan deletion in gp120 V1/V2: one virus strain contained a N160D mutation in gp120 V2 (Balzarini et al., 2005a), and one virus strain had a glycan deletion at amino acid position N186 of gp120 V2 due to mutation of T188 to N188 (Balzarini et al., 2005b). However, the latter mutation created a new glycosylation site (at amino acid position 188; 188NXS190). Moreover, the mutations did not occur as single mutations, but were invariably present in a background of additional glycan deletions in the other domains of gp120.

To elucidate the underlying mechanism of the virtual lack of glycan deletions in gp120 V1/V2, we constructed 31 recombinant mutant virus strains derived from the HIV-$1_{NL4.3}$ strain with

Table 1

a. Co-receptor use of glycan-deficient HIV-1 strains and susceptibility to the inhibitory effect by CBAs

Number	Mutations in gp120 V1/V2 domain						Co-receptor use		Susceptibility to CBA as EC_{50}[a]		
							CCR5	CXCR4	HHA (nM)	UDA (nM)	PRM-A (μM)
NL4.3	–	–	–	–	–	–	–	+	7±1	47±11	2.2±0.4
1	N136Q	–	–	–	–	–	–	+	1.0±0.4	13±6	1.7±0.2
2	–	N141Q	–	–	–	–	–	+	1.2±0.4	13±7	2.1±0.1
3	–	–	N156Q	–	–	–	–	+	6±1	60±23	–
4	–	–	–	N160Q	–	–	–	+	–	–	–
5	–	–	–	N160D	–	–	–	+	4±2	21±13	2.1±0.2
6	–	–	–	–	N186Q	–	–	+	22±1	115±69	2.1±0.06
7	–	–	–	–	–	N195Q	–	+	1.8±0.2	13±2	2.2±0.01
8	N136Q	N141Q	–	–	–	–	–	+	1.0±0.2	13±1	1.6±0.5
9	N136Q	–	–	N160Q	–	–	–	+	4±3	80±34	2.1±0.2
10	N136Q	–	–	–	–	N195Q	–	+	0.8±0.2	5.7±1	2.1±0.1
11	–	N141Q	–	N160Q	–	–	–	+	3±2	47±10	–
12	–	N141Q	–	–	N186Q	–	–	+	1±0.1	14±2	2.0±0.2
13	–	–	N156Q	–	N186Q	–	–	+	1.6±0.8	12±1	2.7±0.0
14	–	–	N156Q	–	–	N195Q	–	+	–	–	–
15	–	–	–	N160D	N186Q	–	–	+	1.0±0.6	6±2	2.3±0.1
16	N136Q	N141Q	N156Q	–	–	–	–	+	1.6±0.8	14±1	2.2±0.01
17	N136Q	N141Q	–	N160Q	–	–	–	+	5±2	28±15	2.1±0.07
18	N136Q	N141Q	–	–	N186Q	–	N.V.[b]	N.V.	N.V.	N.V.	N.V.
19	N136Q	–	–	–	N186Q	N195Q	–	+	1.0±0.2	4.6±0.0	3.7±0.5
20	N136Q	–	N156Q	–	N186Q	–	–	+	3±1	21±13	2.2±0.01
21	N136Q	–	N156Q	–	–	N195Q	–	+	2±1	9±4	2.1±0.1
22	–	–	N156Q	–	N186Q	N195Q	–	+	–	–	–
23	–	N141Q	–	N160Q	–	N195Q	–	+	1.0±0.2	5.6±0.0	1.9±0.2
24	N136Q	N141Q	N156Q	N160Q	–	–	–	+	1.7±0.2	16±10	2.2±0.01
25	N136Q	N141Q	N156Q	–	N186Q	–	–	+	1.6±0.8	5±2	2.1±0.03
26	N136Q	N141Q	–	N160Q	–	N195Q	–	+	1.8±0.6	15±6	2.2±0.4
27	N136Q	–	N156Q	N160Q	N186Q	–	–	+	1.4±0.4	11±7	–
28	N136Q	–	N156Q	–	N186Q	N195Q	N.V.	N.V.	N.V.	N.V.	N.V.
29	N136Q	N141Q	N156Q	N160Q	–	N195Q	–	+	–	–	–
30	N136Q	–	N156Q	N160Q	N186Q	N195Q	N.V.	N.V.	N.V.	N.V.	N.V.
31	N136Q	N141Q	N156Q	N160Q	N186Q	N195Q	N.V.	N.V.	N.V.	N.V.	N.V.

b. Susceptibility of selected glycan-deficient HIV-1 strains to the inhibitory effect of AMD3100 and sCD4

Number	Mutations in gp120 V1/V2 domain						EC_{50}[a] (μg/ml)	
							AMD3100	sCD4
NL4.3	–	–	–	–	–	–	0.006±0.004	0.05±0.03
3	–	–	N156Q	–	–	–	0.007±0.002	0.04±0.02
9	N136Q	–	–	N160Q	–	–	0.006±0.003	0.09±0.06
18	N136Q	N141Q	–	–	N186Q	–	0.004±0.002	0.10±0.04
26	N136Q	N141Q	–	N160Q	–	N195Q	0.002±0.001	0.03±0.01

Data are means of at least 3 independent experiments.

[a] 50% effective concentration.

[b] Non-sufficient viable glycan-deficient virus.

combinations of glycan deletions at one or several glycosylation sites in gp120 V1/V2. All 31 recombinant mutant viruses were evaluated in cell-based infection assays for infectivity, replication fitness, co-receptor usage (CXCR4 or CCR5) and sensitivity to HHA and UDA. HHA and UDA were chosen for their different carbohydrate specificity (α(1,3)/α(1,6)-mannose *versus* GlcNAc). The viruses were also evaluated against the non-peptidic α(1,2)-mannose-specific antibiotic PRM-A.

The observed attenuation of infectivity of the mutant virus strains and their increased sensitivity to the inhibitory effect of HHA and UDA may provide a rational explanation of the virtual lack of appearance of glycan deletions in the gp120 V1/V2 domain under CBA pressure. Our findings revealed that the glycan configuration of the gp120 V1/V2 area of HIV-1 may be considered as a hot spot and selective target for future novel drug development.

Results

Co-receptor usage of the mutant virus strains

A variety of 31 different recombinant virus strains all derived from the X4-tropic HIV-$1_{NL4.3}$ strain have been constructed, containing mutations in either of the six glycosylation motifs in the HIV-1 gp120 V1/V2 domain, and combinations thereof. Multi-site directed mutagenesis of the V1/V2 domain of HIV-1 gp120 resulted in a variety of 7 virus strains with 1 mutation, 8 virus strains with 2 mutations, 8 virus strains with 3 mutations, 5 virus strains with 4 mutations, 2 virus strains with 5 mutations and 1 virus strain containing 6 mutations (Table 1). The viral tropism of these recombinant mutant (deglycosylated) virus strains was evaluated by examining the ability of the mutant viruses to productively infect U87/CD4$^+$/CCR5$^+$, U87/CD4$^+$/CXCR4$^+$ or U87/CD4$^+$/CXCR4$^+$/CCR5$^+$ cells. All recombinant viruses for which replication could have been measured invariably infected solely U87/CD4$^+$/CXCR4$^+$ and U87/CD4$^+$/CXCR4$^+$/CCR5$^+$ cells but not U87/CD4$^+$/CCR5$^+$ cells (Table 1) and thus kept their exclusive CXCR4-tropism. Thus, since none of the recombinant virus strains were able to infect the U87/CD4$^+$/CCR5$^+$ cell cultures, we concluded that glycan mutations that were exclusively located in the gp120 V1/V2 domain did not result in a co-receptor switch of the laboratory strain NL4.3 from CXCR4 to CCR5.

Replication fitness of the mutant virus strains upon normalization of p24 levels

The mutant virus strains lacking the single gp120 V1 domain-associated *N*-glycosylation sites N136, N141 or N156 showed quite similar replication rates compared to HIV-$1_{NL4.3}$ wild-type (Fig. 1A). However, the mutant viruses with single gp120 V2-associated glycan deletions showed a lower replication rate than wild-type virus (i.e. N186Q and N195Q). The N160Q mutant virus was even devoid of meaningful replicating capacity. The N160D mutant virus showed again a recovered replicative potential, although not comparable with wild-type virus (Fig. 1A). Several double mutated virus strains behaved virtually identical as wild-type HIV-1 (Fig. 1B). Remarkable are the very compromised replication rates of the viruses containing V1 N156Q or N136Q in combination with the N195Q mutation that is located in the carboxy-terminal stem of the V2 loop. The decreased replication potential of these double mutated viruses is much more pronounced than expected from the single mutants (Fig. 1B). Thus, the sugar moiety on position N195 could have a supportive role in entry/fusion and is indispensable for proper envelope function and replication competence. This is also confirmed from the data shown in Fig. 1C where the triple mutants containing the N195Q mutation have very low and highly compromised replication rates. In contrast, single (Fig. 1A), double

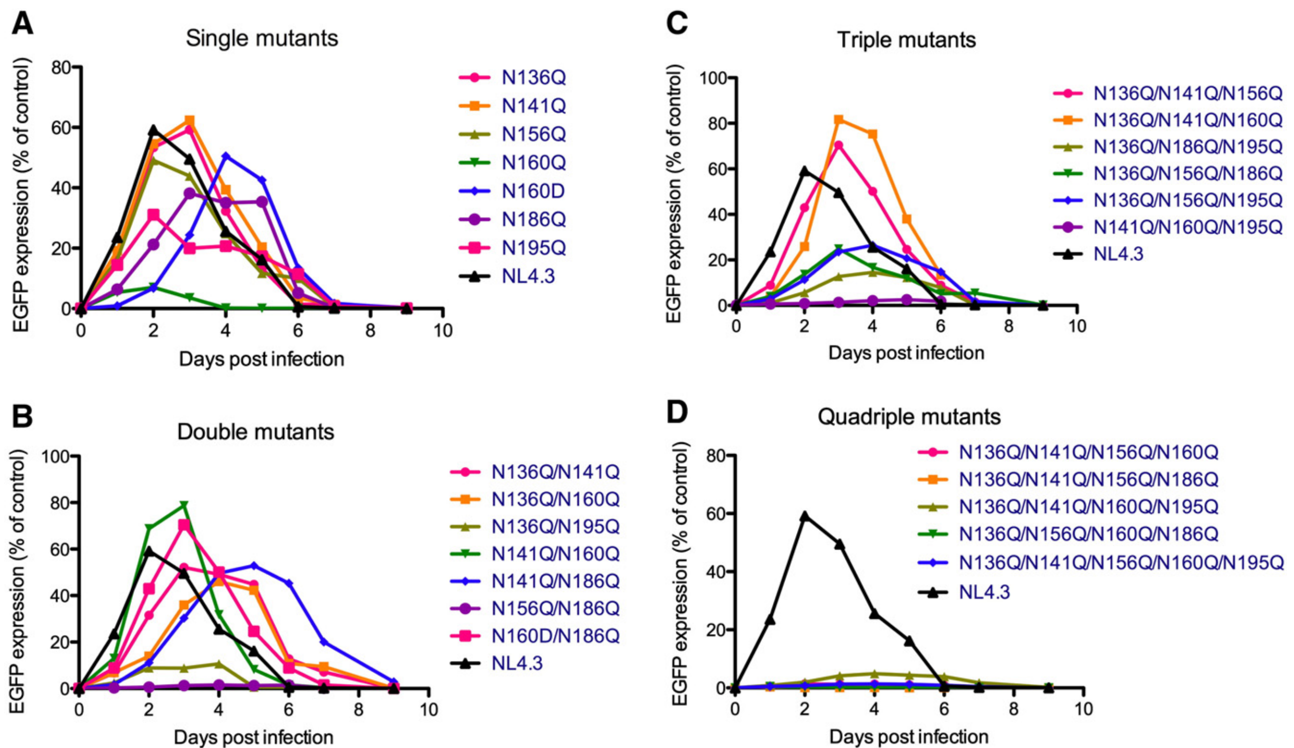

Fig. 1. Viral replication of V1/V2 loop mutant virus strains based on a p24 normalization. The growth of different virus mutants with single (A), double (B), triple (C) and quadruple (D) deleted *N*-glycosylation site(s) in the V1 and/or V2 loop was monitored by inoculation of C8166 cells with equal amounts of HIV-1 p24 antigen. Starting at 1-day post-infection, cells were fixed daily in 2% PFA and monitored on a FACS CantoII flow cytometer for EGFP expression as a measurement of (mutant) HIV-1 replication. Data are the means of at least 3 independent experiments.

(Fig. 1B) and triple (Fig. 1C) glycan mutants in the V1 loop do not have a significant effect on the loss of the viral replication capacity. Quadruple mutant virus strains displayed highly impaired replication rates. Most of the mutant constructs did not even result in replication-competent virus (Fig. 1D). This is also the case for the recombinant viruses with five mutations of their glycosylation sites and with a completely deglycosylated gp120 V1/V2 domain-containing virus strain (Table 1). Interestingly, the budding of these heavily deglycosylated viruses from the transfected cells was still possible as observed by a p24 ELISA on the transfection supernatants (data not shown).

Replication fitness of the mutant virus strains upon normalization of infectious viral particles

The results of the replication capacity assay normalized by the amount of infectious particles were plotted on a log scale on the *Y*-axis versus the time points post-day 3 as depicted in Fig. 2. The amount of infected cells was normalized to the percentage infection on day 3 (which was determined to be approximately 0.1%). The resulted *k*-values of the exponential functions can be used as a tool to compare the replication capacity between mutant virus strains and wild-type NL4.3 and are depicted in Table 2.

The N160Q mutant virus strain was devoid of meaningful replication capacity. However the N160D mutant virus showed a replication rate that was close to wild-type virus (Fig. 2A). The mutant virus strains lacking the single gp120 V2 domain-associated *N*-glycosylation site N195 showed a lower replication rate than wild-type virus. Most double mutant virus strains were endowed with replication capacities that were inferior to wild-type virus. When the N136Q mutation was introduced in the (replication-deficient) N160Q mutant virus the replication capacity was restored to the level of wild-type virus. Double mutant virus strains containing the V2-domain-associated N186 or N195 glycan deletion all showed decreased replication rates compared to wild-type virus (Fig. 2B). Triple mutants containing the N195 deletion showed also a decreased replication rate compared to wild-type. The introduced N195Q mutation resulted in the most impaired virus strains as also

Table 2
k- and R^2-values for the replication graphs of the mutant glycan-deficient HIV-1 strains depicted in Fig. 2

Number	Mutations in gp120 V1/V2 domain						*k*-value	R^2
NL4.3	–	–	–	–	–	–	2.961	0.9999
1	N136Q	–	–	–	–	–	2.933	1.000
2	–	N141Q	–	–	–	–	2.484	1.000
3	–	–	N156Q	–	–	–	2.806	0.9997
4	–	–	–	N160Q	–	–	0.196	0.9999
5	–	–	–	N160D	–	–	3.085	0.9999
6	–	–	–	–	N186Q	–	2.853	0.9995
7	–	–	–	–	–	N195Q	2.158	0.9988
8	N136Q	N141Q	–	–	–	–	2.379	0.9999
9	N136Q	–	–	N160Q	–	–	2.991	0.9999
10	N136Q	–	–	–	–	N195Q	1.998	0.9995
11	–	N141Q	–	N160Q	–	–	2.940	0.9999
12	–	N141Q	–	–	N186Q	–	2.068	0.9972
13	–	–	N156Q	–	N186Q	–	1.643	0.9999
14	–	–	N156Q	–	–	N195Q	–	–
15	–	–	–	N160D	N186Q	–	2.650	0.9998
16	N136Q	N141Q	N156Q	–	–	–	1.791	0.9988
17	N136Q	N141Q	–	N160Q	–	–	2.780	1.000
18	N136Q	N141Q	–	–	N186Q	–	–	–
19	N136Q	–	–	–	N186Q	N195Q	1.927	0.9967
20	N136Q	–	N156Q	–	N186Q	–	2.646	0.9987
21	N136Q	–	N156Q	–	–	N195Q	2.081	0.9977
22	–	–	N156Q	–	N186Q	N195Q	–	–
23	–	N141Q	–	N160Q	–	N195Q	1.997	0.9999
24	N136Q	N141Q	N156Q	N160Q	–	–	1.458	0.9973
25	N136Q	N141Q	N156Q	–	N186Q	–	1.567	0.9986
26	N136Q	N141Q	–	N160Q	–	N195Q	2.034	0.9969
27	N136Q	–	N156Q	N160Q	N186Q	–	1.234	0.9985
28	N136Q	–	N156Q	–	N186Q	N195Q	–	–
29	N136Q	N141Q	N156Q	N160Q	–	N195Q	1.630	0.9995
30	N136Q	–	N156Q	N160Q	N186Q	N195Q	–	–
31	N136Q	N141Q	N156Q	N160Q	N186Q	N195Q	–	–

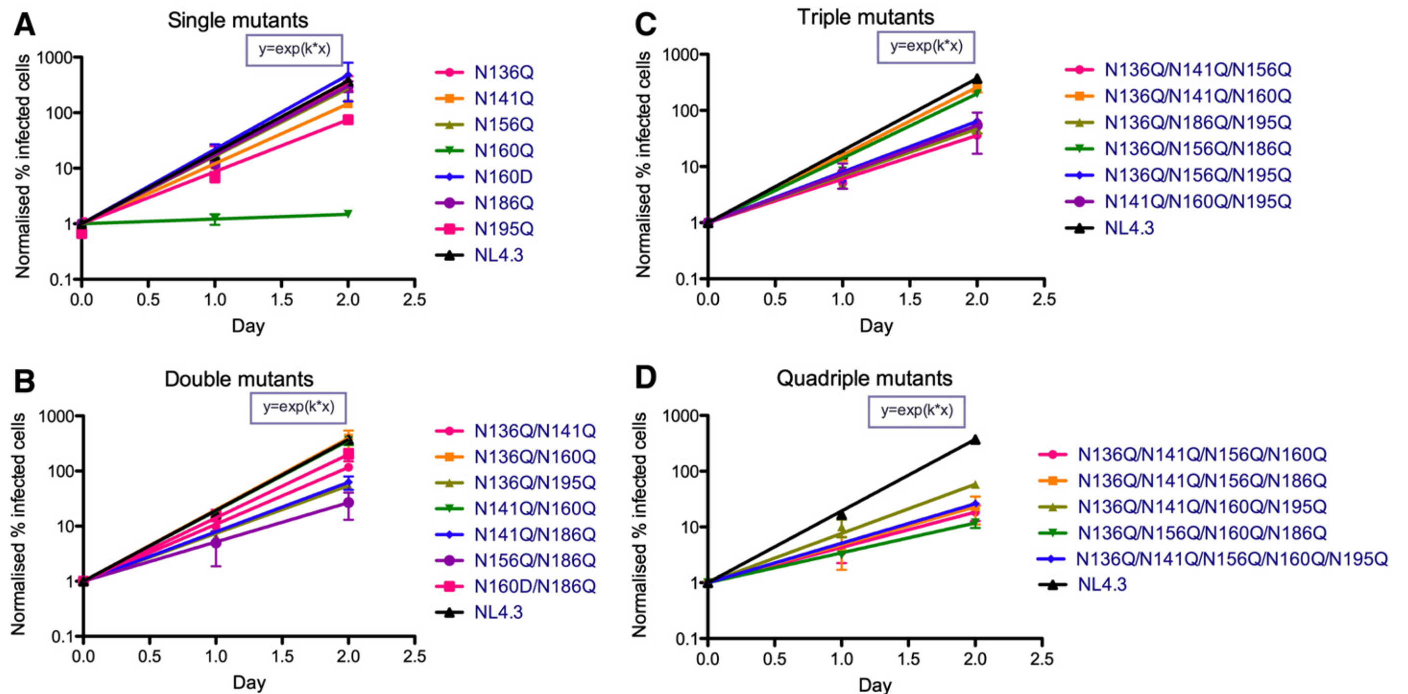

Fig. 2. Comparison of the replication capacity of mutant V1/V2-loop virus strains based on infectious particle normalization. Growth kinetics of different virus mutants with single (A), double (B), triple (C) and quadruple (D) deleted *N*-glycosylation site(s) were recorded in C8166 cells that were infected with an equal amount of infectious particles that result in 0.1% infection on day 3 post-inoculation. Cells were fixed daily after day 3 in 2% PFA and monitored on a FACS CantoII for EGFP expression as a measurement of (mutant) HIV-1 replication. The data were normalized to the day 3 value and plotted on a log scale versus the time points post-day 3. The *k*- and R^2-values are depicted in Table 2. Data are the means of at least 3 independent experiments.

found in the previously described assay where p24 levels were used as the normalized standard. In general all triple mutant virus strains showed a decreased replication rate compared to wild-type (Fig. 2C). This observation is even more pronounced when we introduced 4 or more deletions in the glycosylation sites of the V1/V2 loop, all resulting in virus strains with seriously impaired replication capacity compared to wild-type virus (Fig. 2D).

Susceptibility of deglycosylated HIV-1 strains to the carbohydrate-binding agents (CBAs), AMD3100 and sCD4

The inhibitory effects of the α(1,3)/α(1,6)-mannose-specific HHA, the GlcNAc-specific UDA, the α(1,2)-mannose-specific non-peptidic antibiotic PRM-A, AMD3100 and sCD4 on the expression of EGFP by the different mutant recombinant viruses in human lymphocyte C8166 cell cultures are displayed in Table 1. Wild-type HIV-$1_{NL4.3}$ is inhibited by HHA at 7±1 nM, by UDA at 47±11 nM and by PRM-A at 2.2±0.4 μM. Higher susceptibility to HHA and UDA was invariably observed with only a very few exceptions (i.e. the virus strains containing the single N156 and N186 glycan deletions). The higher susceptibility of the mutant viruses to these CBAs ranged from 2-fold to up to 10-fold. There was a close correlation between the degree of sensitization of the mutant virus strains against the mannose-specific HHA and against the GlcNAc-specific UDA (r=0.853) (Fig. 3). This observation points to a similar mechanism of sensitization of the mutant virus strains against both types of CBAs. In fact, both HHA and UDA bind carbohydrate configurations that are present in the interior structure of the complex-type glycans present in gp120 V1/V2. In contrast to HHA and UDA, PRM-A acts at the surface of the high-mannose-type glycans, and did not show an increased antiviral activity (Table 1). Its antiviral activity ranged between 1.6 and 3.7 μM irrespective the nature of the glycan site mutations in V1/V2 gp120 (EC_{50} wild-type virus: 2.2±0.4 μM).

In addition, the antiviral activity of the CXCR4 antagonist AMD3100 on selected mutant deglycosylated recombinant viruses (EC_{50} ranging from 0.02 to 0.07 μg/ml) did not show significant differences compared to wild-type HIV-$1_{NL4.3}$ (EC_{50} of 0.06 μg/ml). Also, sCD4 showed very similar antiviral activity against the selected mutant deglycosylated strains (EC_{50} ranging from 0.03 to 0.1 μg/ml) compared to wild-type HIV-$1_{NL4.3}$ (EC_{50} of 0.05 μg/ml) (Table 1).

Binding of mutant deglycosylated HIV-1 strains to human sCD4

We determined the binding capacity of a fixed amount of wild-type and mutant deglycosylated viruses to human sCD4 that was immobilized on a 96-well maxisorp surface (Table 3). The observed OD-values related to the binding of the mutant deglycosylated viruses to sCD4 were similar or higher than those observed for wild-type HIV-$1_{NL4.3}$, except for the viruses bearing the N141Q/N160Q/N195Q or N136Q/N141Q/N160Q/N195Q mutations that had a CD4-binding capacity of 71±7% and 82±7% of wild-type, respectively.

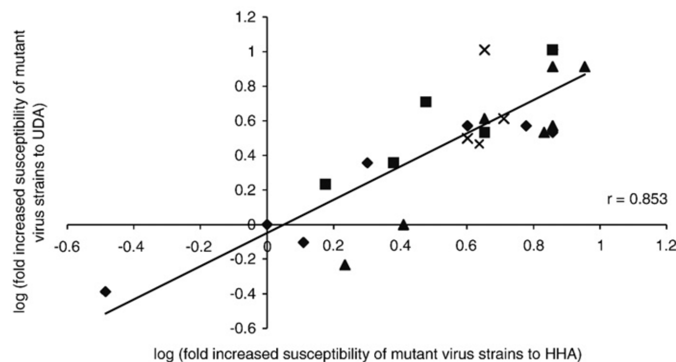

Fig. 3. Correlation between the sensitization of the mutant HIV-1 strains against the mannose-specific HHA and against the GlcNAc-specific UDA. Different symbols were used for single (◆), double (▲), triple (■) and quadruple (X) virus mutants.

Table 3
Binding of glycan-deficient virus strains to human CD4

Number	Mutations in gp120 V1/V2 domain						CD4 binding (% of wild-type)[a]
1	N136Q	–	–	–	–	–	127±9
2	–	N141Q	–	–	–	–	211±49
3	–	–	N156Q	–	–	–	127±21
4	–	–	–	N160Q	–	–	188±42
5	–	–	–	N160D	–	–	140±21
6	–	–	–	–	N186Q	–	94±1
8	N136Q	N141Q	–	–	–	–	237±39
9	N136Q	–	–	N160Q	–	–	158±27
10	N136Q	–	–	–	–	N195Q	159±26
11	–	N141Q	–	N160Q	–	–	180±29
12	–	N141Q	–	–	N186Q	–	117±20
13	–	–	N156Q	–	N186Q		150±25
17	N136Q	N141Q	–	N160Q	–	–	121±20
23	–	N141Q	–	N160Q	–	N195Q	71±7
24	N136Q	N141Q	N156Q	N160Q	–	–	113±26
26	N136Q	N141Q	–	N160Q	–	N195Q	82±7

[a] Data are the means of at least 3 independent experiments.

Discussion

The role of HIV-1 (and SIV) gp120 envelope *N*-glycans in neutralizing antibody response has been investigated by mutagenesis technology (Quinones-Kochs et al., 2002; Reitter and Desrosiers, 1998; Wolk and Schreiber, 2006). These studies revealed that mutant virus strains lacking glycans on position N136 and N141 in V1 and glycans on position N160 and N186 in V2 showed a high sensitivity against human serum antibodies from HIV-1-infected individuals. Viruses with gp120 V1/V2 loop deletions show an impaired ability to form syncytia, however, a deletion of the entire V1/V2 loop of HIV-1 can still result in an infectious virus but with impaired entry capacity in comparison to wild-type virus (Cao et al., 1997), which could indicate that an intact V1/V2 loop is indispensable for optimal viral infectivity and replication.

Mutations in V3 which are sufficient for changing co-receptor use from CCR5 to CXCR4 often entail a high risk for the virus, leading to major loss of entry fitness or even resulting in lethality (Huang et al., 2005; Hwang et al., 1991). Mutations in or near the gp120 V1/V2 loop were able to compensate for the deleterious V3 mutations and may even be needed to precede some V3 mutations to permit efficient virus survival (Pastore et al., 2006). In contrast, deletion of the surrounding glycans of the V3 loop can result in a mutant virus with an increased infectivity (Polzer et al., 2001) and the deletion of the sole complex-type glycan within the V3 loop itself influences viral tropism (Polzer et al., 2002).

In the current study no viral tropism change was observed for any of the replication-competent viruses with single glycan deletions or for any of the multiple combinations of glycan deletions that were introduced in the gp120 V1/V2 loop in the presence of a wild-type gp120 V3 loop genetic background. It can, of course, not be excluded that one or several glycan deletions in gp120 V1/V2 may allow co-receptor switch in the presence of well-defined mutations in the V3 loop, given the communication and interaction between the V3 and V1/V2 domains of gp120 and the co-receptor interaction with the bridging sheet of gp120.

The replication competence of the mutant virus strains have been compared with wild-type virus starting from equal amounts of p24 (Fig. 1, Table 1) and from equal amounts of infectivity units (Fig. 2, Table 2). The results and conclusions from both sets of experiments were in agreement with each other. Analysis of the replication competence of the mutant virus strains revealed striking differences

in the effect of glycan deletions in the gp120 V1 and V2 loop. Whereas V1 loop glycan mutations had little, if any effect on viral infectivity, glycan mutations in the V2 loop (i.e. N186Q and N195Q) had a dramatic impact on the infection capacity of the mutant virus strains. This phenomenon is in agreement with previous findings made by other investigators (Ly and Stamatatos, 2000; Stamatatos et al., 1998; Wu et al., 1995). Moreover, none of the V1 glycan deletions were able to compensate for the infectivity loss of V2 glycan deletions when combined, and multiple paired glycan deletions in V1 synergistically compromised virus infectivity. These observations point to a serious restriction of the glycan configuration in gp120 V1/V2, and a potential limitation of flexibility of this part of the viral envelope in response to gp120-targetting (i.e. CBA) drugs. Most likely, abrogation of glycosylation in the V1/V2 area of HIV-1 gp120 would affect the tertiary structure of the protein (through a direct effect on correct protein folding), resulting in an altered infection capacity (i.e. lower fitness). Indeed, evidence has been presented for a role of specific V2 glycans in the correct folding of the V1/V2 domain (Wu et al., 1995), and lack of proper folding of V1/V2 may eventually result in a less optimal overall envelope conformation directly affecting infectivity efficiency. The different infectivity capacity of the N160Q and N160D mutant virus strains suggests that not only the location of the mutated amino acid site, but also the nature of the mutated amino acid is important to eventually determine its effect on mutant virus infectivity and that the degree of glycosylation of gp120 does not solely account for viral infectivity. Also, the marked fluctuating differences of infectivity potential of the N160Q, N141Q/N160Q, N136Q/N141Q/N160Q and N136Q/N141Q/N156Q/N160Q mutant virus strains cannot be explained if the infectivity potential of the viruses is solely ascribed to the presence or absence of the glycans. Crystal structure studies on the entire gp120 would be needed to distinguish between the effect of amino acid changes on the one hand, or solely glycan deletions on the other hand on the gp120 conformation and related infectivity potential of the mutant viruses.

In an adapted HIV-1_{ADA} strain the deletion of the entire gp120 V1/V2 loop allowed a CD4-independent entry of the virus and gp120 binding to CCR5 (Kolchinsky et al., 2001). In this context it was also shown that the elimination of only one single glycosylation site at position N195 in the V1/V2 stem was sufficient for this CD4-independent gp120 binding to CCR5 and for HIV-1 entry into CD4-negative cells expressing CCR5 (Kolchinsky et al., 2001). This was not observed with the single N195 mutant HIV-$1_{NL4.3}$ in our study. However, we found that the deletion of the *N*-glycosylation site at the gp120 position N195 of the NL4.3 virus strain (the N195 glycan in HIV-$1_{NL4.3}$ corresponds to the N197 glycan in HIV-1_{IIIB} or HIV-1_{ADA}) resulted in a lower replication rate, in particular when the mutation was combined with other glycan deletions in the gp120 V1/V2 domain, such as N136 or N156. Thus, the disappearance of the glycan at N195 seems to compromise the interaction of HIV-$1_{NL4.3}$ with its CD4 or CXCR4 co-receptor, resulting in reduced viral infectivity.

It is currently unknown whether the glycan mutations have an effect on the level of mutant *env* production and/or incorporation of *env* in the virions. Lower *env* levels in the virion may affect CBA sensitivity and infection potential of such mutant viruses. Therefore, determination of the number of *env* molecules in the mutant virus particles would be interesting to address this issue and to obtain better insights in the structural and functional events that are caused by the glycan deletions in the envelope of HIV-1. From the observation that the inhibitory effect of sCD4 and the CXCR4 antagonist AMD3100 on HIV replication is very similar against wild-type and mutant deglycosylated HIV-$1_{NL4.3}$ we can conclude that glycan deletions in the V1/V2 domain have no marked effect on CD4 receptor or CXCR4 co-receptor binding. This observation was confirmed in a CD4-binding assay where the mutant deglycosylated viral strains had a rather minor effect on their CD4-binding capacities compared to wild-type HIV-$1_{NL4.3}$.

Exposure of HIV-1 to escalating CBA concentrations invariably results in the appearance of glycan deletions in gp120 (Balzarini, 2007a; 2007b and references therein). There is a preference for deletions of high-mannose type glycans, and of glycans that are located in areas on gp120 different from V1/V2. Indeed, a glycan deletion in the V1/V2 domain (N160 or N186) was very seldom observed (only 2 times) to have occurred among a total number of more than 50 independently selected mutant virus strains. In all other virus strains, the 6 complex-type glycans in gp120 V1/V2 were kept intact. Such conservation of the glycans in the HIV-1 gp120 V1/V2 domain under CBA drug pressure is unusual given the fact that approximately 25% of all glycans on gp120 resides in this envelope domain. This observation can now be explained by our findings that virtually all mutant virus strains obtained in our study showed an increased sensitivity (2- to up to 10-fold) to the mannose-specific (HHA) and GlcNAc-specific (UDA) CBAs. In addition, given our findings that glycan deletions in the gp120 V1 and particularly in the gp120 V2 loop seriously compromise the viruses' infectivity potential, it seems obvious that such mutant virus strains are highly unlikely to emerge under the continuous pressure of the HHA and UDA CBAs. The only exceptions are the N160 and N186 HIV-1 gp120 mutants that have been (only once) observed to occur in cell culture under CBA pressure. It may not be co-incidence that the N186 gp120 HIV-1 mutant is indeed less sensitive to HHA and UDA suppression than wild-type virus, and thus could be expected to be selected in the presence of HHA or UDA, given its fairly good replication competence. The N156 HIV-1 gp120 mutant has also a good replication competence but its EC_{50} for HHA and UDA is not significantly different from wild-type virus, explaining its absence in CBA-escalating selection experiments. We believe that sensitization to CBAs are not due to the loss of replication fitness because the data in Tables 1 and 3 do not reveal a correlation between both phenomena.

Sensitization to HHA was also very recently observed for a mutant virus strain containing the N295 glycan deletion selected under pressure of the neutralizing, carbohydrate-binding antibody 2G12 (Huskens et al., 2007). It is possible that the disappearance of a particular glycan on gp120 may result in a better accessibility of the other glycans located nearby the deleted glycan position by the relatively large tetrameric HHA protein (4×12.5 kDa). Indeed, HHA has specificity for α(1,3)/α(1,6)-mannose oligomers that are present in those interior parts of the complex-type glycans that are adjacent to $(GlcNAc)_2$ which is linked to the amide function of the glycosylated asparagines. Deletion of one or more *N*-glycans in the dense glycan shield of the gp120 V1/V2 domain may allow better availability of the α(1,3)/α(1,6)-mannoses for HHA interaction. This may also be the case for the smaller monomeric GlcNAc-specific UDA (8.7 kDa). Our observation that the α(1,2)-mannose-specific small non-peptidic PRM-A does not show an increased antiviral activity against the mutant virus strains is in agreement with this interpretation, since the complex-type glycans on gp120 V1/V2 do not contain the α(1,2)-mannose configuration, and thus, could not be targeted by PRM-A. Although it cannot be excluded that CBA binding to V1/V2 gp120 glycans does not lead to neutralization or that the CBAs do not bind at all to the V1/V2 glycans, both the decreased fitness of mutant (V1/V2 glycan-deleted) HIV and the increased sensitivity to CBAs against these virus strains argue for the V1/V2 gp120 domain as a target for specific drug development that forces the virus to delete its glycans in V1/V2 gp120. Thus, the maintenance of the V1/V2 glycans under CBA pressure may indicate an important functional role of the glycans in the V1/V2 domain and suggest that they may represent novel targets for specific therapeutic intervention. This premise is additionally supported by the observation that the glycans at positions 156, 160, 186 and 195 of the V1/V2 loops are highly conserved among a wide

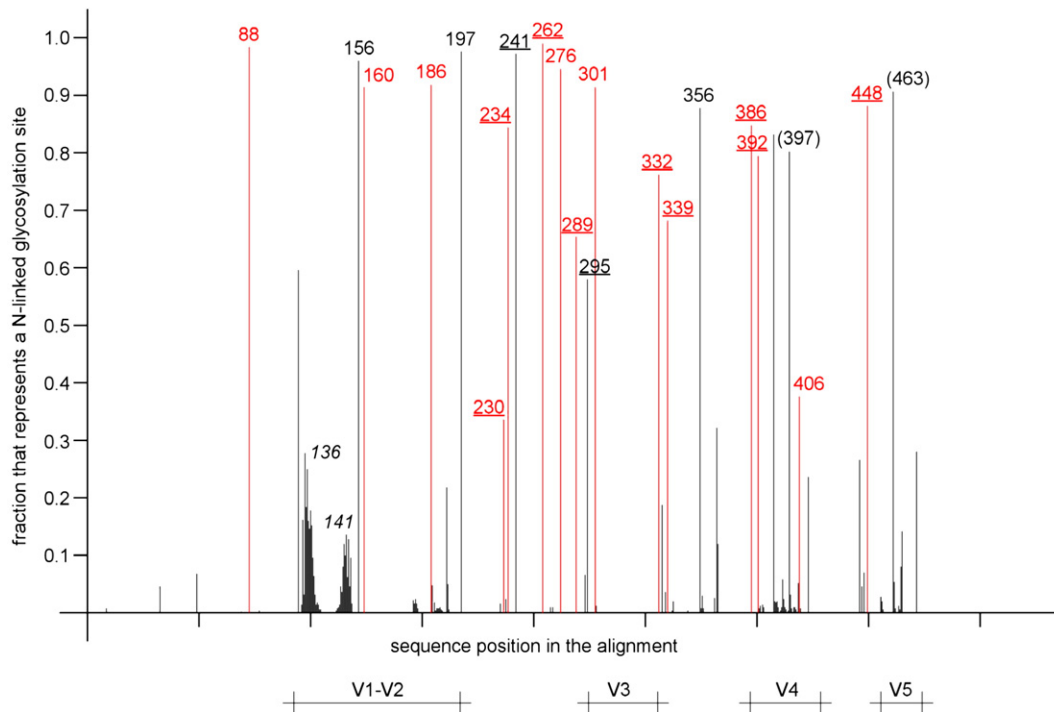

Fig. 4. Allocation of putative *N*-glycosylation sites in group M viruses. Alignment of envelope glycoprotein sequences was obtained from the HIV Sequence Database (http://hiv-web.lanl.gov) and subjected to analysis with the program N-glycosite (Zhang et al., 2004) for the allocation of putative *N*-glycosylation sites. The protein alignments contain all complete gene sequences that were available within the database (September 2007), with the important exception that very similar sequences (e.g. multiple clones from one isolate, multiple sequences from one person) have been deleted. Based on these results the fraction that represents a *N*-linked glycosylation site at each sequence position in the alignment was calculated and visualized. The numbers at the top of each of the vertical lines represent the asparagine positions in gp120 according to Kwong et al. (1998). Underlined numbers represent high-mannose type glycosylation sites. The other positions are complex-mannose type. The exact positions of 136 and 141 are difficult to assign and therefore, they are displayed in italic. Numbers between parentheses represent glycosylation sites that were not present in the HIV-1(III_B) strain that was used in our experiments. Vertical lines are red-colored when mutated in at least one of the isolates that were obtained after resistance selection experiments with CBAs.

variety of group M virus strains (Fig. 4). The glycans on positions 136 and 141 are difficult to assign due to considerable amino acid variability in this area of V1/V2, but it is clear that a glycan must be present on, or in the vicinity of, amino acid positions 136 and 141. This points to the importance of the presence of a glycan at, or near to, positions 136 and 141 of V1/V2. Besides searching for novel CBAs that specifically interact with the glycan configuration on gp120 V1/V2, attempts should also be made to produce monoclonal antibodies (mAb) that selectively recognize the glycans on the gp120 V1/V2 domain. This must be a feasible and achievable goal as examples of specific glycan-recognizing antibodies exist (Pinter et al., 2005; Warrier et al., 1994). Also, the 2G12 mAb is one of the very few known broadly neutralizing anti-HIV antibodies directed against an epitope on gp120 located around the C4–V4 region containing a specific glycan configuration (Calarese et al., 2003, 2005; Scanlan et al., 2002; Trkola et al., 1996).

In conclusion, we believe that both sensitization to CBAs and compromised infectivity upon glycan deletions in the gp120 V1/V2 envelope domain of HIV may explain why glycan deletions in V1/V2 rarely occur in the presence of escalating CBA pressure. This area of HIV-1 gp120 may represent a novel hot spot for rational and specific drug targeting of the HIV entry process due to its highly conserved glycans and a lack of flexibility in response to, and in the presence of, CBA drug pressure.

Material and methods

Test compounds

The mannose-specific plant lectin from *Hippeastrum* hybrid (HHA) and the *N*-acetylglucosamine-specific plant lectin from *Urtica dioica* (UDA) were derived and purified from these plants as described previously (Van Damme et al., 1988a; 1988b) and kindly provided by E. J.M. Van Damme and W. Peumans (Ghent, Belgium). Pradimicin A (PRM-A) was obtained from Prof. T. Oki and Prof. Y. Igarashi (Toyama, Japan). The CXCR4 antagonist AMD3100 was obtained from AnorMed (Langley, BC, Canada) and human soluble CD4 (sCD4) was purchased from Prospec (Rehovot, Israel).

Cells

Human T lymphocytic C8166 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and were cultivated in RPMI-1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS) (Cambrex Bio Science, Verviers, Belgium), 1% streptomycin, 2 mM L-glutamine and 75 mM $NaHCO_3$. Human embryo kidney cells (293T) were purchased from the ATCC and cultivated in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% FCS, 1% streptomycin and 75 mM $NaHCO_3$. U87/$CD4^+$/$CXCR4^+$, U87/$CD4^+$/$CCR5^+$ and U87/$CD4^+$/$CXCR4^+$/$CCR5^+$ cells (Princen et al., 2004) were obtained from Prof. D. Schols (Leuven, Belgium) and cultivated in DMEM containing 10% FCS supplemented with 0.4% geneticin (Invitrogen) and 1% puromycin (Invitrogen) for selection of CD4, CCR5 and CXCR4, respectively.

Viruses

The pNL4.3-Δenv-EGFP construct is used for production of wild-type NL4.3 virus after recombination with *env* and expresses an enhanced version of green fluorescent protein (EGFP) located between *env* and *nef* without affecting the expression of any HIV-1 gene. For this molecular clone, the expression of EGFP in infected cells is a measurement of virus production as described previously (Weber et

al., 2006). The construct pNL4.3-Δenv-EGFP was a kind gift from Dr. M. E. Quiñones-Mateu (Lerner Research Institute, Cleveland, OH, USA).

Construction of mutant molecular virus

The plasmid pBlue-env which encodes the *env* gene (Fikkert et al., 2002; Weber et al., 2006) was used to generate a variety of gp120 mutant viral strains with disrupted glycosylation sites at positions N136, N141, N156, N160, N186 and N195, where Asn was replaced by the closely related Gln. To generate the various multiple glycosylation site-mutant viruses, random combinations of these six glycosylation sites were introduced into pBlue-env using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, Westburg, Leusden, The Netherlands). Briefly, supercoiled double stranded pBlue-env and a combination of six synthetic primers containing the desired mutation were used (Table 4). These primer sets were extended during temperature cycling using *Pfu* Turbo DNA polymerase mixture. Subsequently, the product was digested with DpnI, which selects for the synthesized DNA containing the mutations and transformed in XL-10 Gold ultracompetent bacteria (Stratagene). Plasmid DNA was purified by the QIAgen miniprep kit (Qiagen, Venlo, The Netherlands). The presence of combinations of glycosylation site mutations was confirmed by sequencing the *gp120* gene.

Generation of mutant viruses by env chimeric virus technology

Various mutant PCR fragments were amplified from mutated pBlue-env by means of a hot start PCR using Expand High Fidelity Enzyme blend (Roche, Brussels, Belgium). The cycling conditions were as follows: a first denaturation step of 2 min at 95 °C, followed by 35 cycles consisting of 15 s at 95 °C, 45 s at 55 °C and 3 min at 68 °C. A final extension step was performed at 68 °C for 7 min. The various PCR products were purified and concentrated using the QIAgen PCR Purification Kit (Qiagen). Subsequently, 2 µg of the purified and concentrated PCR product was co-precipitated with 10 µg of pNL4.3-Δenv-EGFP, linearized by XbaI digestion and co-transfected into 293T cells using the calcium phosphate method like that described previously by Fikkert et al. (2002) and Weber et al. (2006). Briefly, 700,000 293T cells were seeded in a 6 cm-dish and 2 M $CaCl_2$ was carefully mixed with the DNA solution and added to an equal amount of HEPES buffered saline (HBS). After incubating 20 min at room temperature the mixture was added directly to the 293T cells. One day after incubation at 37 °C the supernatant was replaced by 2 ml of fresh medium. Positive transfection was detected by fluorescence microscopy. Recombinant replication-competent viruses were harvested and further grown on U87/CD4$^+$/CXCR4$^+$/CCR5$^+$ cells to produce more infectious viruses. After 3 to 5 days post-infection of the U87 cells, aliquots of 1 ml were stored at −80 °C for subsequent infectivity ($CCID_{50}$) and drug susceptibility (EC_{50}) determinations.

After the production of replication-competent viruses a genotyping of the HIV-1 *env* region of interest was performed. Proviral DNA was extracted from cell pellets using the DNeasy Tissue Kit (Qiagen) and sequenced as described previously (Van Laethem et al., 2005).

Table 4
Oligonucleotides used for the multi site-directed mutagenesis of the *env* gene

Mutation	Sense primer sequence
N136Q	5′-GTGCACTGATTTGAAG**CAG**GATACTAATACCAATAGTAG-3′
N141Q	5′-GAATGATACTAATACC**CAG**AGTAGTAGCGGGAGAATG-3′
N156Q	5′-GGAGAAAGGAGAGATAAAA**CAG**TGCTCTTTCAATATCAGC-3′
N160Q	5′-GATAAAAAACTGCTCTTTC**CAG**ATCAGCACAAGCATAAG-3′
N160D	5′-GATAAAAAACTGCTCTTTC**GAC**ATCAGCACAAGCATAAG-3′
N186Q	5′-CTTGATATAGTACCAATAGAT**CAG**ACCAGCTATAGGTTG-3′
N195Q	5′-GGTTGATAAGTTGT**CAG**ACCTCAGTCATTACACAGG-3′

The mutated codon is marked in bold.

Determination of the replication fitness of the deglycosylated HIV-1 strains using similar p24 levels for infection

Various deglycosylated HIV-1 strains containing equal amounts of HIV-1 p24 antigen (40,000 pg p24/ml) were exposed to C8166 cells. Starting at 1-day post-infection, cells were microscopically examined during 8 consecutive days for the appearance of HIV-1-induced cytopathogenicity. In addition, cells were fixed in phosphate buffered saline (PBS) containing 2% paraformaldehyde (PFA) and analyzed on a FACS CantoII flow cytometer (Becton Dickinson, San Jose, CA). Data were analyzed with FACS Diva Software (Becton Dickinson) for EGFP expression.

Determination of the replication fitness of the deglycosylated HIV-1 strains using a similar number of infectious viral particles for infection

Because the use of p24 levels only provides a quantitative determination of the HIV-1 particles, we also performed assays that take the virus infectivity into account. Therefore, we examined the replication capacity of the different mutant virus strains using a similar amount of infectious viral particles as recently described by Brockman et al. (2006). Prior to the assay, the amount of infectious viral particles that were able to infect 0.1% of the C8166 cell culture at 3 days post-infection was determined for wild-type and mutant (deglycosylated) virus strains. This amount of infectious viral particles was used to infect 500,000 C8166 lymphocytes in a 24-well plate. After 3 days of incubation at 37 °C, aliquots of cell suspensions that contain at least 200,000 cells were collected and fixed in 2% PFA. Fresh medium was added to the remaining cell cultures. The fixed cell suspensions were analyzed for EGFP expression on a FACS CantoII and normalized to the infection rate value on day 3 which was determined to be approximately 0.1%. The collection of cell suspensions and determination of EGFP expression was repeated for 2 more days.

Co-receptor usage of the deglycosylated HIV-1 strain

Inoculants of various deglycosylated HIV-1 strains were added to 10^4 U87/CD4$^+$/CCR5$^+$, U87/CD4$^+$/CXCR4$^+$ or U87/CD4$^+$/CXCR4$^+$/CCR5$^+$ cells in a 96-well microtiter plate. Medium was replaced 2 h after infection. The cell cultures were scored 3 days post-virus exposure for viral replication by fluorescence microscopy to detect EGFP expression.

Susceptibility of mutant deglycosylated HIV-1 strains to carbohydrate-binding agents (CBAs), AMD3100 and sCD4

The inhibitory effects of the antiviral compounds on HIV-1 replication in C8166 cell cultures were determined upon measurement of the EGFP expression in a FACS CantoII flow cytometer. The 50% cell culture infective doses ($CCID_{50}$) of the different HIV-1 strains were determined by titration of the virus stocks in C8166 cells. For drug susceptibility assays, C8166 cells were infected with 10 $CCID_{50}$ of the HIV-1 strains in the presence of five-fold serial dilutions of the antiviral drugs. After 3 days post-infection, the concentration of each compound achieving 50% reduction of EGFP expression, defined as EC_{50}, was determined.

Binding of mutant deglycosylated HIV-1 gp120 to human sCD4

96-well Maxi-Sorp plates (Nunc Inc) were coated with 25 ng human soluble CD4 in 50 mM carbonate buffer pH 9.6 for 60 min at 37 °C. Subsequently, the wells were blocked with phosphate buffered saline, pH 7.4 with 0.05% Tween-20 (PBST) containing 5% BSA for

60 min at 37 °C. After four washes with PBST, different mutant deglycosylated virus strains containing a normalized amount of 150,000 pg p24 Ag pre-treated with 10% TritonX-100, were added for 60 min at 37 °C. Subsequently, the wells were washed four times with PBST and incubated with 0.2 μg primary sheep anti-gp120 antibody (Aalto, Dublin, Ireland) for 60 min at room temperature (R. T.). After incubation, the wells were again washed four times with PBST and incubated with a 1/1000 dilution of peroxidase conjugated donkey anti-sheep IgG (Sigma, St.-Louis, MO) for 60 min at R.T. The wells were washed four times with PBST and 0.4 mg/ml *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigma) diluted in 0.05 M phosphate–citrate buffer, pH 5.0 containing 0.03% sodium perborate was added for 5 min and the reaction was stopped with 2 M H_2SO_4. The absorbance at 492 nm was determined using a Safire2 microtiter plate reader (Tecan). Specific absorbance was calculated subtracting the value for substrate and buffer from the measured value.

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