ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

VIROLOGY

Virology 313 (2003) 387–400

www.elsevier.com/locate/yviro

Structure-based, targeted deglycosylation of HIV-1 gp120 and effects on neutralization sensitivity and antibody recognition

Markus Koch,[a] Marie Pancera,[b] Peter D. Kwong,[b,c] Peter Kolchinsky,[a] Christoph Grundner,[a] Liping Wang,[a] Wayne A. Hendrickson,[c,d] Joseph Sodroski,[a,e,f] and Richard Wyatt[a,b,g,*]

[a] *Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, MA 02115, USA*
[b] *Vaccine Research Center, Laboratory of Virology, Building 40, National Institutes of Health, Bethesda, MD 20892, USA*
[c] *Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032, USA*
[d] *Howard Hughes Medical Institute, Columbia University, New York, NY 10032, USA*
[e] *Department of Pathology, Harvard Medical School, Boston, MA 02115, USA*
[f] *Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115, USA*
[g] *Department of Medicine, Harvard Medical School, Boston, MA 02115, USA*

Received 11 December 2002; returned to author for revision 9 January 2003; accepted 21 March 2003

Abstract

The human immunodeficiency virus (HIV-1) exterior envelope glycoprotein, gp120, mediates receptor binding and is the major target for neutralizing antibodies. Primary HIV-1 isolates are characteristically more resistant to broadly neutralizing antibodies, although the structural basis for this resistance remains obscure. Most broadly neutralizing antibodies are directed against functionally conserved gp120 regions involved in binding to either the primary virus receptor, CD4, or the viral coreceptor molecules that normally function as chemokine receptors. These antibodies are known as CD4 binding site (CD4BS) and CD4-induced (CD4i) antibodies, respectively. Inspection of the gp120 crystal structure reveals that although the receptor-binding regions lack glycosylation, sugar moieties lie proximal to both receptor-binding sites on gp120 and thus in proximity to both the CD4BS and the CD4i epitopes. In this study, guided by the X-ray crystal structure of gp120, we deleted four N-linked glycosylation sites that flank the receptor-binding regions. We examined the effects of selected changes on the sensitivity of two prototypic HIV-1 primary isolates to neutralization by antibodies. Surprisingly, removal of a single N-linked glycosylation site at the base of the gp120 third variable region (V3 loop) increased the sensitivity of the primary viruses to neutralization by CD4BS antibodies. Envelope glycoprotein oligomers on the cell surface derived from the V3 glycan-deficient virus were better recognized by a CD4BS antibody and a V3 loop antibody than were the wild-type glycoproteins. Absence of all four glycosylation sites rendered a primary isolate sensitive to CD4i antibody-mediated neutralization. Thus, carbohydrates that flank receptor-binding regions on gp120 protect primary HIV-1 isolates from antibody-mediated neutralization.

Keywords: HIV-1; Exterior envelope glycoprotein; Structure; Deglycosylation; Neutralization; Antibody binding; Correlation

Introduction

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Gallo et al., 1984). The heavily glycosylated envelope glycoproteins of HIV-1, the exterior envelope glycoprotein, gp120 (SU), and the transmembrane glycoprotein, gp41 (TM), are assembled in an oligomeric spike on the virion surface (Earl et al., 1990; Kowalski et al., 1987; Otteken et al., 1996). These glycoproteins are generated from a gp160 precursor glycoprotein and, following cleavage by cellular proteases, remain noncovalently associated on the cell or viral surface. The gp120 glycoprotein is composed of variable regions (V1–V5) and constant regions (C1–C5) and is the major target for neu-

* Corresponding author. Vaccine Research Center, Building 40, Room 4512, National Institutes of Health, Bethesda, MD 20892. Fax: +1-301-480-2658.
E-mail address: richardwyatt@nih.gov (R. Wyatt).

0042-6822/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0042-6822(03)00294-0

tralizing antibodies. The variable loops may contribute to evasion of host immune responses both by permitting the existence of replication-competent viral variants able to evade strain-restricted neutralizing antibodies and by shielding more conserved envelope regions from host antibody responses (Cao et al., 1997).

The heterotrimeric envelope glycoprotein spikes mediate virus entry into susceptible target cells by sequential binding of gp120 to the primary viral receptor, CD4, and to specific proteins of the chemokine receptor family, which serve as coreceptors (Alkhatib et al., 1996; Choe et al., 1996; Feng et al., 1996; Maddon et al., 1986). In vivo, the two major HIV-1 coreceptors are CCR5 and CXCR4. Primary isolates of HIV-1 predominantly use the chemokine receptor CCR5 as a coreceptor (R5 viruses); later in the course of HIV-1 infection isolates that utilize both CCR5 and CXCR4 may appear (R5X4 viruses) (Connor et al., 1997). Viruses that replicate under diminished immune selective pressures may acquire the ability to use CXCR4 exclusively or in addition to other chemokine receptors. Viruses that become CXCR4-dependent are referred to as T cell tropic (X4 viruses) and include T cell line, laboratory-adapted HIV-1 variants (TCLA viruses). TCLA viruses are typically more sensitive to antibody neutralization than the primary viruses from which they are derived. The mechanism by which sensitivity to neutralizing ligands is acquired is not clear and is manifest only in the context of the functional trimeric envelope spike. For example, monomeric gp120 glycoproteins derived from either soluble CD4 (sCD4)-resistant primary isolates or sCD4-sensitive TCLA viruses exhibit similar affinities for soluble CD4 (Brighty et al., 1991; Moore et al., 1991). By contrast, binding of CD4 by the trimeric envelope glycoprotein complex of the TCLA viruses is more efficient than that of the primary isolates (Kabat et al., 1994; Moore et al., 1992; Platt et al., 1997).

A model of virus entry mediated by the HIV-1 envelope glycoproteins has been deduced from the results of numerous in vitro and in vivo investigations, including the crystal structure of the CD4-bound gp120 core glycoprotein (reviewed in Wyatt and Sodroski, 1998). Initially gp120 binds to the primary receptor, CD4, and undergoes a conformational change that allows a high-affinity interaction with the chemokine receptor. The conformational change likely involves movement of the large, disulfide-linked V1/V2 loop and formation of a minidomain bridging sheet (Kwong, 1998, no. 4364; M. Koch and R. Wyatt, unpublished data). The envelope glycoproteins of some HIV-1 variants that have been adapted to replicate on CD4-negative target cells are able to bind chemokine receptor in a CD4-independent manner (Bandres et al., 1998; Kolchinsky et al., 1999). One such gp120 glycoprotein achieves CD4 independence by deleting a single N-linked glycan in the V1/V2 stem of the exterior envelope glycoprotein (Kolchinsky et al., 1999). The chemokine receptor-binding site minimally involves elements of the V3 loop and residues within the C4 region of gp120. Interaction of gp120 with coreceptor is believed to trigger a second conformational change that allows insertion of the hydrophobic amino-terminal fusion peptide of the HIV-1 transmembrane protein, gp41, into the target cell membrane (Bergeron et al., 1992; Gallaher, 1987). Thus, gp41 bridges the viral and target cell membranes. Subsequent conformational rearrangement of this prefusogenic intermediate into a lower energy state presumably brings the two membranes into direct juxtaposition, allowing membrane fusion to ensue (Chan et al., 1997; Chan and Kim, 1998; Tan et al., 1997; Wang et al., 1998; Weissenhorn et al., 1997). Membrane fusion permits entry of the HIV-1 core into the cytoplasm of the target cell and initiates the infection process.

The HIV-1 envelope glycoproteins have evolved a number of features that contribute to evasion of the host immune response. In addition to the variability of some gp120 regions, conformational flexibility and an extremely high degree of carbohydrate may contribute to the masking of functionally constrained gp120 elements (Kwong et al., 2002c; Myszka et al., 2000; Wyatt and Sodroski, 1998). More than half of the molecular weight of gp120 is due to N-linked glycosylation. The extensive glycosylation of the gp120 glycoprotein is likely to be involved in evasion of host immune responses in at least two distinct ways. First, the crystal structure of the gp120 core has revealed that much of the glycosylation lies on the gp120 outer domain, which is likely to be the region most exposed to the host humoral immune system. This glycosylated surface likely appears as “self” to the host immune system. Indeed, this so-called “silent face” of gp120 seldom elicits antibodies and thus appears to avoid the generation of potential neutralizing humoral responses (Wyatt et al., 1998).

A second contribution of glycosylation may be to enhance the ability of the virus to resist envelope-directed, antibody-mediated neutralization by the steric shielding of potential neutralizing determinants with carbohydrate moieties. Consistent with this hypothesis is the observation that sensitivity of TCLA HIV-1 strains to neutralization by V3-directed antibodies is influenced by glycosylation of the V3 loop (Back et al., 1994; Hansen et al., 1996; Schonning et al., 1996b). Primary isolates are usually insensitive to neutralization by most envelope glycoprotein-directed antibodies (Sullivan et al., 1995) and glycosylation may play a role in the resistant phenotype. Presumably, the glycosylation contributes to neutralization resistance by direct steric interference with antibody access to both strain-restricted and more conserved neutralizing determinants. During the preparation of this manuscript, Malenbaum et al. (2000) reported that removal of the 301 glycan resulted in increased neutralization sensitivity of HIV-1 to CD4 BS antibodies, including some primary isolates. In addition, the two viruses lacking the 301 glycan also demonstrated sensitivity to CD4i antibodies. However, no direct studies on antibody recognition of the viral “glycan types” were performed, so the mechanistic basis for the observed changes remains unclear.

Other studies have implicated primate immunodeficiency virus gp120 glycosylation in the evasion of host neutralizing responses or in receptor interaction. In one study, the elimination of N-linked glycosylation in the V1/V2 loop of pathogenic SIV mac239 rendered the virus more sensitive to host antibody responses (Reitter et al., 1998). Other work has mapped the ability of the HIV-1 primary R5 isolate ADA to achieve CD4 independence to a single sugar residue in this same V1/V2 stem loop region (Kolchinsky et al., 1999). The resulting CD4-independent R5 virus becomes extremely sensitive to antibody-mediated neutralization (Kolchinsky et al., 2001). Indeed, several studies have shown that complete deletion of the V2 or V1/V2 regions, and the several glycosylation sites present in these regions increased sensitivity to antibody-mediated neutralization (Cao et al., 1997; Stamatatos and Cheng-Mayer, 1998; Stamatatos et al., 1998). However, since both peptide and sugars were eliminated in these studies, it was not possible to assess the relative contribution of each modification to neutralization sensitivity.

The crystal structure of the HIV-1 gp120 core glycoprotein has been solved in complex with CD4 and the CD4i antibody 17b (Kwong et al., 1998, 2000a; Wyatt et al., 1998). Subsequent studies based in part upon the observed 17b-gp120 contacts have determined that the 17b epitope overlaps with the elements of the gp120 chemokine receptor-binding site (Rizzuto et al., 1998). Thus, resolution of both gp120 receptor-binding sites, analyzed in the context of mutagenic mapping of gp120 antibody epitopes, has allowed us to deduce that HIV-1 neutralizing antibodies are often directed against receptor-binding regions. Thereby, the CD4 binding site (CD4BS) antibodies recognize epitopes near the CD4 binding region, whereas the CD4-induced (CD4i) and V3 antibodies recognize epitopes implicated in chemokine receptor binding (Wyatt et al., 1998).

The V3 loop, not present in the available crystal structures, also contributes to chemokine receptor interaction and has been schematically modeled onto the gp120 core in the context of a trimeric model of gp120 (Wyatt et al., 1998). The enzymatically deglycosylated gp120 core in the X-ray crystal structure retained the peptide-proximal *N*-acetylglucosamine residues, which were resolved in the structure. The mannose cores of these sugars have been computationally modeled in the context of monomeric and trimeric gp120 (Kwong et al., 2000b; Wyatt et al., 1998).

This structure-based analysis has allowed us to target several N-glycans in close proximity to conserved receptor binding sites that might potentially limit the access of neutralizing antibodies to these functionally conserved regions (Fig. 1). We examined the effects of these structure-based glycan deletions on the neutralization sensitivity of primary isolates by CD4BS and CD4i antibodies and a V3-loop directed antibody. Compared with wild-type viruses, primary isolates lacking the N-glycan at residue 301 in the V3 loop were more sensitive to neutralization by CD4BS antibodies and a V3 loop-directed antibody, but not to CD4i antibodies. Increased antibody recognition of the envelope glycoproteins lacking the 301 glycan was observed, indicating a potential mechanism of immune evasion. In contrast to the isolates examined in the Mallenbaum study, we report that only in the absence of four N-glycans proximal to receptor binding regions did the primary isolate examined in our study become sensitive to CD4i antibodies.

Results

Selection of N-linked glycosylation sites for modification

Primary isolates of HIV-1, including the R5 viruses JR-FL and YU2 used in this study, are relatively resistant to most CD4BS antibodies; the one exception is the CD4BS antibody IgGb12 (Burton et al., 1994; Roben et al., 1994; Sullivan et al., 1995). Even in the case of the more potent IgGb12 antibody, primary isolates are more resistant to neutralization when compared to TCLA viruses (Sullivan et al., 1995). Many primary HIV-1 isolates have also been shown to exhibit a decreased sensitivity to sCD4 neutralization relative to that of TCLA viruses (Sullivan et al., 1995). We sought to examine the sensitivity to neutralization by several CD4BS and CD4i monoclonal antibodies and sCD4 of primary viruses with envelope glycoproteins lacking specific N-linked glycosylation sites. The decision of which glycosylation sites to eliminate was based upon molecular modeling of the HXBc2 gp120 core crystal structure (Kwong et al., 1998). The validity of this model for primary isolates has subsequently been demonstrated by the crystal structure of the gp120 core of the clinical HIV-1 isolate, YU2 (Kwong et al., 2000a). In the solved crystal structures, many of the peptide-proximal *N*-acetylglucosamine residues were retained. The mannose core moieties were then modeled onto this gp120 core structure as previously described (Kwong et al., 1998; Wyatt et al., 1998). Inspection of this model led to the observation that sugar moieties at asparagines 197 and 276 were proximal to the CD4 binding site (Fig. 1, top). Carbohydrate residues at asparagines 301 and 386 were in relatively close proximity to the putative chemokine receptor-binding site (Fig. 1, bottom). Therefore, we eliminated these glycosylation sites both singly and in combination in the HXBc2 virus. The carbohydrate addition site at asparagine 197 in the HXBc2 isolate is not present in the JR-FL strain and so only the glycosylation sites at 276, 301, and 386 were removed from the envelope glycoproteins of this virus. For the YU2 virus we eliminated the glycan at residue 301.

Antibody recognition of monomeric gp120 and replicative ability of gp120 glycosylation mutants

Soluble, monomeric HXBc2 and JR-FL gp120 molecules with the sugar deletions were analyzed for recognition by several CD4BS antibodies and CD4i antibodies, by soluble

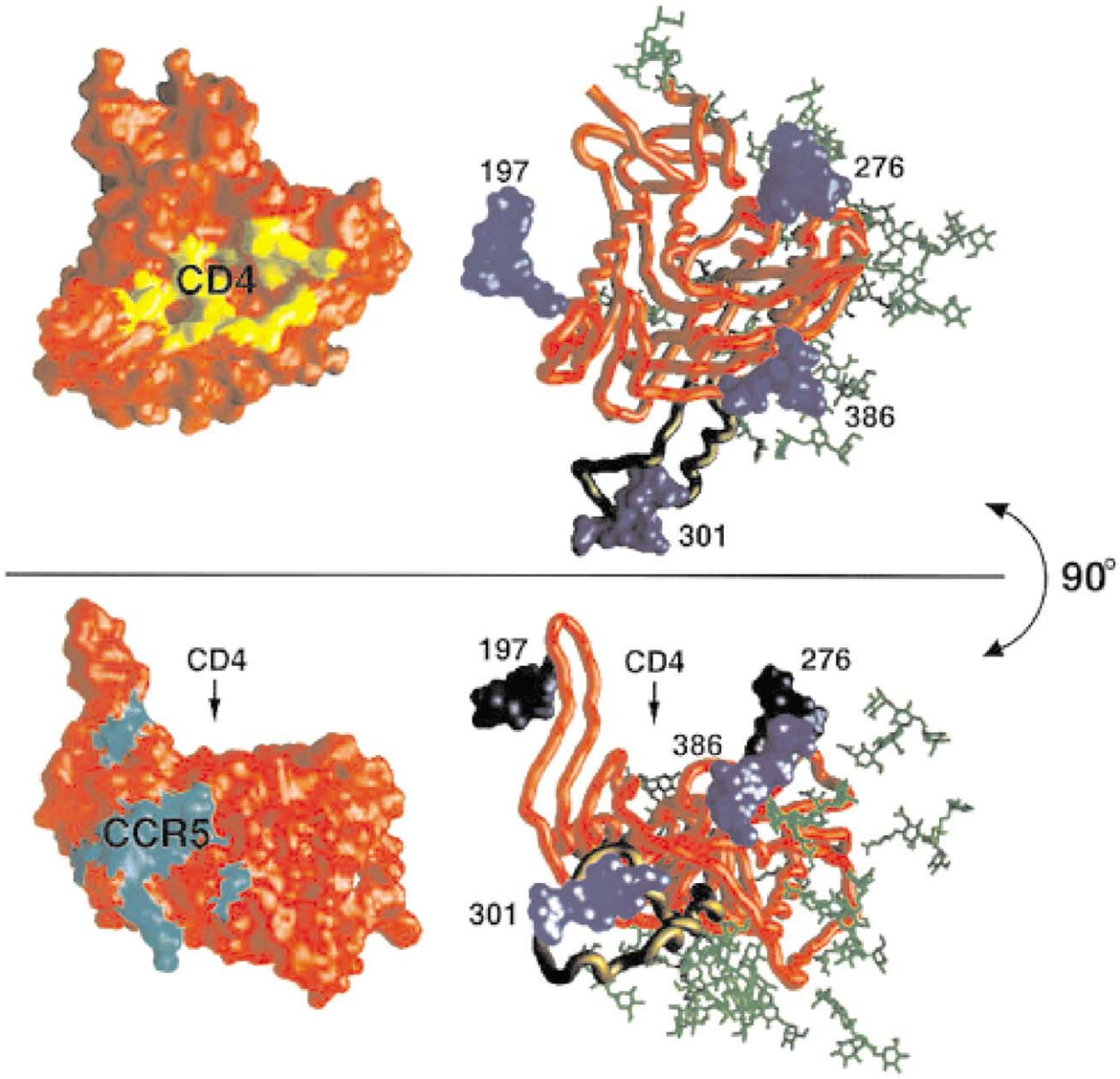

Fig. 1. Top: on the left is shown the molecular surface of the gp120 core in red with the CD4 contacts shown in yellow. In this orientation, the viral cell membrane would be at the top of the page and the target cell membrane would be toward the bottom of the page. To the right is the α-carbon chain of the gp120 core (in red) shown in the same orientation. The NMR V3 loop structure has been modeled on to the gp120 core as previously described and is shown in brown (Kwong et al., 2000b). The molecular surface of the mannose cores of the four targeted glycans at residues 197, 276, 301, and 386 previously modeled in the Kwong study are shown in blue. The molecules of the other gp120 sugars previously modeled are shown in green. Bottom: on the left is shown the molecular surface of the core gp120 glycoprotein rotated 90° relative to the top and viewed from the perspective of the target cell. The protruding V1/V2 stem projects toward the top of the page. The gp120 core residues implicated in CCR5 binding are shown in cyan, while the remaining surface residues are colored in red. The CD4 binding site is located on the plane of the molecule that projects into the page and is marked with an arrow. To the right is the α-carbon chain of the gp120 core in the same orientation, the sugars colored as described above. As can be seen in the model, the glycans at asparagines 301 and 386 lie most proximal to the residues implicated in chemokine receptor binding.

CD4 and by a mixture of sera from HIV-1-infected individuals in immunoprecipitation assays. No significant differences in ligand recognition between the glycan-deleted gp120 glycoproteins and wild-type glycoproteins were observed (data not shown).

To assess the effect of the sugar deletions in the context of the functional envelope oligomer, recombinant virions with the wild-type and carbohydrate-deleted envelope glycoproteins were produced in 293T cells. Infectivity of viruses with the YU2 and JR-FL Δ301 envelope glycoproteins was similar to that of viruses with the respective wild-type envelope glycoproteins (data not shown). Viruses with the JR-FL Δ276/301/386 envelope glycoproteins infected target cells at roughly 10% the efficiency of the viruses with the

Table 1
The concentration of antibody or sCD4 (μg/ml) required to neutralize 50% of the infection of recombinant HIV-1 with the indicated envelope glycoprotein is shown

Envelope glycoprotein strain	Envelope glycoprotein variants	Ligands							
		CD4BS				CD4i		α gp41	
		F105	IgGb12	15e	21h	17b	48d	2F5	sCD4
JR-FL	Wild-type	>50	0.05	≫	≫	≫	≫	0.7	1.0
	Δ276	>50	0.05	ND	ND	≫	≫	ND	0.5
	Δ301	1.0	0.017	0.7	10.8	≫	≫	0.7	0.12
	Δ386	>50	0.04	ND	ND	≫	≫	ND	2.0
	Δ301/386	3.0	0.01	ND	ND	≫	≫	ND	0.21
	Δ276/301/386	0.4	0.007	ND	ND	10	0.6	0.6	0.18
YU2	Wild-type	≫	2.3	≫	≫	ND	ND	3.0	0.35
	Δ301	9.0	0.2	41.0	32.0			2.8	0.024
HXBc2[a]	Wild-type	1.1	0.029	3.0	44	ND	ND	ND	ND
	Δ197	0.4	0.01	ND	ND				
	Δ276	0.2	0.028	ND	ND				
	Δ301	0.026	0.01	0.01	0.01				
	Δ386	0.2	0.032	ND	ND				
	Δ301/386	0.036	0.007	ND	ND				

Note. Entry of viruses containing envelope glycoprotein variants from the HXBc2 strain was determined using CXCR4 and CD4 expressing Cf2Th cells as target cells while entry of viruses with YU2 or JR-FL envelope glycoproteins was determined using CCR5 and CD4 expressing Cf2Th cells as target cells. It is not possible to quantitatively compare the activity of inhibitory ligands between viruses that enter different target cells. >50, Greater than all concentrations tested; previous studies report neutralization by this ligand at higher concentrations. ≫, Greater than all concentrations tested; previous studies report neutralization resistance to this ligand. ND, not determined.

[a] HXBc2 Δ197/276/301/386 viruses (similar to the JR-FL Δ276/301/386 viruses, which naturally lack the 197 glycan) infected CXCR4-expressing target cells at a level that was too low to generate reproducible entry and neutralization data.

respective wild-type envelope glycoproteins (data not shown). The lower level of infectivity may be, in part, due to a slight increase in shedding of gp120 from the envelope glycoprotein complex, which was observable in 293T cells transiently transfected with the JR-FL Δ276/301/386 envelope glycoprotein expressor plasmids and analyzed by immunoprecipitation (data not shown). Nevertheless, the viruses with the JR-FL Δ276/301/386 envelope glycoproteins were able to infect target cells at a significant and reproducible level. HXBc2 Δ197/276/301/386 viruses infected CXCR4-expressing target cells at a level that was too low to generate reproducible entry and neutralization data (not shown).

Neutralization sensitivity of glycosylation mutants

To determine effects of the sugar deletions on neutralization sensitivity, viruses were incubated with selected antibodies or sCD4, prior to the addition to either $CD4^+$/$CCR5^+$ or $CD4^+$/$CXCR4^+$ Cf2Th target cells. Viruses with the JR-FL and YU2 Δ301 envelope glycoproteins were tested for neutralization sensitivity by selected CD4BS antibodies, IgGb12, F105, 15e, and 21h and by the V3 loop-directed antibody 39F. Of the set of CD4BS antibodies, IgGb12 alone had been shown to neutralize the wild-type JR-FL or YU2 viruses (Moore et al., 1995; Roben et al., 1994; Sullivan et al., 1995).

As anticipated, in this study IgGb12 was the sole CD4BS antibody able to inhibit the entry of recombinant viruses with the wild-type JR-FL envelope glycoproteins (Table 1). However, in contrast to wild-type JR-FL viruses, viruses containing the JR-FL Δ301 envelope glycoproteins were sensitive to neutralization by all of the CD4BS antibodies tested. In addition, viruses with the JR-FL Δ301 envelope glycoproteins were three-fold more sensitive to IgGb12 neutralization, compared to viruses with the wild-type JR-FL envelope glycoproteins (Table 1).

Similar effects were observed for viruses containing the mutant envelope glycoproteins of the primary isolate YU2. In the presence of the 15e or 21h CD4BS antibodies, the entry of viruses with the YU2 Δ301 envelope glycoproteins was inhibited by nearly 50% under the conditions tested (Fig. 2 and Table 1). This effect was significantly different from that observed for viruses containing the wild-type YU2 envelope glycoproteins, which were not neutralized under the test conditions. In fact, the viruses with the wild-type YU2 envelope glycoproteins were not only resistant to neutralization by the 15e and 21h antibodies, but demonstrated enhanced entry in the presence of these antibodies (Fig. 2 and Table 1), consistent with previous studies (Sullivan et al., 1998). The YU2 wild-type virus was resistant to the V3 loop-directed antibody 39F, whereas the Δ301 virus was rendered sensitive to 39F-mediated neutralization by removal of the 301 glycan (Fig. 2).

Increased sensitivity of viruses with envelope glycoproteins lacking the N-glycan at residue 301 to neutralization by CD4BS antibodies was not restricted to primary isolates, but was also observed for TCLA viruses possessing HXBc2

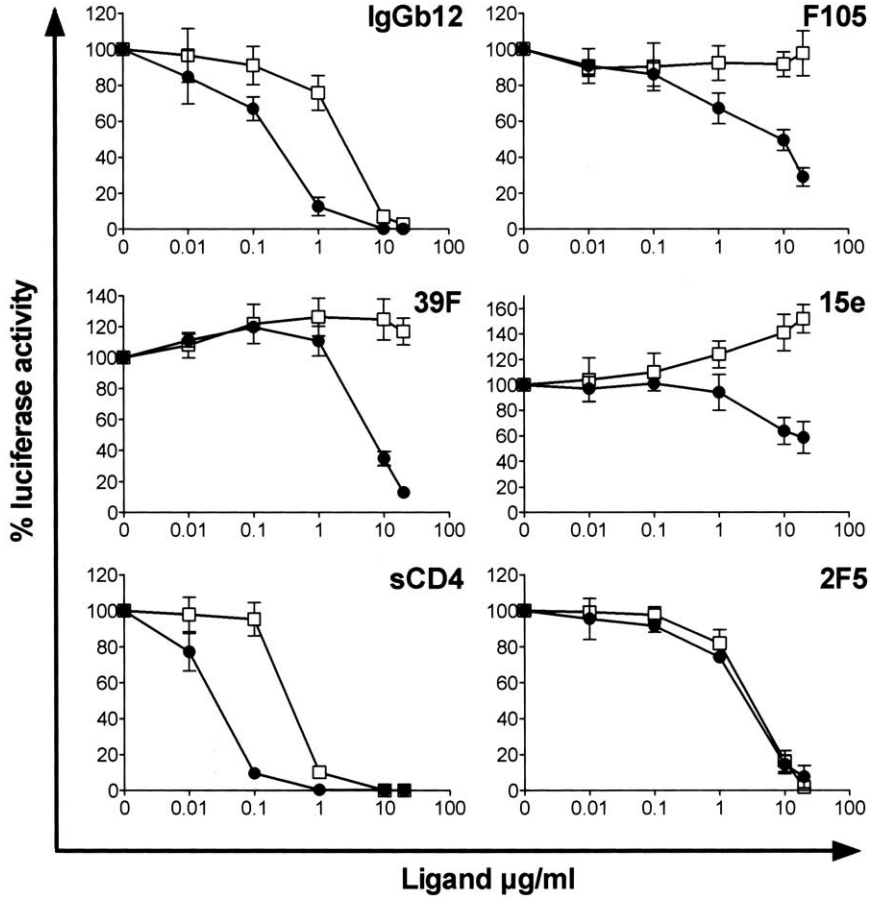

Fig. 2. Neutralization curves of wild-type YU2 luciferase viruses (□) and Δ301YU2 luciferase viruses (●) with the CDBS antibodies IgGb12, F105, and 15e, the V3 loop antibody 39F, soluble CD4, and the gp41 antibody 2F5. Values are presented as percentage luciferase activity and are normalized to 100% virus entry into Cf2Th target cells in the absence of any ligand. The data points presented represent the average of three independent experiments (39F two experiments) with the error bars representing the standard deviation between these experiments.

envelope glycoproteins. The viruses with the HXBc2 Δ301 envelope glycoproteins were 30- to 40-fold more sensitive to neutralization by F105, 15e, and IgGb12 compared to viruses with the wild-type HXBc2 envelope glycoproteins. These data support previous reports that have shown greater neutralization sensitivity of TCLA viruses lacking the 301 glycosylation site for both V3 loop antibodies and one CD4BS antibody (Back et al., 1994; Schonning et al., 1996b).

Viruses with envelope glycoproteins lacking the N-glycan at position 301 were also more sensitive to neutralization by sCD4 (Table 1). The viruses with the JR-FL Δ301 envelope glycoproteins were fivefold more sensitive than viruses with the wild-type JR-FL envelope glycoproteins. An even greater effect was observed for the viruses containing the YU2 Δ301 glycan envelope glycoproteins. Here a 10-fold increase in sensitivity to neutralization by sCD4 was observed relative to viruses with the wild-type YU2 envelope glycoproteins (Fig. 2 and Table 1).

The individual removal of N-glycans from asparagines 276 and 386 did not affect the sensitivity of viruses with these envelope glycoproteins to neutralization by the antibodies tested. However, viruses with envelope glycoproteins lacking N-glycans at three residues (asparagines 276, 301, 386) were more sensitive to neutralization by a range of antibodies. Viruses with JR-FL envelope glycoproteins lacking the glycosylation sites at 276, 301, and 386 were not only more sensitive to CD4BS antibodies and sCD4, presumably due to the missing N-glycan at residue 301, but were also more sensitive to the two examined CD4i antibodies, 17b and 48d (Fig. 3 and Table 1). These antibodies are relatively ineffective at neutralizing primary HIV-1 isolates, even at the highest concentrations tested (Sullivan et al., 1998; Thali et al., 1992). Even for TCLA HIV-1 viruses, more than 30 μg/ml of 17b was reported to be required to achieve 90% inhibition of a representative isolate (Thali et al., 1993). In this study, 17b and 48d were able to neutralize viruses with the JR-FL Δ276/301/386 envelope glycoproteins with IC50s of 10 and 0.6 μg/ml, respectively (Fig. 3).

To examine if viruses with envelope glycoproteins lacking the three N-glycans were generally sensitive to antibody-mediated neutralization, we examined the sensitivity of these viruses to neutralization by 2F5, an antibody directed against a gp41 epitope. No difference was observed

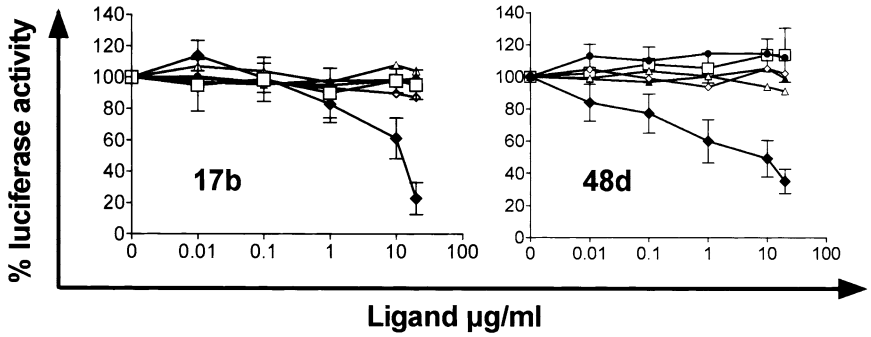

Fig. 3. Neutralization curves of JR-FL wild-type and selected glycan-deleted luciferase viruses by the CD4i antibodies 17b and 48d presented as percentage luciferase activity normalized to 100% entry of each virus into Cf2Th target cells in the absence of antibody. The symbols represent the following viruses: JR-FL wild-type (□, containing a natural 197 glycan deletion); Δ301 JR-FL (●); Δ276 JR-FL (△); Δ386 JR-FL (◇); Δ301/386 JR-FL (▲), and Δ276/301/386 JR-FL (◆). The data points presented represent the average of three independent experiments with the error bars representing the standard deviation between these experiments.

in the sensitivity to 2F5 neutralization of viruses lacking N-glycans when compared to viruses with wild-type envelope glycoproteins (Figs. 2 and 3 and Table 1).

Antibody binding to cells expressing envelope glycoprotein

To determine if a higher level of antibody binding to oligomeric envelope glycoproteins derived from the neutralization-sensitive Δ301 glycan JR-FL virus, as opposed to the neutralization-resistant wild-type JR-FL viruses, could be observed, quantitative cell-surface staining was performed. Equivalent levels of cell-surface expression of the two JR-FL oligomers (wild-type and Δ301 glycan) were confirmed by cell-surface staining using a pool of IgG proteins from HIV patients (HIVIgG) and the 2F5 antibody (Fig. 4), which neutralized both viruses equivalently (Fig. 4 and Table 1). In some experiments, staining with the 2G12 antibody was also included to assess equivalent expression of the two glycoproteins (representative data shown in Fig. 4).

The IgGb12 antibody bound to both the wild-type and the Δ301 oligomeric glycoproteins at significant levels in a dose-dependent manner (Fig. 4). In the experiment shown, for both envelope glycoproteins, saturation binding of b12 was achieved at 10 μg/ml and half-maximal binding at less than 1 μg/ml. Saturation binding of b12 at these concentrations was observable in all experiments ($n = 5$). The V3 loop-directed antibody 39F, which could neutralize the YU2 Δ301 virus but not wild-type virus, bound the Δ301 oligomeric glycoproteins approximately fourfold better at 100 μg/ml (10 μg/well; Fig. 4). Saturation binding of 39F was not achieved to the Δ301 glycoproteins at the highest antibody concentrations tested, so this significantly increased binding may be an underestimate of differential recognition by 39F of the two glycoproteins. The F105 antibody also bound the envelope glycoproteins derived from F105-sensitive Δ301 JR-FL virus at a higher level (nearly threefold greater binding at 100 μg/ml) than the glycoproteins derived from the F105 neutralization-resistant wild-type JR-FL isolate (Fig. 4). The level of F105 staining, as well as that of 2F5, was significantly lower than that of IgGb12 to either wild-type or Δ301 glycoproteins or that of 39F to the Δ301 glycoproteins (Fig. 4).

Although the observed staining of F105 and 2F5 to wild-type oligomers was at a low level, it increased with increasing antibody concentrations and was significantly different from negative controls. When we used an anti-RSV human IgG1 antibody as a negative control, a much lower level of background staining was observed. The level of the anti-RSV staining remained below 10 mean fluorescence intensity (MFI) units up to concentrations of 250 μg/ml (Fig. 4 and data not shown). For F105, 2F5, and the V3 loop antibody 39F, binding to the wild-type oligomers did not achieve saturation (Fig. 4). To accurately assess half-maximal binding levels of these antibodies as compared to the levels of IgGb12 and 2G12, it is necessary that each antibody achieve saturation binding. Presumably this is due to full-site occupancy of each particular epitope. The IgGb12 antibody achieved saturation binding to wild-type oligomers at 10 μg/ml and half-maximal binding at less than 1 μg/ml, but F105 continues to increase its binding as the antibody concentration is increased (Fig. 4). Even at 100 μg, the highest level of F105 antibody, the F105 achieves an MFI of 40, fivefold less than that of the binding that IgGb12 achieves (and maintains) at a 10-fold lower dosage (>200 MFI, Fig. 4). Therefore this large difference in the level of binding achieved by the two antibodies, while only an underestimate of the actual binding differences, is consistent with the distinct neutralizing properties of the antibodies.

Discussion

Due to its location on the surface of the virus, HIV-1 gp120 is the major target for neutralizing antibodies. The extreme degree of gp120 glycosylation has led to the frequent suggestion that this extensive carbohydrate content

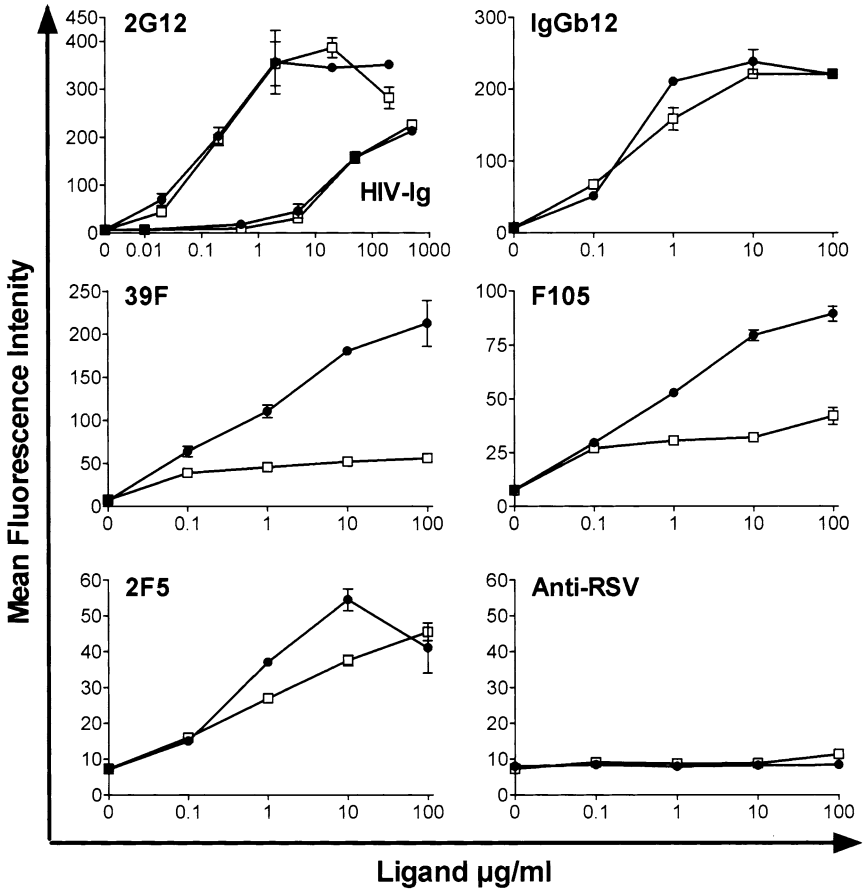

Fig. 4. FACs-based binding curves to JR-FL cleavage-competent wild type envelope glycoproteins (□) and Δ301 glycan proteins (●) expressed on the surface of transiently transfected 293T cells with the anti-HIV-1 human antibodies, 2G12, HIVIgG, IgGb12, F105, 39F, 2F5, and the negative control human IgG1 antibody, Anti-RSV. The data shown are from a representative experiment performed with duplicate samples with the exception of the 2G12 and anti-RSV, which are from an independent but similar experiment. Error bars indicate the range of values obtained for duplicate samples. The analysis of IgGb12, F105, 39F, and 2F5 were repeated in five independent experiments and the staining with 2G12, anti-RSV, and HIVIgG were repeated three times with similar results.

may contribute to escape from immune selective forces imposed by neutralizing antibodies (Cheng-Mayer et al., 1999; Moore et al., 1995; Olofsson and Hansen, 1998; Reitter et al., 1998; Roben et al., 1994; Schonning et al., 1996a). The solution of the gp120 core crystal structure has afforded testable hypotheses in this regard. In this study, we sought to determine if removal of specific sugars proximal to receptor-binding regions would affect the neutralizability of HIV-1 or recognition by HIV-1 monoclonal antibodies.

We observed two distinct effects of targeted gp120 sugar removal on the neutralization sensitivity of primary HIV-1 isolates. The first and major effect was that removal of an N-linked glycosylation site at the base of the V3 loop rendered two primary viruses significantly more sensitive to neutralization by a panel of CD4BS antibodies as well as to neutralization by sCD4 and a V3 loop-directed antibody. These observations are in agreement with previous reports demonstrating that removal of the 301 glycan in two TCLA viruses and two primary isolates increased their sensitivity to inhibition by CD4BS antibodies (Back et al., 1994; Malenbaum et al., 2000). The second effect that was observed in our study was an increase in HIV-1 sensitivity to the CD4i antibodies 17b and 48d.

These observations then lead to the question of mechanism: does the removal of the 301 glycan remove a steric (or conformational) blockade and allow access to neutralizing determinants not accessible on the wild-type oligomer? We have addressed this issue by performing FACS-based binding assays. To date it has been difficult to demonstrate a correlation between antibody recognition and neutralization sensitivity/resistance of primary isolates. This has been accomplished for lab-adapted strains (Sattentau and Moore, 1995) and more recently for SHIV-derived Envs (Si et al., 2001) and for one primary isolate (Fouts et al., 1997). Using a method similar to Si et al., we have studied antibody binding to envelope glycoproteins expressed on the cell surface of transfected cells to generate binding curves that allow a relative binding comparison and, for some antibodies where saturation binding is achieved, an affinity estimate.

These data reveal several observations relevant to antibody recognition of oligomeric Env. A rank order of bind-

ing to wild-type JR-FL oligomers by the gp120-directed antibodies, as determined by MFI levels at increasing antibody concentrations, is 2G12 > b12 ≫ 39F, F105. Since the data correlate with neutralization sensitivity, they suggest that both 2G12 and b12 can achieve higher levels of site occupancy than the less potent gp120-directed neutralizing antibodies. That the potent, gp41-directed antibody 2F5, which binds at relatively low levels, does not fit this general pattern may reflect a distinct mechanism of neutralization for this antibody. Perhaps lower site occupancy of its epitope is still sufficient to achieve neutralization by interfering with the fusion process in a manner yet to be defined.

The same rank order is observable on Δ301 oligomeric glycoproteins, except that (a) 39F binding now approaches b12 MFI at the highest concentrations tested (but does not fully saturate) and (b) F105 binds at a slightly but reproducibly higher level compared to wild-type JR-FL Env and also does not achieve saturation binding (five independent experiments). Because F105 does not achieve saturation, an accurate quantitative comparison between F105 and b12 binding is not possible. The general trend that higher levels of binding correlate with increased potency of gp120-directed antibody neutralization is consistent with these observations. This again suggests that an increased degree of "site occupancy" is required to achieve neutralization for the gp120 epitopes. By site occupancy we mean either a greater number of antibody binding events per oligomer or a greater number of oligomers recognized on either the cell surface or the virus. A spike occupancy model does not reconcile the data in its entirety since F105 does not closely achieve MFI levels of b12 or 2G12 under the conditions tested, yet is still able to neutralize the Δ301 virus. Perhaps this reflects differences in binding kinetics, potency, or mechanisms of neutralization for each antibody or epitope.

How might the available information on HIV-1 gp120 structure explain the observed increase in neutralization sensitivity of the virus and increased antibody recognition engendered by deletions of the V3 loop glycan at asparagine 301? That the V3 loop is capable of conformational rearrangement is consistent with the observation that the V3 loop becomes more sensitive to proteases following CD4 binding (Sattentau and Moore, 1991). A previous study on the lab-adapted isolate BRU-2 showed that BRU Δ301 oligomeric glycoproteins, but not monomers, were better recognized by a V3 loop antibody than wild-type BRU oligomers (Schonning et al., 1996b). In a structural context, these results can be viewed in at least two ways: potential effects confined to interactions within a single monomeric gp120 subunit or potential effects occurring between adjacent monomers within the context of a functional trimer.

Within the gp120 monomer, both direct and indirect effects are possible. For purposes of interpreting the data, we have utilized the previously described model in which an NMR-derived V3 loop structure was affixed to the CD4-bound gp120 core crystal structure (Kwong et al., 2000b and Fig. 4). Because this is a model, there is uncertainty in defining both the orientation and the conformation of the V3 loop and assigning the orientation of the V3 loop 301 glycan. Despite this caveat, direct effects of modification of glycan 301 on the CD4 binding site itself appear unlikely based upon the trimeric model derived from the known structures. In this model, the V3 loop resides on the "bottom" of the envelope spike and the 301 glycosylation site is relatively close to the disulfide-linked V3 base (Kwong et al., 2000b). For the V3 loop to reposition proximal to the CD4 binding site would require an unlikely and extensive rotational movement of the outer domain relative to the inner domain. That there was no effect of removing 301 N-linked glycosylation on CD4 binding site antibody recognition in the context of monomeric gp120 (data not shown) supports the notion that 301 glycosylation removal does not exert a direct effect on exposure of the CD4 binding site. However, these observations should be interpreted with some caution for the following reasons. Although immunoprecipitation experiments did not reveal any difference in antibody recognition of monomeric gp120 with or without the targeted sugars, such effects may not be readily apparent within the context of the free monomer. Thermodynamic analysis indicates that monomeric gp120 is extremely flexible and exhibits considerable entropy that is greatly reduced upon binding of gp120 to CD4 (Myszka et al., 2000). Due to these properties, the assessment of some structural relationships by antibody binding analysis may not be easily achieved in the context of monomeric gp120. The flexibility of gp120 may allow a ligand to reposition proximal gp120 elements without observable steric hindrance. However, in the oligomeric context, the individual monomers are likely more constrained in conformation. Thereby, although the structural proximity of two elements within a gp120 monomer may be more or less the same, steric hindrance will be more manifest in the context of the oligomer. With this cautious note in mind, it is possible that deletion of carbohydrate at residue 301 repositions the V1/V2 region, or some other gp120 element, within the same monomer and increases neutralization sensitivity in an indirect manner.

Another interpretation of these data is suggested by the trimeric gp120 model previously described (Kwong et al., 2000b). This model is based upon the optimization of quantifiable surface parameters of the gp120 monomeric core crystal structure to create a trimer. Three different V3 loop models were then constructed using steric hindrance as the only constraint. Each of these models suggests that the V3 base of one monomer is located in close proximity to the V1/V2 stem of an adjacent monomer (Kwong et al., 2000b and Fig. 5). Therefore, removal of the sugar on asparagine 301 in the base of the V3 loop might allow a repositioning of the V1/V2 stem on the adjacent monomer. In this interpretation, the V1/V2 loop itself or some other element of gp120 influenced by the stem rearrangement now becomes less effective at sterically shielding the CD4 binding site (see Fig. 5). If this conjecture were valid, then one would

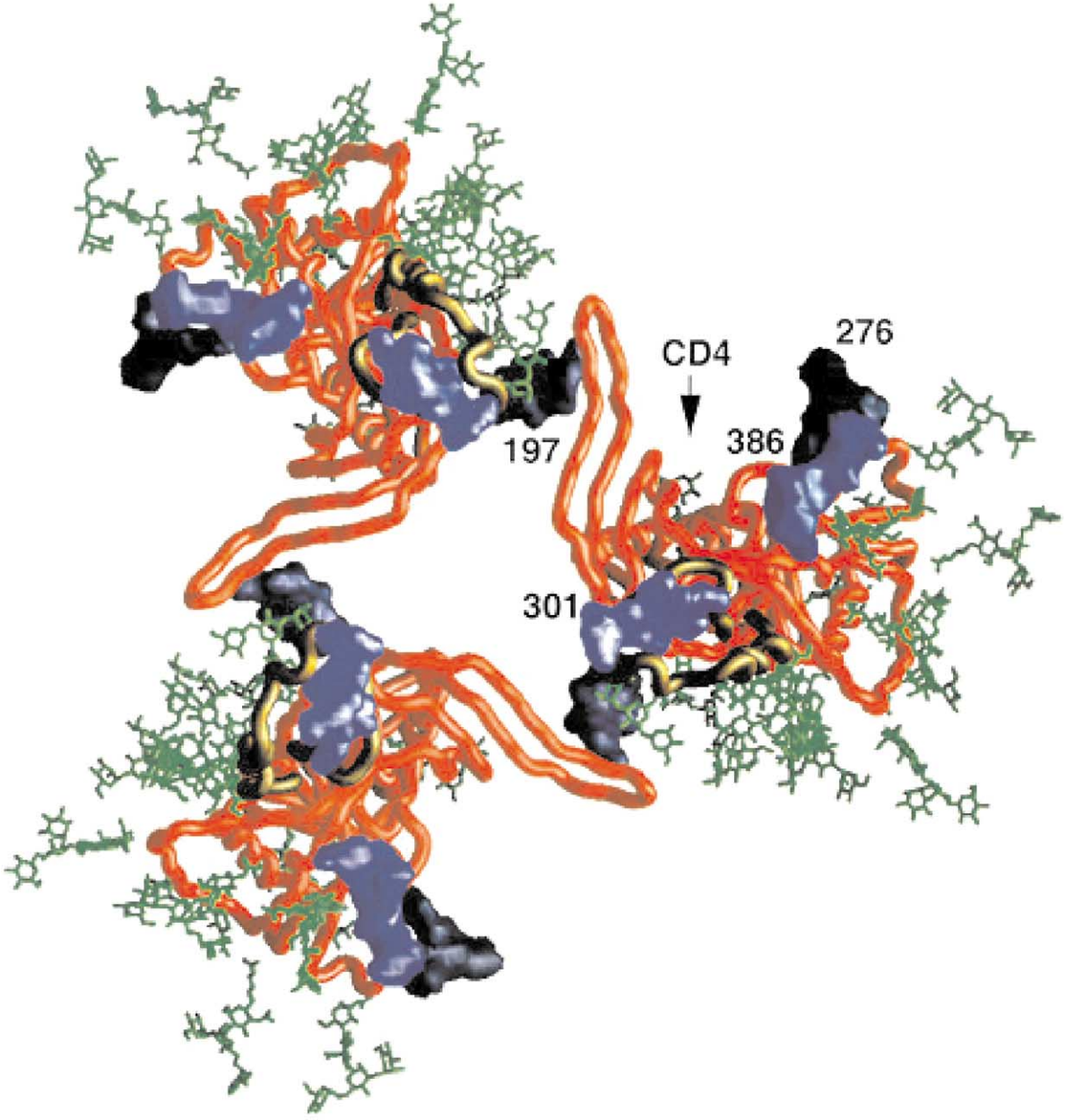

Fig. 5. A trimeric model of the gp120 core glycoprotein shown from the perspective of the target cell (same as in Fig. 1, bottom) is based upon quantifiable surface parameters as previously described (Kwong et al., 2000b). The α-carbon chain of the gp120 core is shown in red and the V3 loop α-carbon chain is in brown. The molecular surfaces of the targeted glycans are shown in blue and labeled on one gp120 monomer. The proximity of the 301 glycan to the V1/V2 stem and 197 glycan on an adjacent monomer is apparent. The other sugar molecules are colored green. The CD4 binding site is labeled (CD4) and marked with an arrow on one gp120 monomer.

expect that the effects of the removal of carbohydrate at position 301 on antibody accessibility to the CD4 binding site would be observed only in the context of the oligomer. The interpretation is consistent with the data presented here.

Yet another potential explanation of the data is that many or most CD4BS antibodies bind gp120 with an orientation that results in much of the antibody mass being positioned between the viral envelope glycoproteins and the target cell. This might be the case even if the antibody epitope itself is located on the side of the viral spike overlapping the CD4 binding site. Removal of the sugar moieties at position 301 in the base of the V3 loop would better allow such an orientation by removing a steric hindrance. This orientation of some CD4BS antibodies might allow these antibodies to interfere with both CD4 binding and chemokine receptor interaction. Indeed, antibody competition analyses have revealed that CD4BS antibodies compete with sCD4 and antibodies directed against the chemokine receptor-binding surface of gp120 (Moore and Sodroski, 1996). Data recently obtained with CD4-independent HIV-1 isolates supports the

notion that CD4BS antibodies interfere with CCR5 binding (Raja et al., 2003).

If the carbohydrate at position 301 in the V3 loop was positioned proximal to the chemokine receptor-binding site in the CD4-free state, one might expect that deletion of this sugar might affect chemokine receptor binding. Enhancement of chemokine receptor binding by 301 glycan removal has been reported (Malenbaum et al., 2000), although this was not observed in another study (Losman et al., 1999). Another prediction might be an increase in 17b neutralizing capacity, since this epitope overlaps the chemokine receptor-binding site and the neutralization capacity of 17b is increased by subneutralizing concentrations of sCD4 (Sullivan et al., 1998). In the Malenbaum study, the viruses lacking the 301 glycan described did become more neutralization sensitive to three CD4i antibodies. However, for the isolates used in our study, 301 glycan deletion did not enhance CD4i antibody-mediated neutralization and the JR-FL isolate, already lacking the glycan at 197, required the additional deletion of glycans at positions 276 and 386 to achieve sensitivity to CD4i antibody neutralization. Here, the absence of these sugars resulted in no apparent increase in 17b recognition of monomeric gp120, indicating that the effects of sugar removal are manifest only in the context of the oligomeric spike. Further structural studies of oligomeric HIV envelope glycoproteins should clarify the role of these sugars in occluding the 17b epitope in the context of the functional oligomer.

Although we cannot explain all of the data in a precise structural manner, a general pattern emerges consistent with the following scenario. Selective pressures drive the evolution of viral spikes that best maintain a contiguous steric barrier or "protective shell" composed of sugars and variable loops. Presumably these barriers are required to obscure functionally conserved regions from potentially neutralizing antibodies, consistent with the recently proposed evolving glycan shield mechanism (Wei et al., 2003). For the more broadly neutralizing CD4BS antibodies, the removal of a single sugar at the base of the V3 loop, either indirectly or directly, allows these antibodies to access the CD4 binding site and neutralize primary HIV-1 isolates. This is consistent with the correlation to neutralization we observed in our binding studies: the more potent gp120 antibodies bound to a greater extent and all gp120 neutralizing antibodies bound 301-glycan-deleted oligomers more efficiently than wild-type oligomers. For the viruses studied here, the CD4i antibodies require larger "holes" in this protective shell, provided by removal of the three glycans, to access their epitopes and neutralize virus. Previously, we have shown that the removal of a single N-linked carbohydrate in the V1/V2 stem of the primary ADA HIV-1 isolate results in CD4 independence and increased neutralization sensitivity (Kolchinsky et al., 2001a, 2001b). In this case, the phenotypes associated with the presence or absence of the carbohydrate were dependent on the integrity of the V1/V2 variable loops. Thus, even a single sugar can exert major effects on the positions of large variable loops in the context of either monomeric or trimeric envelope glycoproteins.

Removal of the 301 glycan could involve rearrangement with both the same monomer and the adjacent monomer. In this sense, a trimer from a given HIV-1 strain can be viewed as a unique "jig-saw puzzle" that has been selected to achieve a proper "fit" by functional constraints and by host immune selection pressures. Particular perturbations of this specific fit can cause rearrangements of trimeric elements and alter exposure of normally concealed determinants. Effects of some of these perturbations may be strain-specific if they, for example, involve variable region interfaces. Others, such as removal of the 301 glycan and exposure of the CD4 binding site, may exert effects on multiple envelope strains if they are involved in common mechanisms used to maintain trimeric spike fitness, although some HIV-1 isolates retain function and neutralization resistance even in the absence of the N-glycan at asparagine 301. The dynamic process of mutational selection leads to a myriad of strain-specific envelope structures that are unique in their subtle variations within the boundaries aforementioned: mutation, compensation, maintenance of envelope function, and survival of the fittest viral forms selected by this process. Further studies of the HIV envelope glycoproteins will likely reveal the molecular details of this dynamic process. The structural details of glycan-mediated neutralization resistance/sensitivity and masking/exposure of neutralizing determinants are important considerations for the selection of particular envelope glycoproteins as subunit vaccine candidates.

Materials and methods

Plasmids

The plasmid pSVIIIenv, expressing the HIV-1 envelope glycoproteins from the HXBc2, JR-FL, or YU2 isolate, has been described previously (Helseth et al., 1990). For production of viruses that contain mutant or wild-type envelope glycoproteins and express luciferase upon infection, the plasmid pSVIIIenv was cotransfected with pCMVΔP1ΔenvpA and pHIvcc2.luc. The plasmid pHIvec2.luc is derived from pHIvec2.gfp that was supplied by Wolfgang Hofmann, Dana-Farber Cancer Institute (Hofmann et al., 1999). It contains the two exons of *tat* and the luciferase gene in an HIV-1 proviral vector. The *gag*, *pol*, and *env* genes and the genes for the accessory proteins are either deleted in total or are rendered nonfunctional by partial deletion of coding sequences. The luciferase gene was "excised" from the plasmid pGL3b (Promega, Madison, WI) using the restriction sites *Bgl*II and *Xba*I. The luciferase gene was then inserted in place of the *gfp* gene normally contained in the pHIvec2.gfp using the restriction sites *Bam*HI (3830) and *Xba*I (5535). The plasmid pCMVΔP1ΔenvpA contains the functional HIV-1 *gag* and *pol*

genes under the control of the CMV promoter. This plasmid also contains the functional genes encoding the accessory proteins Vif and Tat, whereas both the *env* and the *nef* genes are deleted. Due to the deleted packaging signal (Δpsi) in this construct, the *gag/pol* messages are not incorporated into a budding virion, eliminating the possibility of further rounds of viral replication.

Mutagenesis

The glycosylation sites (NX(T/S)) at residues 276, 301, and 386 were deleted in the JR-FL and HXBc2 envelope glycoproteins by genetic mutation. The glycosylation site at residue 301 in the YU2 envelope glycoprotein was deleted. To achieve these alterations, either the sequence coding for N was changed to code for Q (at residue 301) or the sequence for T was changed to code for L or A (at residues 386 and 276). Amino acid substitutions were guided by changes found in primate immunodeficiency viruses possessing envelope glycoproteins that lack those particular glycosylation sites and thus naturally harbored the same changes. Amino acid changes were introduced by site-directed mutagenesis, utilizing a combination of the QuikChange (Stratagene) and megaprimer protocols (Brons-Poulsen et al., 1998). Briefly, the *env* gene was PCR amplified using a 5′ forward oligonucleotide harboring the desired mutagenic changes (and as well, possessing a unique restriction site) and a 3′ reverse oligonucleotide complementary to the wild-type *env* sequences. The PCR product containing the desired mutant sequence was then purified on an agarose gel and isolated from the gel by spin columns (Qiagen). The isolated set of complementary oligonucleotides was then utilized as a megaprimer pair to perform QuickChange site-directed mutagenesis. The successful deletion of the particular glycosylation site was confirmed by restriction enzyme analysis and sequencing.

Neutralization assays

Complementation of a single round of replication of the *env*-deficient luciferase-expressing proviruses by the various envelope glycoproteins was performed as described previously (Hofmann et al., 1999; Kolchinsky et al., 2001b). To inhibit viral replication, monoclonal antibodies were incubated with recombinant virus for 1 h at 37°C in DMEM containing 10% heat-inactivated fetal bovine serum. Viruses and antibody were then added to Cf2Th target cells expressing CD4 and CCR5 or CD4 and CXCR4. Three days after infection, the cells were lysed with passive lysis buffer (Promega), and luciferase activity was measured as relative luminescence. The data shown are the arithmetical averages of at least five independent experiments with the exception of neutralization experiments using the 39F anti-V3 loop antibody, which were performed twice.

Fluorescence activated cell sorting (FACS)

Cell-surface, FACS-based binding curves with antibodies were generated as follows. 293T cells transiently expressing JR-FL gp160 glycoproteins or JR-FL Δ301 glycan glycoproteins were stained and analyzed by FACS. Initially, the cells were plated onto 150-mm tissue culture dishes at a density of 10×10^6/plate. The following day the cells were transfected with the pSVIII*env* expressor plasmids and pSVIII*tat* using Fugene6 as per the manufacturer's instructions (Roche; 5–10 μg plasmid DNA/10^6 cells, DNA/Fugene6 mass ratio 1/3). The JR-FL cleavage-competent glycoproteins contained stop codons in sequences encoding the cytoplasmic tails to enhance cell-surface expression of the oligomeric glycoproteins (at codon position 712, HXBc2 numbering). Forty-eight hours following transfection, the cells were removed from the tissue culture dish using PBS containing 5 mM EDTA, washed with FACS buffer (PBS with 5% FCS and 0.02% azide) to remove the EDTA, resuspended in FACS buffer, and dispensed into 96-well microtiter flat-bottom plates at 10^6 cells/well. Antibodies were added to the wells at 0 to 200 μg/ml (HIVIgG to 2 mg/ml) in volume of 100 μl/well and incubated for 1 h at room temperature. For each antibody tested, duplicate wells were done. The cells were washed extensively in FACS buffer and a 1:150 dilution of anti-human IgG-R-Phycoerythrin (Sigma) in FACS buffer was added to all wells for 1 h at room temperature. The cells were then washed extensively in FACS buffer, fixed in 2% paraformaldehyde, and analyzed on a FACS Calibur from Becton–Dickinson. We have reported the data as the MFI of the total cells. The data shown in Fig. 4 are from a single representative experiment with the exception of the 2G12 and anti-RSV. The FACS analysis of IgGb12, F105, 39F, and 2F5 was repeated in at least five independent experiments and the staining with 2G12, anti-respiratory syncytial virus (anti-RSV; negative control human IgG1, Medimmune), and HIVIgG were repeated three times, with similar results.

Acknowledgments

We thank James Robinson, Marshall Posner, Hermann Katinger, and Dennis Burton for antibodies and Katina Bryan and Karen Stroud for help in preparation of the manuscript. This study was supported in part by the NIH Grants 5 R21 AI-44328-02 and 1 R21 AI-44328-02 and by the Mathers Foundation, the Friends 10, William F. McCarty-Cooper, and Douglas and Judith Krupp.

References

Alkhatib, G., Combadiere, C., Broder, C.C., Feng, Y., Kennedy, P.E., Murphy, P.M., Berger, E.A., 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 272 (5270), 1955–1958.

Back, N.K., Smit, L., De Jong, J.J., Keulen, W., Schutten, M., Goudsmit, J., Tersmette, M., 1994. An N-glycan within the human immunodeficiency virus type 1 gp120 V3 loop affects virus neutralization. Virology 199 (2), 431–438.

Bandres, J.C., Wang, Q.F., O'Leary, J., Baleaux, F., Amara, A., Hoxie, J.A., Zolla-Pazner, S., Gorny, M.K., 1998. Human immunodeficiency virus (HIV) envelope binds to CXCR4 independently of CD4, and binding can be enhanced by interaction with soluble CD4 or by HIV envelope deglycosylation. J. Virol. 72 (3), 2500–2504.

Barre-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W., Montagnier, L., 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220 (4599), 868–871.

Bergeron, L., Sullivan, N., Sodroski, J., 1992. Target cell-specific determinants of membrane fusion within the human immunodeficiency virus type 1 gp120 third variable region and gp41 amino terminus. J. Virol. 66 (4), 2389–2397.

Brighty, D.W., Rosenberg, M., Chen, I.S., Ivey-Hoyle, M., 1991. Envelope proteins from clinical isolates of human immunodeficiency virus type 1 that are refractory to neutralization by soluble CD4 possess high affinity for the CD4 receptor. Proc. Natl. Acad. Sci. USA 88 (17), 7802–7805.

Brons-Poulsen, J., Petersen, N.E., Horder, M., Kristiansen, K., 1998. An improved PCR-based method for site directed mutagenesis using megaprimers. Mol. Cell. Probes 12 (6), 345–348.

Burton, D.R., Pyati, J., Koduri, R., Sharp, S.J., Thornton, G.B., Parren, P.W., Sawyer, L.S., Hendry, R.M., Dunlop, N., Nara, P.L., et al., 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science 266 (5187), 1024–1027.

Cao, J., Sullivan, N., Desjardin, E., Parolin, C., Robinson, J., Wyatt, R., Sodroski, J., 1997. Replication and neutralization of human immunodeficiency virus type 1 lacking the V1 and V2 variable loops of the gp120 envelope glycoprotein. J. Virol. 71 (12), 9808–9812.

Chan, D.C., Fass, D., Berger, J.M., Kim, P.S., 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell 89 (2), 263–273.

Chan, D.C., Kim, P.S., 1998. HIV entry and its inhibition. Cell 93 (5), 681–684.

Cheng-Mayer, C., Brown, A., Harouse, J., Luciw, P.A., Mayer, A.J., 1999. Selection for neutralization resistance of the simian/human immunodeficiency virus SHIVSF33A variant in vivo by virtue of sequence changes in the extracellular envelope glycoprotein that modify N-linked glycosylation. J. Virol. 73 (7), 5294–5300.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P.D., Wu, L., Mackay, C.R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., Sodroski, J., 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 85 (7), 1135–1148.

Connor, R.I., Sheridan, K.E., Ceradini, D., Choe, S., Landau, N.R., 1997. Change in coreceptor use coreceptor use correlates with disease progression in HIV-1-infected individuals. J. Exp. Med. 185 (4), 621–628.

Earl, P.L., Doms, R.W., Moss, B., 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. Proc. Natl. Acad. Sci. USA 87 (2), 648–652.

Feng, Y., Broder, C.C., Kennedy, P.E., Berger, E.A., 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 272 (5263), 872–877.

Fouts, T.R., Binley, J.M., Trkola, A., Robinson, J.E., Moore, J.P., 1997. Neutralization of the human immunodeficiency virus type 1 primary isolate JR-FL by human monoclonal antibodies correlates with antibody binding to the oligomeric form of the envelope glycoprotein complex. J. Virol. 71 (4), 2779–2785.

Gallaher, W.R., 1987. Detection of a fusion peptide sequence in the transmembrane protein of human immunodeficiency virus. Cell 50 (3), 327–328.

Gallo, R.C., Salahuddin, S.Z., Popovic, M., Shearer, G.M., Kaplan, M., Haynes, B.F., Palker, T.J., Redfield, R., Oleske, J., Safai, B., et al., 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 224 (4648), 500–503.

Hansen, J.E., Jansson, B., Gram, G.J., Clausen, H., Nielsen, J.O., Olofsson, S., 1996. Sensitivity of HIV-1 to neutralization by antibodies against O-linked carbohydrate epitopes despite deletion of O-glycosylation signals in the V3 loop. Arch. Virol. 141 (2), 291–300.

Helseth, E., Kowalski, M., Gabuzda, D., Olshevsky, U., Haseltine, W., Sodroski, J., 1990. Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. J. Virol. 64 (5), 2416–2420.

Hofmann, W., Schubert, D., LaBonte, J., Munson, L., Gibson, S., Scammell, J., Ferrigno, P., Sodroski, J., 1999. Species-specific, postentry barriers to primate immunodeficiency virus infection. J. Virol. 73 (12), 10020–10028.

Kabat, D., Kozak, S.L., Wehrly, K., Chesebro, B., 1994. Differences in CD4 dependence for infectivity of laboratory-adapted and primary patient isolates of human immunodeficiency virus type 1. J. Virol. 68 (4), 2570–2577.

Kolchinsky, P., Kiprilov, E., Bartley, P., Rubinstein, R., Sodroski, J., 2001a. Loss of a single N-linked glycan allows CD4-independent human immunodeficiency virus type 1 infection by altering the position of the gp120 V1/V2 variable loops. J. Virol. 75 (7), 3435–3443.

Kolchinsky, P., Kiprilov, E., Sodroski, J., 2001b. Increased neutralization sensitivity of CD4-independent human immunodeficiency virus variants. J. Virol. 75 (5), 2041–2050.

Kolchinsky, P., Mirzabekov, T., Farzan, M., Kiprilov, E., Cayabyab, M., Mooney, L.J., Choe, H., Sodroski, J., 1999. Adaptation of a CCR5-using, primary human immunodeficiency virus type 1 isolate for CD4-independent replication. J. Virol. 73 (10), 8120–8126.

Kowalski, M., Potz, J., Basiripour, L., Dorfman, T., Goh, W.C., Terwilliger, E., Dayton, A., Rosen, C., Haseltine, W., Sodroski, J., 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science 237 (4820), 1351–1355.

Kwong, P.D., Doyle, M.L., Casper, D.J., Cicala, C., Leavitt, S.A., Majeed, S., Steenbeke, T.D., Venturi, M., Chaiken, I., Fung, M., Katinger, H., Parren, P.W., Robinson, J., Van Ryk, D., Wang, L., Burton, D.R., Freire, E., Wyatt, R., Sodroski, J., Hendrickson, W.A., Arthos, J., 2002c. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature 420 (6916), 678–682.

Kwong, P.D., Wyatt, R., Majeed, S., Robinson, J., Sweet, R.W., Sodroski, J., Hendrickson, W.A., 2000a. Structures of HIV-1 gp120 envelope glycoproteins from laboratory-adapted and primary isolates. Struct. Fold Des. 8 (12), 1329–1339.

Kwong, P.D., Wyatt, R., Robinson, J., Sweet, R.W., Sodroski, J., Hendrickson, W.A., 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature 393 (6686), 648–659.

Kwong, P.D., Wyatt, R., Sattentau, Q.J., Sodroski, J., Hendrickson, W.A., 2000b. Oligomeric modeling and electrostatic analysis of the gp120 envelope glycoprotein of human immunodeficiency virus. J. Virol. 74 (4), 1961–1972.

Losman, B., Biller, M., Olofsson, S., Schonning, K., Lund, O.S., Svennerholm, B., Hansen, J.E., Bolmstedt, A., 1999. The N-linked glycan of the V3 region of HIV-1 gp120 and CXCR4-dependent multiplication of a human immunodeficiency virus type 1 lymphocyte-tropic variant. FEBS Lett. 454 (1–2), 47–52.

Maddon, P.J., Dalgleish, A.G., McDougal, J.S., Clapham, P.R., Weiss, R.A., Axel, R., 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell 47 (3), 333–348.

Malenbaum, S.E., Yang, D., Cavacini, L., Posner, M., Robinson, J., Cheng-Mayer, C., 2000. The N-terminal V3 loop glycan modulates the interaction of clade A and B human immunodeficiency virus type 1 envelopes with CD4 and chemokine receptors. J. Virol. 74 (23), 11008–11016.

Moore, J.P., Cao, Y., Qing, L., Sattentau, Q.J., Pyati, J., Koduri, R., Robinson, J., Barbas 3rd, C.F., Burton, D.R., Ho, D.D., 1995. Primary

isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. J. Virol. 69 (1), 101–109.

Moore, J.P., McKeating, J.A., Huang, Y.X., Ashkenazi, A., Ho, D.D., 1992. Virions of primary human immunodeficiency virus type 1 isolates resistant to soluble CD4 (sCD4) neutralization differ in sCD4 binding and glycoprotein gp120 retention from sCD4-sensitive isolates. J. Virol. 66 (1), 235–243.

Moore, J.P., McKeating, J.A., Norton, W.A., Sattentau, Q.J., 1991. Direct measurement of soluble CD4 binding to human immunodeficiency virus type 1 virions: gp120 dissociation and its implications for virus-cell binding and fusion reactions and their neutralization by soluble CD4. J. Virol. 65 (3), 1133–1140.

Moore, J.P., Sodroski, J., 1996. Antibody cross-competition analysis of the human immunodeficiency virus type 1 gp120 exterior envelope glycoprotein. J. Virol. 70 (3), 1863–1872.

Myszka, D.G., Sweet, R.W., Hensley, P., Brigham-Burke, M., Kwong, P.D., Hendrickson, W.A., Wyatt, R., Sodroski, J., Doyle, M.L., 2000. Energetics of the HIV gp120-CD4 binding reaction. Proc. Natl. Acad. Sci. USA 97 (16), 9026–9031.

Olofsson, S., Hansen, J.E., 1998. Host cell glycosylation of viral glycoproteins—a battlefield for host defence and viral resistance. Scand. J. Infect. Dis. 30 (5), 435–440.

Otteken, A., Earl, P.L., Moss, B., 1996. Folding, assembly, and intracellular trafficking of the human immunodeficiency virus type 1 envelope glycoprotein analyzed with monoclonal antibodies recognizing maturational intermediates. J. Virol. 70 (6), 3407–3415.

Platt, E.J., Madani, N., Kozak, S.L., Kabat, D., 1997. Infectious properties of human immunodeficiency virus type 1 mutants with distinct affinities for the CD4 receptor. J. Virol. 71 (2), 883–890.

Raja, A., Venturi, M., Kwong, P., Sodroski, J., 2003. CD4 binding site antibodies inhibit human immunodeficiency virus gp120 envelope glycoprotein interaction with CCR5. J. Virol. 77 (1), 713–718.

Reitter, J.N., Means, R.E., Desrosiers, R.C., 1998. A role for carbohydrates in immune evasion in AIDS. Nat. Med. 4 (6), 679–684.

Rizzuto, C.D., Wyatt, R., Hernandez-Ramos, N., Sun, Y., Kwong, P.D., Hendrickson, W.A., Sodroski, J., 1998. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. Science 280 (5371), 1949–1953.

Roben, P., Moore, J.P., Thali, M., Sodroski, J., Barbas 3rd, C.F., Burton, D.R., 1994. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. J. Virol. 68 (8), 4821–4828.

Sattentau, Q.J., Moore, J.P., 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J. Exp. Med. 174 (2), 407–415.

Sattentau, Q.J., Moore, J.P., 1995. Human immunodeficiency virus type 1 neutralization is determined by epitope exposure on the gp120 oligomer. J. Exp. Med. 182 (1), 185–196.

Schonning, K., Jansson, B., Olofsson, S., Hansen, J.E., 1996a. Rapid selection for an N-linked oligosaccharide by monoclonal antibodies directed against the V3 loop of human immunodeficiency virus type 1. J. Gen. Virol. 77 (Pt 4), 753–758.

Schonning, K., Jansson, B., Olofsson, S., Nielsen, J.O., Hansen, J.S., 1996b. Resistance to V3-directed neutralization caused by an N-linked oligosaccharide depends on the quaternary structure of the HIV-1 envelope oligomer. Virology 218 (1), 134–140.

Si, Z., Cayabyab, M., Sodroski, J., 2001. Envelope glycoprotein determinants of neutralization resistance in a simian-human immunodeficiency virus (SHIV-HXBc2P 3.2) derived by passage in monkeys. J. Virol. 75 (9), 4208–4218.

Stamatatos, L., Cheng-Mayer, C., 1998. An envelope modification that renders a primary, neutralization-resistant clade B human immunodeficiency virus type 1 isolate highly susceptible to neutralization by sera from other clades. J. Virol. 72 (10), 7840–7845.

Stamatatos, L., Wiskerchen, M., Cheng-Mayer, C., 1998. Effect of major deletions in the V1 and V2 loops of a macrophage-tropic HIV type 1 isolate on viral envelope structure, cell entry, and replication. AIDS Res. Hum. Retroviruses 14 (13), 1129–1139.

Sullivan, N., Sun, Y., Binley, J., Lee, J., Barbas 3rd, C.F., Parren, P.W., Burton, D.R., Sodroski, J., 1998. Determinants of human immunodeficiency virus type 1 envelope glycoprotein activation by soluble CD4 and monoclonal antibodies. J. Virol. 72 (8), 6332–6338.

Sullivan, N., Sun, Y., Li, J., Hofmann, W., Sodroski, J., 1995. Replicative function and neutralization sensitivity of envelope glycoproteins from primary and T-cell line-passaged human immunodeficiency virus type 1 isolates. J. Virol. 69 (7), 4413–4422.

Tan, K., Liu, J., Wang, J., Shen, S., Lu, M., 1997. Atomic structure of a thermostable subdomain of HIV-1 gp41. Proc. Natl. Acad. Sci. USA 94 (23), 12303–12308.

Thali, M., Furman, C., Ho, D.D., Robinson, J., Tilley, S., Pinter, A., Sodroski, J., 1992. Discontinuous, conserved neutralization epitopes overlapping the CD4-binding region of human immunodeficiency virus type 1 gp120 envelope glycoprotein. J. Virol. 66 (9), 5635–5641.

Thali, M., Moore, J.P., Furman, C., Charles, M., Ho, D.D., Robinson, J., Sodroski, J., 1993. Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. J. Virol. 67 (7), 3978–3988.

Wang, W.K., Dudek, T., Zhao, Y.J., Brumblay, H.G., Essex, M., Lee, T.H., 1998. CCR5 coreceptor utilization involves a highly conserved arginine residue of HIV type 1 gp120. Proc. Natl. Acad. Sci. USA 95 (10), 5740–5745.

Wei, X., Decker, J.M., Wang, S., Hui, H., Kappes, J.C., Wu, X., Salzar, J.F., Salzar, M.G., Kilby, J.M., Saag, M.S., Komarova, N.L., Nowak, M.A., Hahn, B.H., Kwong, P.D., Shaw, G.M., 2003. Antibody neutralization and escape by HIV-1. Nature 422 (6929), 307–312.

Weissenhorn, W., Dessen, A., Harrison, S.C., Skehel, J.J., Wiley, D.C., 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature 387 (6631), 426–430.

Wyatt, R., Kwong, P.D., Desjardins, E., Sweet, R.W., Robinson, J., Hendrickson, W.A., Sodroski, J.G., 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. Nature 393 (6686), 705–711.

Wyatt, R., Sodroski, J., 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science 280 (5371), 1884–1888.