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REVIEW

# Involvement of Envelope-Glycoprotein Glycans in HIV-1 Biology and Infection

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**Abstract** Infection of host cells with HIV-1 depends on a highly glycosylated virus envelope glycoprotein (Env) and host-cell receptors. Glycans participate substantially in Env folding and in the binding of virions to the host-cell surface and indirectly affect cellular uptake of HIV-1. Moreover, Env glycans could protect HIV-1 from host's neutralizing antibodies, but some glycans, on the other hand, represent targets for neutralizing antibodies. Variability of Env and its glycans in the HIV-1 strains from around the world as well as in patients during disease progression contributes substantially to further HIV-1 spreading in spite of the progress in basic HIV-1 research, vaccine development, and highly active antiretroviral therapy of HIV-1 infections.

**Keywords** HIV-1 · gp120 glycosylation · gp120 folding · gp120 antibody recognition

**Abbreviations**

| | |
|---|---|
| aa | Amino acid |
| CBA | Carbohydrate-binding agents |
| CD4i | CD4-induced |
| CRD | Carbohydrate-recognition domain |
| DC | Dendritic cell |
| DC-SIGN | Dendritic cell-specific intercellular adhesion molecule 3 (ICAM3)-grabbing nonintegrin (CD209) |
| DC-SIGNR | Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin related or sinusoidal endothelial cell receptor (CD299; L-SIGN; CD209L) |
| Fuc | Fucose |
| Gal | Galactose |
| GalCer | Galactosyl ceramide |
| GlcCer | Glucosylceramide |
| GlcNAc | *N*-acetylglucosamine |
| GM-CSF | Granulocyte–macrophage colony-stimulating factor |
| GSL | Glycosphingolipid |
| HS | Heparan sulfate |
| Man | Mannose |
| MBL | Mannan-binding lectin |
| MDDC | Monocyte-derived dendritic cells |
| MDLC | Monocyte-derived Langerhans cells |
| MR | Man receptor |
| PNGS | Potential *N*-glycosylation sites |
| SA | Sialic acid |
| SAG | Salivary agglutinin |

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## Introduction

HIV-1 Env is presented on virion surfaces as a trimer of gp120/gp41 complexes. Env, a heavily glycosylated glycoprotein, is a ligand for several classes of cell-surface receptors, including glycan-dependent C-type lectins. Binding to other cellular receptors, including entry receptors (CD4, CCR5, and CXCR4), can be indirectly affected by glycans as well, because glycans contribute to Env folding. Env glycans play a principal role during host's humoral immune response because they shield Env against

host's neutralizing antibodies, but also serve as a target for rarely occurring glycan-specific neutralizing antibodies. Furthermore, Env glycans modulate also cell-mediated response, because they are involved in antigen processing and presentation to CD4 T cells. During the course of infection, mutations leading to changes in glycan-attachment sites contribute to transition from CCR5-tropic HIV-1 viruses to CXCR4-tropic viruses that could infect naïve T cells as the last possible target. This manuscript reviews various aspects of glycobiology of HIV-1 infection. Many studies underscore the notion that understanding to HIV-1 Env-glycans biology and immunology is important for effective vaccine design or novel therapeutics development.

## Oligosaccharide Structures Detected on HIV-1 Env

About one half of gp120 glycoprotein molecular mass is represented by glycans, predominantly *N*-linked glycans, with a small and variable contribution from *O*-linked glycans (Zhu et al. 2000). *N*-glycans are composed of a core, i.e., two *N*-acetylglucosamine (GlcNAc) residues and three mannose (Man) residues, with variable content of additional glycans, Man, GlcNAc, galactose (Gal), fucose (Fuc), or sialic acid (SA). *N*-glycans can be divided into three basic structures: high-Man, hybrid, and complex glycans (Fig. 1); all of them were detected on HIV-1 Env glycoprotein (Kwong et al. 1998; Zhu et al. 2000). *N*-linked glycan is transferred en bloc as a lipid-bound precursor and attached to the asparagine (Asn, N) residue in the amino acid (aa) backbone of nascent protein at positions predetermined by short aa motifs (N-X-S/T-X′) (N, Asn; S, Ser; T, Thr). X and X′ represent any aa with the following exceptions: for N-X-S-X′ motif, W (Trp), D (Asp), or E (Glu) are excluded at the position X; for N-X-T-X′motif, P (Pro) is excluded at the position X; and for both motifs, P is excluded at the position X′ (Gavel and von Heijne 1990; Kasturi et al. 1997; Marshall 1972; Mellquist et al. 1998). After the attachment, the *N*-glycan precursors are modified to give rise to high-Man glycans, with up to 6 Man residues in addition to the core glycan, $GlcNAc_2Man_3$. Further trimming of the Man residues and addition of GlcNAc, Gal, SA, and Fuc residues leads to the formation of hybrid and, finally, complex *N*-glycans. Complex glycans may have 2, 3, or 4 antennas and a bisecting GlcNAc. The number of *N*-glycosylation motifs varies among HIV-1 virus clades from 18 to 33, with a median of 25 (Korber et al. 2001; Zhang et al. 2004). Recent mass spectrometric analyses revealed that not all *N*-glycosylation motifs are occupied by glycans and therefore it is more accurate to designate these motifs as potential *N*-glycosylation sites (PNGS), unless confirmed experimentally (Go et al. 2008; Irungu et al. 2008). The distribution of PNGS on Env among various HIV-1 isolates is not random. Some PNGS locations are conserved in most HIV-1 strains, whereas other PNGS are highly variable (Fig. 2). This phenomenon

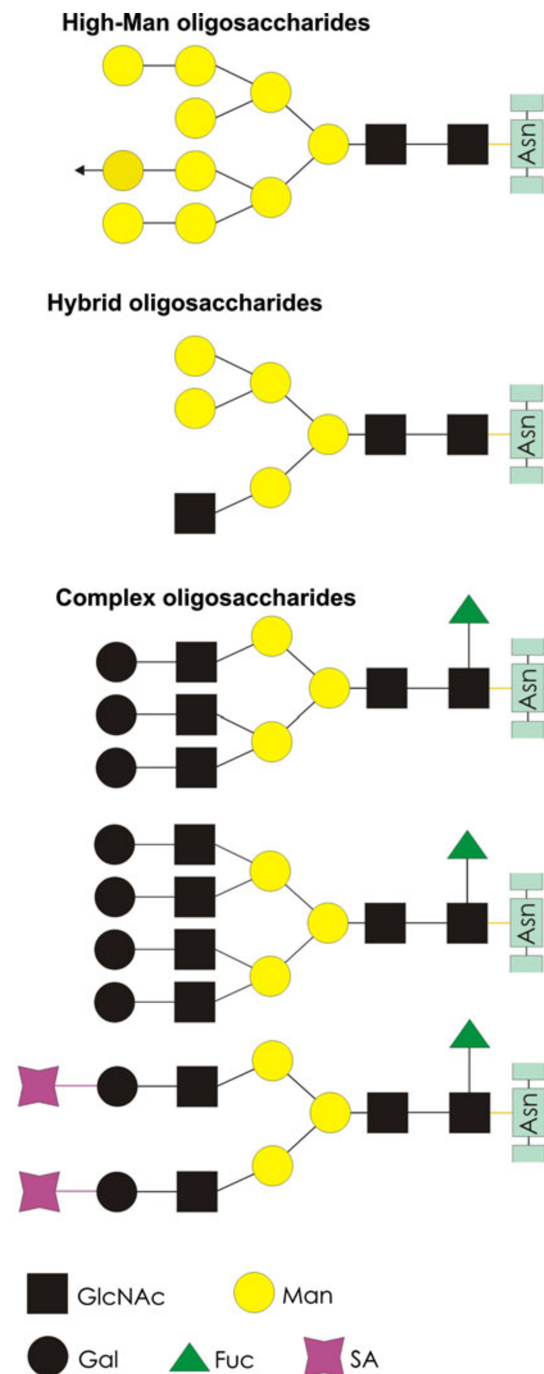


**Fig. 1** Examples of *N*-glycan structures on HIV-1. *N*-glycans are composed of *N*-acetylglucosamine (*GlcNAc*), mannose (*Man*), with variable contributions of other saccharides: galactose (*Gal*), fucose (*Fuc*), or sialic acid (*SA*). The branching of the Man and attachment of GlcNAc, Gal, SA, and Fuc characterizes type of *N*-glycans (high-Man, hybrid, and complex). Complex glycans contain two to four antennas

can be described as a movement (or shift) of PNGS and is typical for variable loops V1/V2, V4, and V5, but not V3 (Bunnik et al. 2008; Mascola and Montefiori 2003; Samleerat et al. 2008; Zhang et al. 2004; Zolla-Pazner 2005). The mechanism of PNGS movement is associated with general HIV-1 mutation mechanism due to the infidelity of the HIV-1 reverse transcriptase enzyme that generates mutations in viral genome; consequently, new PNGS occur and former PNGS disappear. This mechanism is further facilitated by characteristically conserved accumulation of PNGS-coding aa N, T, and S in V1/V2, V4, and V5 loops (McNearney et al. 1992; Zhang et al. 2004).

In contrast to explicitly defined PNGS, *O*-linked-glycan sites of attachment are difficult to predict. *O*-glycans are synthesized in a stepwise process and are attached to surface-exposed aa S or T in S/T/P-rich domains of already folded, *N*-glycosylated, and oligomerized glycoproteins (Julenius et al. 2005). The prediction of occurrence of *O*-glycosylated sites is based on modeling of surface accessibility, estimation of secondary structure and distance constraints followed by integration of these factors by computer analysis based on neural-network. The accuracy of *O*-glycan site prediction is 76% (Julenius et al. 2005). Therefore, unless experimentally confirmed, it is difficult to assess the *O*-glycan contribution to the biology of HIV-1 Env (Julenius et al. 2005). Using the CBS NetOGlyc prediction server (http://www.cbs.dtu.dk/services/NetOGlyc), most Env sequences are predicted to have one or two potential *O*-glycans (Julenius et al. 2005). The presence of *O*-glycans on the Env protein was confirmed by binding of specific antibodies, some of which neutralize HIV-1 infectivity (Hansen et al. 1990, 1991, 1996).

## Biological Functions of the Env Oligosaccharides

### Folding of Env

Env "spikes" on the surface of HIV-1 virions, composed of gp120 and gp41 subunits, are arranged into multimers, predominantly trimers. The Env precursor, gp160, is synthesized in the rough endoplasmic reticulum where *N*-glycan precursors are attached, with a subsequent transport to the Golgi apparatus where the proper folding of each monomer is completed with further processing of *N*-glycans to generate high-Man, hybrid, and complex *N*-glycans. The process is completed by cleavage of the gp160 precursor into gp120 and gp41 subunits by host furin protease and multimerization and folding into a functional conformation of the envelope spikes (Hallenberger et al. 1992). Many studies described that the *N*-glycans play a unique role in establishing the Env structure, surface exposure, antigenicity, and immunogenicity (Kwong et al. 1998, 2000; Wyatt and Sodroski 1998).

Env or its gp120 subunit used for structural and functional analyses or for immunization experiments are produced in cells of various origin (predominantly Chinese hamster ovary line-CHO or human embryonic kidney cells-293). Expression of gp120 in different cell types influences significantly the number, type, and composition of *N*-glycans and, thus, the Env properties (Cutalo et al. 2004; Go et al. 2008; Irungu et al. 2008; Leonard et al. 1990; Raska et al. 2008b; Zhu et al. 2000). It is important to stress that significant differences in the composition of *N*-glycans (high-Man, hybrid and/or complex with various number of antennas) have been observed on the same gp120 aa sequence expressed in different human cell types, i.e., cells originating from various tissues (Raska and Novak, unpublished observation). Consequently, at least some of the differences in various structural, functional, and immunological studies of HIV-1 Env can be attributed to differential glycosylation due to the cell types and culture conditions used for Env production (Raska et al. 2008b).

### Binding of HIV-1 to the Entry Receptors or to the Attachment Receptors

#### *Env Glycans and Entry Receptors—cis-Infection*

HIV-1 infection of target cells involves attachment, fusion of HIV-1 envelope with target cell, releasing nucleocapsid into cytoplasm, reverse transcription, integration and viral RNA replication, and release of new virions from the cell. This process is termed *cis*-infection (Wu and KewalRamani 2006). The attachment of HIV-1 to cell surface and the entry involves interactions between gp120/gp41 and two major cell-surface receptors: CD4 receptor and a chemokine CCR5 or CXCR4 co-receptor (Hoffman et al. 1999). The differential usage of co-receptors distinguishes two types of HIV-1. CCR5-restricted (R5) HIV-1 strains also referred to as nonsyncytia-inducing or macrophage-tropic viruses which are responsible for HIV-1 transmission. R5 HIV-1 types are most prevalent, particularly in the intestine, during primary infection (Keele et al. 2008; Meng et al. 2002; Polzer et al. 2002; Salazar-Gonzalez et al. 2009; Smith 1997; Smith et al. 2003). CXCR4 (X4)-using HIV-1 types, also termed syncytia-inducing or T-cell-tropic viruses, are more associated with later stage disease and appear with disease progression (Hoffman et al. 1999; Pollakis et al. 2001). X4-tropic variants of gp120 feature highly positively charged V3 loop sequences. In contrast, V3 loops with low charge are characteristic for R5 variants (Kwong et al. 2000; Zhang et al. 2002). Analysis of HIV-1 DNA sequences suggested a close association between the V3 loop glycans and CCR5 usage

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HXB2_LAI-III  MRVK---EKYQHLWRWGWRWGTMLLGMLMICSATEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLV 87
WEAU transm   ....GIRKN.....K..----I....I......A.N.................................................E 86
WEAU d391     ....GIRKN.....K..----I....I..........................................................E 86
WEAU d1166    ....GIRKN.....K..----I....I....N...N.................................................ME 86
CH40 transm   ...MGIRKN......E.----IL...I......ADN...........R................A...................E.K 86
CH40 d412     ...MGIRKN......E.----IL...I......ADN...........R................A...................E.K 86
Con B         ....GIRKN.......----............A....................................................E 86
Con C         ...RGILRNC.QW.I..----ILGFW.....NVVGN.............K............EK..................I..E 86
Con S         ...RGIQRNC.......----.LI.........A.N.............N...................................I..E 86
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              β1        α1          β2              V1                          V1  V2
HXB2_LAI-III  NVTENFNMWKNDMVEQMHEDIISLWDQSLKPCVKLTPLCVSLKCT-----DLKNDTNTNSSSGRMIMEKGEIKNCSFNISTSIRGKVQKE 172
WEAU transm   .........N...........................T.N..NVNVTN...E.......GEK..E..M.....VT.L..N.RKT. 176
WEAU d391     .........N...........................T.N..NVNVTN...E...K....GEK..E..M.....VT.L..NERKT. 176
WEAU d1166    .........N...........................T.N..NVNVTN...E.....R..GEK.KE..M.....VT.L..N.RKT. 176
CH40 transm   .......E.N...........................T.N..-----..G.V...TN.N-GEM.....V.....K.T.D.KDRTR.. 170
CH40 d412     .......E.N...........................T.N..-----..G.V.T.TN.N-GTL.....V.....K.T.D.KDRTR.. 170
Con B         .........N...........................T.N..-----..M.A...TT--IIYRWR..........T.....D..... 169
Con C         ...........D.........................T.N..----NAT.A..----------TM...........T.EL.D.K..V 161
Con S         .........N...........................T.N..NVNVTNTT.N.E----------..............T.E..D.K..V 166
                                                 CCR5 CD4
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                          V2      β3                β4      β5        β6  β7   β8
HXB2_LAI-III  YAFFYKLDIIPIDNDTTS---YKLTSCNTSVITQACPKVSFEPIPIHYCAPAGFAILKCNNKTFNGTGPCTNVSTVQCTHGIRPVVSTQL 259
WEAU transm   ..L.....VM...H.N...---.T.IN..S.T.......................D.K...K...K................. 263
WEAU d391     ..L.....VM.....N...---.T.IN..S.T..........F............D.K.......K................. 263
WEAU d1166    ..L.....VM.....D...---.T.RN..S.T..........F............D.K.......K................. 263
CH40 transm   ..L.....VV..N--D.R---.R.V..............................D.Q.I........................ 255
CH40 d412     ..L.....VV..N--D.R---.R.V..............................D.Q.I........................ 255
Con B         ..L.....VV.....N...---.R.I.............................D.K.......................... 256
Con C         ..L..R...V.LNENNS----.R.IN...A.........D...........Y.............N.........K....... 247
Con S         ..L..R..VV...DNNNNSSN.R.IN...A.........................D.K.......K.........K....... 256
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                β9      β10         β11   β12 V3                           V3  β13     α2
HXB2_LAI-III  LLNGSLAEEEVVIRSVNFTDNAKTIIVQLNTSVEINCTRPNNNTRKRIRIQRGPGRAFVTIG-KIGNMRQAHCNISRAKWNNTLKQIASK 348
WEAU transm   ....N....DI....E.........N.....V.I...........K.TL--....VLY.T.EI..DI.R.....L..TS.......E. 351
WEAU d391     .........DI....E.........N.....V.IG...I.........TL--....VLY.T.EI..DI.R.....L..TS......VK. 351
WEAU d1166    .........DI....E..M......N.....A.IK...I........G.TL--....VLY.T.EI..DI.......L..TS......VK. 351
CH40 transm   ..............S..........K....T.........S.PM--...K..YAR.DIT.DI.K.Y.E.NGTE.HS...LVVE. 343
CH40 d412     ..............S..........K....T.........S.PM--...K..YAR.DI..DI.K.S.K.NGTE.HS...LVVE. 343
Con B         ...............E.................E................S.H.--.......Y.T.EI..DI.................VK. 344
Con C         .........II...E.L.N.......H..E....V.......S..--...QT.YAT.DI..DI.........ED....K..QKVSK. 335
Con S         .........II...E.I.N...........E.............S..--...Q..YAT.DI..DI.........GT....K..Q.V.K. 344
                           CD4              2G12 R5-N X4-H         CCR5            2G12   2G12
                                                  *
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                       β14    β15 α3 β16       β17  α4  β18 V4          V4       β19      β20      β21
HXB2_LAI-III  LREQFGN-NKTIIFKQSSGGDPEIVTHSFNCGGEFFYCNSTQLFNSTWFN-STWSTEGSNNTEGSDT-ITLPCRIKQIINMWQKVGKAMY 435
WEAU transm   ...IKQFK...V...........M..................HANG..-----K....A.NN.........R..E...... 436
WEAU d391     ...MEQFK...V...........M..................NATG..-----.K...A.NN.........R..E...... 436
WEAU d1166    ...IEQFK...V...........M..................NATG..-----.D...A.NN.........R..E...... 436
CH40 transm   ....Y---...V.NR........MY..........K.......PWND.K---..---HDTNGTLI...K.........G...... 424
CH40 d412     ....Y---...V.NR........MY.........T.K.......PWND.K---..---HDTNDTLM...K.........G...... 424
Con B         .......-...V.N..........M.........T............--NG.-----.......---N.............E...... 422
Con C         .K.H.P--...K.EP....L..T.......R....TSK.......Y--N..------------NST..............E..R.... 409
on S          ...H.N--.......P.....L..T......R......TSG.........IGNG.K-----..NNTN..-...........G..Q... 426
                             CD4                     2G12  2G12                          CCR5     CD4
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                        β22     β23    V5    β24       α5      β25
HXB2_LAI-III  APPISGQIRCSSNITGLLLTRDGGNSN-NESEIFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQREKR 515
WEAU transm   ....E.....L..........S.EE.QT.......N.K.................................... 518
WEAU d391     ....E.....L..........S.KD.QT.......N.K.................................... 518
WEAU d1166    ....E.....L..........-EG.QT.......N.K.................................... 517
CH40 transm   ....E.K..............YESNETD.................................................. 505
CH40 d412     ....E.I..............YESNETD.................................................. 505
Con B         ....R................---..T.................................................. 500
Con C         ....A.N.T.K..........KN.T---.T...................E.K....I...........E.... 487
Con S         ....E.K.T.K...........N.T..T.........................................E.... 507
                CD4              CD4
```

(Clevestig et al. 2006; Decker et al. 2005; McCaffrey et al. 2004; Pollakis et al. 2001; Salazar-Gonzalez et al. 2008). Comparison of R5 with R5X4 (HIV-1 types using both co-receptors) or pure X4 viruses in HIV-1-infected patients as well as experimental modeling of R5 to X4 transition revealed that R5 to X4 transition is linked to loss of PNGS

◀ **Fig. 2** Alignment of gp120 sequences and potential *N*-glycosylation sites (PNGS) among various HIV-1 isolates. Alignment includes subtype B HIV-$1_{HXB2_LAI\text{-}III}$—sequence commonly used for numbering of aa (and nucleotides) in HIV-1. Furthermore, three additional sequences (transmitted—Fiebig stage II, and estimated day 391 and 1166 after transmission) from an HIV-1 subtype B-infected male WEAU (WEAU transmitted Env pseudotyped virions are highly sensitive to neutralization by 2G12 and use CCR5/CXCR4 coreceptor) (Keele et al. 2008), and two more sequences identified from two time points (transmitted—Fiebig stage V, estimated day 412 after transmission) from an HIV-1 subtype B-infected male 700010040 (CH40), (CH40 transmitted Env pseudotyped virions are resistant to neutralization by 2G12 and use CCR5 coreceptor) (Keele et al. 2008). Finally, subtype B consensus (Con B), subtype C consensus (Con C), and global consensus envelope sequence (Con S) are included. Con S is considered to be one of the most promising vaccine candidates (Santra et al. 2008). All sequences are obtained from Los Alamos National Library HIV sequence database. Secondary structure $\alpha$1-$\alpha$5 helices (*light blue*) and $\beta$1-$\beta$25 strands (*dark blue*) are indicated above the sequences according to published crystal structure of HIV-$1_{HXBc2}$ gp120 (Kwong et al. 1998). Variable loops V1–V5 are in *light-green color* (Leonard et al. 1990). PNGSs identified by server (www.cbs.dtu.dk/services/NetNGlyc) are in *pink*, and shifting PNGSs are in orange color. CD4-, CCR5-, and CXCR4-binding regions in *brown*, *yellow*, *light-pink*, resp. (Clevestig et al. 2005, 2006; Coetzer et al. 2008; Kolchinsky et al. 2001; Kwong et al. 2000; Pollakis et al. 2001; Sarkar et al. 2002; Surman et al. 2001; Wyatt et al. 1993, 1998; Wyatt and Sodroski 1998) and 2G12-binding sites in *red color* (the alternative 2G12-binding sites are indicated in *grey text*) (Calarese et al. 2003, 2005; Go et al. 2008; Scanlan et al. 2002) are below the sequences. * Indicates PNGS 301 site associated with R5-to-X4 switch (Clevestig et al. 2005, 2006; Pastore et al. 2004, 2006, 2007)

in V3 loop (N301H), according to the HIV-$1_{HXB2}$ strain numbering further used throughout this article (Clevestig et al. 2006; Pastore et al. 2004, 2007). These studies, however, were based on analysis of Env DNA sequences and thus have not assessed whether the PNGS were occupied in each Env variant or not.

### *Env Glycans and Attachment Receptor—trans-Infection*

In addition to the canonical CD4 and the chemokine co-receptors (CCR5 and/or CXCR4), HIV-1 can bind to cells through a variety of other cell-surface structures (Table 1). All of them are involved in *trans*-infection of target cells that, in turn, transmit and spread the HIV-1 through the body but, initially, do not include virus replication.

The initial step of the HIV-1 infection includes the transmission of the virus across a stratified epithelium of the vagina, ectocervix, foreskin, lower rectum, and oral cavity or across the single-layer columnar epithelium of the endocervix and gastro-intestinal tract by transcytosis or para-cellular transport (Haase 2005; Hladik and Hope 2009; Hladik and McElrath 2008; Maher et al. 2005). In the close proximity with epithelial cells are the first leukocytes—dendritic cells (DCs) which are considered to be further transporters of the HIV-1 toward target CD4 T cells (Wu and KewalRamani 2006).

Many studies demonstrated contribution of various surface receptors to HIV-1 transmission by DCs. DCs present heterogeneous population of either myeloid or lymphoid origin—myeloid DCs, plasmocytoid DCs in the blood and Langerhans cells (LCs) or interstitial DCs in the mucosal or lymphoid tissues (Merad et al. 2008; Wu and KewalRamani 2006). Each of these major DC types consists of several subsets with distinct functional and maturation stages and different ability to bind, transport, release, and eventually replicates HIV-1 (Hladik and Hope 2009; Hladik and McElrath 2008; Turville et al. 2002, 2003; Wu and KewalRamani 2006). The first line of DCs which could interact with HIV-1 on mucosal surfaces are LCs located within the basal or suprabasal layers of the stratified squamous epithelium followed by immature interstitial DCs located in the stroma or lamina propria (Lenz et al. 1993; McLellan et al. 1998; Merad et al. 2008; Nestle et al. 1993; Pavli et al. 1990, 1993; Turville et al. 2002, 2003). The direct contact of LCs with HIV-1 is still an unsolved question (Wu and KewalRamani 2006).

To assess the role of DCs in HIV-1 infection, several sources of DCs have been used. The best source (biopsy samples) for *ex vivo* experiments is compromised by phenotype instability, associated with loss of surface receptors and de-differentiation of DCs (Turville et al. 2003). One of the most studied DC models presents immature monocyte-derived dendritic cells (MDDC), derived from peripheral blood monocytes after treatment with interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) or monocyte-derived Langerhans cells (MDLC), derived from peripheral blood monocytes after treatment with IL-4, GM-CSF, and transforming growth factor-$\beta$ (Geissmann et al. 1998; Turville et al. 2002). MDDCs used as an in vitro model of immature interstitial or dermal DC express high levels of Man receptor (MR) and DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN). MDLCs express intermediate level of Langerin, MR, and DC-SIGN (de Witte et al. 2007; Geissmann et al. 1998; Turville et al. 2002; Veron et al. 2006). LCs, on the other hand, express Langerin, but no MR or DC-SIGN (Merad et al. 2008; Turville et al. 2002, 2003). Due to these issues with DC stability receptors expression, the conclusions from above studies should be viewed as an approximation of the in vivo status.

### *Galactosyl Ceramide*

The initial step in establishing HIV-1 infection is the binding of HIV-1 to monostratified mucosa epithelial cells of the gastrointestinal tract, or endocervix (Maher et al. 2005; Meng et al. 2002; Phillips 1994). Such cells are rich in galactosyl ceramide (GalCer) in their apical membrane. HIV-1 binds Gal in GalCer through gp41 domain that

**Table 1** Glycan-dependent cellular receptors for HIV-1

| Cell | | | Virus | | | Note | References |
|---|---|---|---|---|---|---|---|
| Cellular receptor | Expression | Function | Env-independent | Env aa dependent | Env glycan-dependent | | |
| CD4 | CD4 T cell, monocyte, macrophage | TcR co-receptor | | Multiple epitops | | *cis*-infection | |
| CCR5 (CD195) | Monocyte, CD4 T cell, PVEC | Chemokine receptor | | Multiple epitops | | *cis*-infection | Bobardt et al. (2007) |
| CXCR4 (CD184) | Naïve CD4 T cell, B cell, DC, monocyte, PVEC | Chemokine receptor | | Multiple epitops | | *cis*-infection | Bobardt et al. (2007) |
| DC-SIGN (CD209), DC-SIGNR (CD299) | Immature DC, rectal mucosal DC, MDDC | C-type lectin, binds ICAM3 | | | Terminal Man, Fuc, GlcNAc | *trans*-infection, enhances *cis*-infection of DC in vitro | de Witte et al. (2007), Geijtenbeek et al. (2000), Jameson et al. (2002), Pohlmann et al. (2001) |
| MR (CD206) | Immature DC, MDDC, monocyte, macrophage | C-type lectin | | | Terminal Man, Fuc, GlcNAc, GalNAc? | *trans*-infection through tetraspanin plasma membrane compartment | Lai et al. (2009), Nguyen and Hildreth (2003), Yu et al. (2008b) |
| Langerin (CD207) | Langerhans cell | C-type lectin | | | Terminal Man, Fuc, GlcNAc | *trans*-infection, | Lai et al. (2009), Turville et al. (2003) |
| MBL | Soluble | C-type lectin | | | Man, GlcNAc, Fuc | Competes with C-type lectins, protects HIV-1 infection | Hansen and Holmskov (1998), Hart et al. (2002), Ji et al. (2005) |
| GSL-RAF | Mature DC, immature DC, vaginal epithelial sheets | Receptor for membrane GlcCer | GlcCer of host cells origin | | | *trans*-infection surface capturing 600 GlcCer molecules per 1 HIV-1 virion | Hatch et al. (2009), Hladik et al. (2007) |
| HSPG | PVEC, immature DC, BMVEC | Syndecan | | V3 CD4i | | *trans*-infection, enhances *cis*-infection of DC in vitro, *cis*-infection of BMVEC? | Bernfield et al. (1999), Bobardt et al. (2007), de Witte et al. (2007), Kumar et al. (2006), Saidi et al. (2007), Vives et al. (2005) |
| CSPG | BMVEC | Syndecan | | gp120 | | Contributes to *cis*-infection of BMVEC | Argyris et al. (2003), Bobardt et al. (2004) |
| gp340 | Endometrial and endocervical epithelial cell, macrophage | Scavenger | | V3 base | | *trans*-infection, cell-to-cell spreading | Cannon et al. (2008), Stoddard et al. (2007) |
| GalCer | Apical membrane of monostratified mucosal epithelia, PVEC, immature DC | Membrane lipid | | gp41 650-661 ELDKWA 668-685 | | *trans*-infection GalCer is recognized as sugar by gp41 moiety with C-type lectin function, conformational epitope, | Alfsen and Bomsel (2002), Bobardt et al. (2007), Kumar et al. (2006), Yu et al. (2008a) |
| LFA-1 CD11a | Leukocyte | α integrin | Host cellular origin—ICAM-1 | | | Cell-to-cell interaction, ICAM-1-specific antibody reduces 100x virus entry to PBMC | Rizzuto and Sodroski (1997) |
| $\alpha 4\beta 7$ | CD4 T cell, Nk cell | Adhesion molecule integrin | | V2 loop Leu-Asp-Val | | Activation of LFA-1, cell-to-cell spread | Arthos et al. (2008) |

*BMVEC* brain microvascular endothelial cells, *CSPG* chondroitin sulfate proteoglycan, *GSL-RAF* GSL-recognizing attachment factor, *HSPG* heparan sulfate proteoglycan, *PBMC* peripheral blood mononuclear cells, *PVEC* primary vaginal epithelial cells

possesses the C-type lectin activity (Simons and van Meer 1988; van Meer and Simons 1988; Yu et al. 2008a). GalCer was found also on immature DCs, the cells that are speculated to be the first target for HIV-1 in the stratified epithelium of vagina, anus, and foreskin (Magerus-Chatinet et al. 2007). GalCer binding induces transcytosis of HIV-1 through epithelial barriers.

*Glucosylceramide*

Related glucosylceramide (GlcCer) was recently identified in the lipid envelope of HIV-1 (Hatch et al. 2009). HIV-1 harbors approximately 600 GlcCer molecules per total 250,000 lipid molecules originating from cholesterol and glycosphingolipid (GSL)-enriched plasma membrane lipid rafts through which HIV-1 buds (Brugger et al. 2006; Chazal and Gerlier 2003). Binding of GSL to DCs can be inhibited by exosomes that bud from lipid raft-like plasma membrane suggesting similar origin of HIV-1 lipid envelope (Izquierdo-Useros et al. 2009). GlcCer is recognized by yet unknown GSL-recognizing attachment factor(s) within lipid rafts-like membrane microdomains of DCs and perhaps in vaginal epithelial sheets or skin explants. GlcCer contributes to *trans*-infection of DCs and perhaps to transcytosis across epithelial layers (Hatch et al. 2009). GlcCer interaction is therefore a gp120-independent mechanism if HIV-1 transmission.

*Syndecans*

Initial interaction of HIV-1 with mucosal surfaces is also mediated by scavenger receptors, such as syndecans or a related gp340 (Cannon et al. 2008) which are expressed by many cell types, including immature DCs (syndecan 3), primary vaginal epithelial cells (syndecan 1, 2), cervical epithelial cells (syndecan 1), and brain microvascular endothelial cells (predominantly syndecan 2, 4) (Bobardt et al. 2004, 2007; de Witte et al. 2007). Heparan sulfate (HS) and chondroitin sulfate (CS) contribute to attachment of HIV-1 to the cell surface, extending HIV-1 viability, and transcytosis of HIV-1 enhancing *trans*-infection or *cis*-infection of permissive cells (de Witte et al. 2007; Patel et al. 1993). HS and CS bind amino acids of the gp120 V3 loop and conformational CD4-induced (CD4i) epitope in $\beta$20–$\beta$21 strands which present highly positively charged region of gp120. The X4-tropic variants of gp120 feature highly positively charged V3 loop sequences which interact with HS. In contrast, low V3-loop charge is characteristic for R5 variants (Kwong et al. 2000; Zhang et al. 2002). Therefore, HS contributes preferentially to the transcytosis of X4-specific HIV-1 (Vives et al. 2005).

gp340 is a scavenger receptor on macrophages and endometrial and endocervical epithelial cells (Cannon et al. 2008; Stoddard et al. 2007). Splice variant of gp340, called salivary agglutinin (SAG), binds HIV-1 and inhibits infection (Wu et al. 2003, 2004). gp340/SAG binds HIV-1 gp120 near to the base of V3 loop at the site which contributes to CDi epitope formation, including the peptide CTRPNYNKRKRIHIG (Stoddard et al. 2007; Wu et al. 2004). This interaction seems to be protein–protein based, without direct contribution of gp120 glycans. Indirectly, however, glycans affect the folding of the HIV-1 Env protein. gp340, together with HS, plays a dominant role in mediating HIV-1 adhesion and *trans*-infection of endometrium and endocervical epithelial cells (Stoddard et al. 2007). In macrophages, it was shown that gp340 enhances *cis*-infection by HIV-1, likely through increasing the local concentration of HIV-1 at the cell surface (Cannon et al. 2008).

*C-type Lectins*

During later stages of infection where monocyte/macrophages and DCs represent the dominant cellular populations harboring the virus, gp120-binding C-type lectins—DC-SIGN, DC-SIGN related (CD-SIGNR), MR, and Langerin are involved in virus transmission. Interaction of lectins with gp120 glycans is $Ca^{++}$-dependent. These lectins contain carbohydrate-recognition domain (CRD) specifically binding Man, Fuc, and GlcNAc (Lai et al. 2009). In general, DC-SIGN is involved in the immune response by binding naïve T cells expressing ICAM-3 and aiding in the sampling of MHC-peptide complexes and in the accumulation of pathogens by antigen-presenting cells (Geijtenbeek et al. 2000). For binding of oligosaccharide-bearing ligands, such as gp120, oligomerization of C-type lectins is essential (Lai et al. 2009). DC-SIGN, DC-SIGNR, and Langerin contain only one CRD on each molecule and their oligomerization is mediated by the neck regions consisting of seven and a half copies of 23 aa motif repeats forming $\alpha$-helical coiled-coil structures (Lupas 1996; Mitchell et al. 2001; Pohlmann et al. 2001; Yu et al. 2009). In contrast, MR contains eight CRDs but no neck domain. MR is a glycoprotein and its oligomerization is thought to be mediated either through SA residues or through interaction of other glycans with CRD of the neighboring MR molecule (Lai et al. 2009; Su et al. 2005). It was demonstrated recently, that MR binds gp120 through CRD in both $Ca^{++}$-dependent and $Ca^{++}$-independent manner and probably also through N-terminal cysteine-rich domain of MR which binds *N*-acetylgalactosamine-4-$SO_4$ (GalNAc)-4-$SO_4$ (Lai et al. 2009). Although GalNAc has been identified on gp120 (Hansen et al. 1991; Merkle et al. 1991), it is not known whether it is sulfated at the C-4 position, a requirement for the high-affinity binding.

C-type lectins do not mediate *cis*-infection (i.e., do not initiate membrane fusion) of DC or monocytes/macrophages, but they contribute significantly to the HIV-1 attachment and transport to permissive cells (Bobardt et al. 2003; Geijtenbeek et al. 2000). In MDDC, which express CD4, CCR5 and low level of CXCR4, it was demonstrated in vitro that DC-SIGN as well as the syndecan 3 contribute significantly to *cis*-infection, probably by enhancing HIV-1 binding to the cell surface (de Witte et al. 2007). Binding of HIV-1 to C-type lectins is followed by several possible events. First and the most important event is the degradation of HIV-1 virions in the endosomal/lysosomal compartments. During a non-degradative transport (*trans*-infection), the HIV-1 virus is transported through the cells and released according to the exosomal model. Endocytosed HIV-1 are concentrated in late endosome/multivesicular bodies which transport virus to the immunological synapses with T cells and after fusion of virus membrane with donor cell membrane the virions are exocytosed toward canonical receptors on T cell surface. Recently, non-endosomal "cell membrane pockets" were described that remain contiguous with the plasma membrane (Yu et al. 2008b) and which are typically co-localized with tetraspanin (CD81). Non-endosomal pathway was described in vitro on DCs and macrophages (Deneka et al. 2007; Welsch et al. 2007; Yu et al. 2008b). Another mechanism is the surface transport of HIV-1 bound by C-type lectins or other surface molecules. This is likely associated with non-endosomal transfer because HIV-1 is accessible to various antiviral molecules, such as soluble CD4, antibodies, and other membrane-impermeable molecules similarly as in the non-endosomal pathway (Yu et al. 2008b). On the other hand, both pathways stabilize HIV-1 in vitro, because HIV-1 bound to DCs remains infectious for several days in contrast to the well-known labile nature of free HIV-1 in vitro (Geijtenbeek et al. 2000).

In addition to the cell-surface-localized C-type lectins, a soluble C-type lectin designated mannan-binding lectin (MBL) is involved in HIV-1 infection. MBL is present in serum in multimeric forms ranging from dimers to hexamers (Hansen and Holmskov 1998). MBL specifically recognizes Man, GlcNAc, and Fuc and binds HIV-1 through high-Man or hybrid *N*-glycans of gp120 (Ji et al. 2005). After binding to purified gp120 in vitro, MBL initiates complement activation through the lectin pathway (Holmskov et al. 2003). However, gp120 expressed on infected cells does not appear to effectively activate the lectin complement pathway (Saarloos et al. 1995). On the other hand, MBL can bind free HIV-1 virions and, thus, mediate uptake of HIV-1 by macrophages expressing collagen receptors, which also bind MBL (Ying et al. 2004). Ability of MBL to neutralize the HIV-1 is controversial. Laboratory-adapted viruses are sensitive to MBL-mediated neutralization, whereas primary isolates are much less sensitive (Hart et al. 2003; Ying et al. 2004). Treatment of the cells producing HIV-1 with deoxymannojirimycin, an inhibitor of Mannosidase I that elevates the content of high-Man glycans, increases sensitivity of some primary HIV-1 to MBL. Similarly, removal of SA increased the neutralization capacity of MBL (Hart et al. 2002, 2003). The mechanisms underlying these observations remain unknown, but they could include differences in diseases stage from which the primary isolates were isolated, differences in gp120 aa sequence, differences in density of *N*-glycans on gp120, and differences in the proportion of high-Man vs. complex *N*-glycans. MBL selects for HIV-1 virions with lower content of high-Man *N*-glycans which are more sensitive to neutralizing antibodies and therefore HIV-1 is under reciprocal pressure during infection and when escape mutants are formed (Ji et al. 2005). Finally, MBL could compete with cell surface-localized C-type lectins, such as DC-SIGN, for HIV-1 binding and, thus, MBL could limit transmission of HIV-1 by DCs or by monocyte/macrophages (Ji et al. 2005; Spear et al. 2003).

Results of genetic association studies have not confirmed neither protectivity nor negative effect of high or low serum levels of MBL, perhaps due to confounding factors, such as differences among particular studies concerning cohorts, methods, and parameters selection. Thus, additional studies are needed to obtain conclusive results (Boniotto et al. 2000; Hundt et al. 2000; Maas et al. 1998; McBride et al. 1998).

After transmission across mucosal barrier, host integrins (LFA-1, $\alpha 4\beta 7$) further stabilize the binding of HIV-1 to target CD4-positive cells in a glycan-independent manner (Arthos et al. 2008). The ligands on HIV-1 are either of cellular origin (LFA-1 ligand ICAM-1 is integrated into the viral envelope from host cellular phospholipide membrane) (Rizzuto and Sodroski 1997) or the ligand is specific epitope on Env protein: Activated form of $\alpha 4\beta 7$ binds Env V2-loop tripeptide Leu-Asp-Val. This interaction induces rapid activation of LFA-1 and efficient cell-to-cell spreading of HIV-1 (Arthos et al. 2008).

## Involvement of Env Glycoprotein in the Host Immune Response

Oligosaccharides of gp120 were described soon after HIV discovery, but their contributions to the virus life cycle and immune evasion were not fully recognized until later (Montefiori et al. 1988).

### Env Glycans as a Shield Against Neutralizing Antibodies

Glycans of gp120 serve as a shield against neutralizing antibodies specific for V1, V2, V3, CD4i, CD4-binding

site, membrane-proximal gp41, and also *N*-linked glycans. Moreover, glycans contribute to structure and function of the recognized epitopes by affecting the Env folding (Balzarini 2005; Decker et al. 2005; Li et al. 2008). HIV escape variants characterized by diverse *env* sequences were detected in the acute and chronic stages of infection, before occurrence of the full-blown AIDS (Bunnik et al. 2008; Decker et al. 2005; Derdeyn et al. 2004; Huang et al. 1997; Keele et al. 2008; Kothe et al. 2007; Li et al. 1993, 2008; Reitz et al. 1994; Rong et al. 2007; Salazar-Gonzalez et al. 2008, 2009; Wain et al. 2007; Wei et al. 2003). These escape variants emerge due to the pressure of humoral immune response and are resistant to the patients' circulating neutralizing antibodies (Wei et al. 2003).

In the longitudinal study of five HIV-1 subtype B-infected patients, the gp160 length and the number of PNGS increased during the acute and early chronic phases of the infection. Later in the infection, positive selection pressure declined as the neutralizing antibodies response subsided, coinciding with the reversion of changes in gp160 length and the number of PNGS (Table 2) (Bunnik et al. 2008).

## Env Glycans as a Target for Neutralizing Antibodies

Several monoclonal antibodies specific for *N*- or *O*-glycans that block viral infection and/or syncytia formation have been described (Hansen et al. 1990; Hansen et al. 1996; Hansen et al. 1991; Sanders et al. 2002). Some of them display broadly neutralizing properties, i.e., neutralize many different strains of HIV-1. Although such antibodies are difficult to generate by immunization of experimental animals, it is reasonable to predict that appropriate vaccine design, formulation, and route of administration could enhance their production (Astronomo et al. 2008; Dosenovic et al. 2009; Gray et al. 2007; Hansen 1992; Pantophlet and Burton 2003; Raska et al. 2008a, b). Examples of such approaches include immunization with recombinant glycoprotein and DNA vaccines encoding for glycans-mimicking polypeptides. Such polypeptides identified by phage display or anti-idiotype antibody technology could mimic glycan structures and, thus, could elicit good neutralizing antibodies accompanied by sufficient immunological memory (Cunto-Amesty et al. 2001; Monzavi-Karbassi et al. 2001a, b; Prinz et al. 2003; Weintraub 2003).

### *2G12 Monoclonal Antibody*

2G12 represents a unique *N*-glycan-specific human IgG1 antibody with a broad neutralizing activity. Its unique conformation, where both Fab regions form a tightly packed dimer via a three-dimensional swap of their $V_H$ domains, is responsible for its trivalency; in addition to the two conventional binding sites, an additional unique binding site is formed by swapped $V_H/V_H'$ domains (Calarese et al. 2003). This antibody recognizes the high-Man *N*-glycans on gp120 at positions 332, 392, and, to a lesser degree, 339 (Calarese et al. 2003). All three PNGS are highly conserved in all HIV-1 subtypes. The loss of PNGS 332 is associated with the loss in neutralization sensitivity of such gp120. It could be speculated that neutralization sensitivity of most HIV-1 subtypes to 2G12 is associated with fitness penalty in majority of 2G12-HIV-1-gp120 escape variants. Furthermore, mass spectrometric analysis of glycopeptides from consensus S gp140 ΔCFI confirmed that PNGS 339 is occupied in only 50%. This observation could be explained by substitution of 339 *N*-glycan by the *N*-glycan in position 295; this site is occupied only partially (Go et al. 2008). Interestingly, the 295 *N*-glycan was formerly considered as one of the two principal 2G12-binding glycans (Scanlan et al. 2002). These *N*-glycans were studied *in silico* by disequon analysis; the results showed strong negative (mutually excluding) interaction between PNGS 332 and 295, indicating that PNGS 295 substitution to 339 is potentially associated with loss of PNGS 332 and, thus, loss of 2G12 binding (Poon et al. 2007). By determining competition efficacy of various glycans for binding of 2G12 to gp120, two structures derived from high-Man *N*-glycans were identified as the most effective: Manα1-2Man and $GlcNAc_2Man_9$. Such moieties are present at the above-mentioned *N*-glycan sites (Calarese et al. 2003; Scanlan et al. 2002). Interestingly, strong competition with 2G12 binding was also observed for fructose (Calarese et al. 2003). Therefore, it is not clear whether 2G12 can inhibit HIV-1 transmission from the seminal fluid where fructose is an abundant sugar.

In addition to 2G12, several other antibodies specific to Env glycans were able to neutralize several HIV-1 isolates. Such antibodies recognize *O*-glycan structures composed of Core 1 terminal GalNAc, known as Tn antigen (Hansen et al. 1991), and sialylated GalNAc, known as sialyl-Tn antigen, and/or terminal glycan structures of *O*- and *N*-linked glycans known as $Le^y$, Fuc(α1-2)-Gal (β1-4) [Fuc(α1-3)]GlcNAc-R, and A1, [Fuc(α1-2)][GalNAc (α1-3)] Gal(β1-3)GlcNAc-R (Hansen et al. 1990). Such Core 1 *O*-glycans and fucosylated glycan structures are not commonly exposed on surface of normal human cells and were thus considered as possible epitopes for eliciting neutralizing antibodies (Hansen et al. 1990).

## Env Glycans as a Barrier or a Facilitator of Specific CD4 T-Cell Response: Antigen Processing

Heavy glycosylation of gp120 has been shown to hinder intracellular transport and processing of antigens for MHC II presentation (Botarelli et al. 1991). Respective CD4-T-cell epitopes of HIV-1 and SIV were shown to be clustered at

**Table 2** gp160 PNGS localization, type of the attached *N*-glycans and their function

| Position | Structure[a] | Type | Conser-vancy | Position[b] | Function or effect of single PNGS deletion | Effect of multiple PNGS mutations | CBA[c] | References |
|---|---|---|---|---|---|---|---|---|
| 88 | | C | High | • | | PRM-A, GNA, HHA[c] UDA[d] | UDA[d] | |
| 130 | V1 | C | Modest | • | | | ND | |
| ~136 | V1 | C | High | ←•→ | Low impact on viral infectivity | In combination with deletions of some V2 PNGS could restore virus infectivity | – | Auwerx et al. (2008) |
| ~141 | V1 | C | High | ←•→ | Low impact to viral infectivity | In combination with some V2 PNGS deletion could restore virus infectivity | – | Auwerx et al. (2008) |
| 156 | V1/V2 | C | High | • | No effect on virus infectivity | | – | |
| 160 | V2 | C | Modest | • | N160Q—non-viable virus. N160D—no effect on infectivity | Spontaneous deletion after CBA treatment occurred in combination with other PNGS | UDA | Auwerx et al. (2008), Balzarini et al. (2005a) |
| 186 | V2 | C | High | ←•→ | Decreased replication capacity | Spontaneous deletion after CBA treatment but compensates by new N188 site occurred in combination with other PNGS | HHA | Auwerx et al. (2008), Balzarini et al. (2005b) |
| 197 | V2–$\beta$3 | C | High | • | Decreased replication capacity | 197 + 156 PNGS deletion results in poor replication capacity | – | Auwerx et al. (2008), Kolchinsky et al. (2001) |
| | | | | | CD4-independent CCR5-dependent HIV-1 entry | 197 + 136 PNGS deletion results in poor replication capacity | | |
| 230 | loop A | HM | Low | ←•→ | | | + | |
| 234 | loop A | HM | Low | ←•→ | | | + | |
| 241 | $\beta$7 | HM | High | • | | | – | |
| 262 | $\beta$9 | HM | High | • | | | – | |
| 276 | loop D | C | High | • | | | HHA | |
| 289 | $\beta$11-$\beta$12 | HM | High | • | | | + | |
| 295 | B12-V3 | HM | Low | • | Alternative 2G12-binding site | | + | Scanlan et al. (2002) |
| 301 | V3 | C | High | • | Increases neutralization sensitivity | PNGS deletion could be compensated by mutations in or near to V1/V2 to maintain virus fitness | + | Pastore et al. (2006), Pollakis et al. (2001), Polzer et al. (2002) |
| | | | | | Predetermines viral CCR5 tropism | | | |
| 332 | V3-$\beta$13 | HM | High | • | One of 2G12-binding sites | | + | Calarese et al. (2003) |
| 339 | $\alpha$2 | HM | High | • | One of 2G12-binding sites | | + | Calarese et al. (2003) |
| 356 | loopE-$\beta$14 | C | High | • | | | – | |
| 362 | $\beta$14 | | Low | • | | | ND | |
| 386 | $\beta$17 | HM | High | • | | | HHA | |
| 392 | $\alpha$4 | HM | High | • | One of the 2G12-binding sites | | + | Calarese et al. (2003) |
| ~397 | V4 | C | High | ←•→ | | | – | |
| ~406 | V4 | C | High | ←•→ | | | UDA | Li et al. (2008) |
| 448 | $\beta$22-$\beta$23 | HM | High | • | | | + | Li et al. (2008) |
| ~463 | V5-$\beta$24 | C | High | ←•→ | | | – | Li et al. (2008) |
| 611 | gp41 | | High | • | | | – | |
| 616 | gp41 | | High | • | | | – | |
| 624 | gp41 | | High | ←• | | | – | |

**Table 2** continued

| Position | Structure[a] | Type | Conser-vancy | Position[b] | Function or effect of single PNGS deletion | Effect of multiple PNGS mutations | CBA[c] | References |
|---|---|---|---|---|---|---|---|---|
| 637 | gp41 | | High | • | | | – | |
| 816 | gp41 | | Modest | • | | | – | |

HHA, *Hyppeastrum hybrid* agglutinin (α1-3, α1-6 Man-specific); PRM-A, Pradimicin A; UDA, *Urtica dioica* agglutinin (GlcNAc-specific)

[a] PNGS positions numbered according to HIV-$1_{HXB2}$ (Kwong et al. 1998)

[b] Positional PNGS stability (•) or frequently occurring shift (←•→) in various HIV-1 isolates

[c] PNGS deletion induced by HIV-$1_{IIIB}$ exposure to particular CBAs occurs in combination with PNGS mutation on other gp120 position (Auwerx et al. 2008; Balzarini et al. 2007; Balzarini et al. 2006); (+) PNGS deletion was induced by several CBAs, *ND* not determined

[d] PNGS 88 deletion induced by HIV-$1_{IIIB}$ exposure to UDA occurs as a single gp120 mutation (Auwerx et al. 2008; Balzarini et al. 2007; Balzarini et al. 2006)

regions lacking PNGS, surrounded, nevertheless, by multiple PNGS (Sarkar et al. 2002; Surman et al. 2001). Although some *N*-glycans are obstructive (Botarelli et al. 1991), others were identified to positively contribute to the epitope processing and subsequent presentation on MHC II, as shown for gp120 constant region C4 (Sjolander et al. 1996). Detailed study revealed that the high-Man *N*-glycan at position 448 is necessary for trypsin digestion and for C4-specific CD4-T-cell stimulation by antigen-presenting cells in vitro. Although loss of PNGS 448 is associated with HIV-1 escape from C4 region-specific CD4 T-cell surveillance, the PNGS 448 is highly conserved among the majority of primary isolates (93.2% of sequences in Los Alamos National Laboratory HIV Sequence Compendium 2009). The lack of PNGS 448 is probably associated with the occurrence of neutralizing antibodies or CD8 T cells which could control potential escape variants of HIV-1 (McCaffrey et al. 2004; Mori et al. 2005). Furthermore, after proper folding of gp120, the PNGS 448 is flanked by two PNGS at position 262 and 295. Therefore, the loss of PNGS 448 may cause buckling of 262 and 295 glycans, leading to blockade of access of the proteolytic enzymes and loss of presentation of C4 epitopes to CD4 T cells (Li et al. 2008). These positive and negative effects of particular PNGS during specific CD4-T-cell activation are difficult to predict and must be experimentally confirmed for each modification of gp120 antigen used for immunization experiment. It remains to be elucidated whether this phenomenon plays important role during intra-host HIV-1 evolution or not.

## Intra-Host Evolution of Env Glycoprotein—Preserving the Biological Functions Under Pressure of Slowly Declining Immune System

Viruses isolated from patients early after clade A and C HIV-1 infection encode compact Env (i.e., Env with fewer PNGS and condensed loops) that are more sensitive to neutralization by donor plasma antibodies than viruses from chronic infection. Such phenomenon was not observed for clade B HIV-1 envelope (Chohan et al. 2005; Derdeyn et al. 2004; Frost et al. 2005; Li et al. 2005).

### Individual Env PNGS Evolution

Spontaneous occurrence of PNGS mutations after HIV-1 replication in the presence of carbohydrate-binding agents (CBA) or site-directed mutagenesis of individual PNGS provide a good model to study the importance of particular PNGS (or their combinations) for HIV-1 infectivity, fitness, CCR5/CXCR4 co-receptor usage, or sensitivity to various CBA (Auwerx et al. 2008; Balzarini 2007; Balzarini et al. 2006, 2007).

Importance of V1/V2 loop for HIV-1 infectivity, resistance to neutralizing antibodies, syncytia formation, and co-receptor usage was underscored in several studies (Auwerx et al. 2008; Cao et al. 1997; Kolchinsky et al. 2001; Quinones-Kochs et al. 2002; Wolk and Schreiber 2006). V1/V2 glycans contribute to about 25% of total glycans on gp120. In vitro replication of HIV-$1_{NL4.3}$ or HIV-$1_{IIIB}$ strains in the presence of CBA induces various PNGS deletions or movements, but seldom such mutations affect V1/V2 loop (Auwerx et al. 2008; Balzarini 2007; Balzarini et al. 2006, 2007). Partial or total deletion of V1/V2 loop aa severely affect virus infectivity and replication capacity (Cao et al. 1997); the deletion of entire V1/V2 loop allows CD4-independent CCR5 binding and entry of HIV-$1_{ADA}$ strain (Kolchinsky et al. 2001). HIV-1 chimeras lacking PNGS in V1 (positions N136 and N141) and V2 (N160 and N186) show a high sensitivity to serum antibodies from HIV-1-infected individuals and overall decreased infectivity (Quinones-Kochs et al. 2002; Reitter and Desrosiers 1998; Wolk and Schreiber 2006). In the recent experiment, successive PNGS deletions showed that V1-loop glycans have minimal influence on viral infectivity; in contrast, deletions of PNGS in V2 (especially 186

or 197) severely compromised HIV-1 infectivity. This alteration could be restored in some double or multiple V1/V2 PNGS deletion mutants (Auwerx et al. 2008). For example, when the PNGS 136 deletion was introduced in the replication-defective N160Q mutant virus, the replication capacity was restored to the level of the wild-type virus. Moreover, although the deletion of PNGS 197 in HIV-$1_{ADA}$ allowed CD4-independent CCR5 binding and virus entry to CD4-negative cells, the same mutation in HIV-$1_{NL4.3}$ was associated with lower replication rate in vitro (Auwerx et al. 2008; Kolchinsky et al. 2001) and probably lower virus fitness in vivo. The above-mentioned in vitro replication of HIV-$1_{NL4.3}$ and HIV-$1_{IIIB}$ strains in the presence of CBA (GlcNAc-specific *Urtica dioica* agglutinin, α1-3 and α1-6 Man-specific *Hyppeastrum hybrid* and *Galanthus nivalis* agglutinins, or α1-2 Man-specific Cyanovirin-N and Pradimicin A) selects only two V1/V2 PNGS deletion mutants (N160 and/or N186) from 50 PNGS mutants obtained (Auwerx et al. 2008). Such mutants, nevertheless, were invariably present in a background of additional PNGS deletions in the other domains of gp120. Furthermore, such a rare CBA resistance is probably associated with diminished viral infectivity or fitness and therefore it is not likely to occur in vivo (Auwerx et al. 2008). CBA-based drugs may thus be promising for future therapy of HIV-1 infection.

V3 loop contains only one internal PNGS at position 331, which is occupied by complex *N*-glycan. Both N- and C-termini contain other glycosylation sites. Although it was reported that V1/V2 loop glycans confer CCR5 or CXCR4 usage, the N-terminal stem part of V3 is probably the principal site for co-receptor specificity (Huang et al. 2007) and also for the resistance to several neutralizing antibodies (including 2G12) (Clevestig et al. 2006; Pollakis et al. 2001). The PNGS surrounding the V3 loop are associated with the commonly observed HIV-1 infectivity. Deletion of PNGS at position 276, 289, or 339 increased the infectivity of the HIV-1 mutants (Polzer et al. 2002).

For the V4 and V5 variable regions, no definitive roles have been ascribed; however, the deletion of the V4 region was shown to disrupt the folding of gp160 (Pantophlet and Burton 2006; Pollard et al. 1992). Moreover, the truncated variants of gp120 still bind CD4 (Wyatt et al. 1993) indicating that the glycans in V4 are apparently not essential for CD4 binding (Hemming et al. 1994). Therefore, the V4 region may exist solely to facilitate viral escape as a component of the evolving glycan shield (Ren et al. 2005).

## Involvement of Env Glycans in R5 to X4 HIV-1 Transition

Intra-host evolution toward CXCR4 use is characterized by diminished binding to CCR5, increased binding to CD4, and slowly increasing use of CXCR4 (Pastore et al. 2007). Switch from the CCR5 toward CXCR4 tropism is a process associated with increased HIV-1 vulnerability and probably proceeds via V1/V2 mutations that could compensate such temporal loss of HIV-1 fitness (Pastore et al. 2006; Polzer et al. 2002). Co-receptor switching from R5 to X4, typically occurring in human hosts after 5–10 years since the infection, is common in clade B and D HIV-1 infection and less common in clade C infection (Pastore et al. 2004, 2007). This complicated process occurs with such a long delay probably because dominant selective forces, such as the availability of the target CCR5-positive cell and CCR5 co-receptor density, prevail later in the HIV-1 infection when viral load reaches high titers. The R5X4 or X4 mutants arise slowly probably because several events must occur in parallel during the transition process: increased susceptibility to co-receptor inhibitors, diminished replication compared to parental R5 isolates, insertion of charged amino acids (R, D, E, K, H) at specific locations in V1/V2, C2, V3, and the strong bias in favor of G-to-A mutation (Coetzer et al. 2008; Pastore et al. 2004, 2007; Yi et al. 2003). This strong bias toward G-to-A transition mutations is likely linked to the activity of host cellular protein APOBEC3G, the cellular factor associated with non-permissivity to HIV-1 infection (Marin et al. 2003; Pastore et al. 2004). The biological sense of such highly complicated process lays in the acquired ability of R5X4 or pure X4 HIV-1 mutants to productively infect naïve T cells that do not express CCR5 but express CXCR4.

Mutation of PNGS 301 in the HIV-$1_{DH12}$—dual tropic HIV-1 envelope disrupted CCR5 usage whereas CXCR4 usage was only reduced by 50%. The complete restoration of CXCR4 usage was enabled by an increase in the net positive charge of the V3 loop without restoring the PNGS at position 301 (Ogert et al. 2001).

CCR5 tropism of HIV-1 is further associated with presence of PNGS at position 331. Deletion of this PNGS results in a shift from CCR5 tropism to dual co-receptor usage, CCR5/CXCR4, or solely CXCR4 tropism and is further associated with increase of overall positive V3-loop charge (+3 to +6) (Pollakis et al. 2001). Furthermore, PNGS 331 deletion is associated with broadening the co-receptor repertoire and includes CCR3 (Pollakis et al. 2001; Polzer et al. 2002). Site-directed mutagenesis study that used step-by-step deletion of all singular or multiple PNGS in V1/V2 loop of HIV-$1_{NL4.3}$ strain did not identify any potential R5- to X4-switch sites in V1/V2 loop (Auwerx et al. 2008). However, V3 loop remained in the wild-type form and therefore it could not be excluded that some PNGS deletions in V1/V2 loop could contribute to co-receptor switch (Auwerx et al. 2008). Such conclusion is in agreement with previous study demonstrating that appearance of additional PNGS in V1 loop (during the course of

infection) could contribute to dual CCR5/CXCR4 tropism of HIV-1. This phenomenon is again influenced by the overall charge of V3 loop (Pollakis et al. 2001). V1/V2 loop contributes to HIV-1 virus tropism not only through its glycans. Replacing the entire V1/V2 loop from the acute or chronic phase of HIV-1 infection demonstrated that the entire amino acid composition of V1/V2 loop is important for co-receptor usage and that intra-host V1/V2-loop evolution allows the virus to utilize CXCR4 while not diminishing its ability to use CCR5. In contrast, entire envelope from the acute and chronic stages of infection from the same patient demonstrated the switch from solely R5 (acute) to solely X4 (chronic) usage; thus, other features in the envelope of the chronic isolates contribute to the co-receptor usage (Pollakis et al. 2001).

Recent *in silico* comparative analysis of 171 gp120 V3 aa sequences from HIV-1 group M detected significant association between CCR5 tropism and presence of PNGS at position 301. On the other hand, dual CCR5/CXCR4 tropic strains or pure CXCR4 are characterized by increased positive aa charge of V3 rather than loss of PNGS 301. A breaking-point charge for V3 was determined +4.2, below which there were no CXCR4 sequences and above which only 10 of 133 analyzed CCR5 sequences were identified. The authors of the study concluded that the dual-tropic sequences should posses both PNGS 301 and a positive aa charge >4.2 (Clevestig et al. 2006).

Longitudinal study of subtype B HIV-1-infected patient from Multicenter AIDS Cohort Study characterized time course of mutations in V1/V2, V3 and V4/V5 domains before and during transition toward R5X4 and X4 HIV-1 variants. All of the aforementioned changes were accompanied in this patient by PNGS addition (PNGS 465) and shifts in V4 and V5 loops (PNGS 397, 462) and by addition of positively charged aa to V4 loop (Coetzer et al. 2008).

In summary, the most important factors influencing the HIV-1 CCR5/CXCR4 coreceptor usage include V1/V2 aa sequence, glycosylation of V1/V2, V3, and V4/V5 loops, overall charge of V3, and V4, V5 sequences (Auwerx et al. 2008; Coetzer et al. 2008; Pollakis et al. 2001; Polzer et al. 2002).

### Computer-Assisted Modeling of Env-Glycan Evolution

During the last decade, great efforts have been made to analyze the *N*-glycan evolution of gp120 in main HIV-1 subtypes M (A, B, C, D) and O using protein translation of HIV-1 DNA sequences and aided by computer-assisted modeling (Clevestig et al. 2005, 2006; Poon et al. 2007). Such approaches assume that glycosylation occurs at each potential *N*-glycosylation site and does not add any discriminating value to potential differences in types of glycans. Based on the alignment of A, B, C, D, and O sequences, 224 potential PNGS have been identified that were present in at least one sequence. However, an individual gp160 sequence has an average of 29.9 PNGS. The highest estimated average PNGS number was 31.9, detected in the O group. According to the experimental observations that the influence of particular PNGS on the fitness of HIV-1 is dependent on presence or absence of other PNGS, the computer modeling showed that mutually distant PNGS are in positive (inclusive, co-occurrence) interaction in contrast to close PNGS, which are in negative (exclusive) interaction. In these analyses, the spatial distances of particular PNGS asparagine residues were calculated from three-dimensional model derived from X-ray crystal structure of truncated gp120 (Kwong et al. 1998). In other words, glycans on the gp120 tend to create a shield which is fully functional once it achieves complete spatial coverage of folded HIV-1 Env and additional PNGS do not provide additional protection of HIV-1 from neutralizing antibodies but rather present a debilitating cost to the normal function of Env glycoprotein (Derdeyn et al. 2004; Poon et al. 2007).

## Consequences for HIV-1 Vaccine Design and Antigen Production: Env Structure Determines the Immune Response and Vice Versa

Several vaccination experiments have been based on expectations that removing the critical PNGS from wild Env antigen will uncover formerly glycan-shielded neutralization epitopes and will increase their immunogenicity (Back et al. 1994; Chakrabarti et al. 2002; Cole et al. 2004; Pollard et al. 1992; Puffer et al. 2002). Env-specific antibodies elicited in the sera of immunized animals were expected to contain significantly higher proportion of neutralizing antibodies and therefore such sera would neutralize HIV-1 viruses better than sera from animals immunized with wild-type gp120 or gp160. One such example was the immunization experiment with immunogen constructed by deletion of highly conserved PNGS in the V3 loop at position 301 (Back et al. 1994; Bolmstedt et al. 2001). Animals immunized twice with the mutated gp160 (T303A) DNA and boosted with wild type rgp160 protein developed higher serum titers of binding IgG (wild-type rgp160 coated on ELISA plates) than those immunized twice with wild-type gp160 DNA and boosted with wild rgp160. However, the neutralization activity of these antibodies was not significantly different. Surprisingly, when neutralization experiments were performed with a mutated T303A virus, the antibodies from T303A gp160 DNA-primed animals exhibited about three times lower neutralization activity than those from wild type gp160 DNA-primed animals (Bolmstedt et al. 2001). This experiment indicated that the

removal of a particular glycan substantially increases the immunogenicity of Env antigen by: (1) uncovering several new (most probably peptide-based) epitopes previously shielded by flexible glycan(s), (2) removal of the glycan which is tollerogenic for the host immune system (because it is produced by host cells, it tends be tolerated), and (3) induction of deviations in the Env folding toward more immunogenic and probably vulnerable states (Balzarini 2005). The observed discrepancy between the antibody binding and the neutralization activity is not easily explainable. One factor which could play an important role is probably the structural difference between free Env antigen (used for immunization) and virion-bound trimeric Env (used during in vitro neutralization assay) (Back et al. 1994). In summary, all these factors should be considered for a design of a future successful Env-based vaccine.

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