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Crystal Structure of the Neutralizing Llama VHH D7 and Its Mode of HIV-1 gp120 Interaction

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Abstract

HIV-1 entry into host cells is mediated by the sequential binding of the envelope glycoprotein gp120 to CD4 and a chemokine receptor. Antibodies binding to epitopes overlapping the CD4-binding site on gp120 are potent inhibitors of HIV entry, such as the llama heavy chain antibody fragment VHH D7, which has cross-clade neutralizing properties and competes with CD4 and mAb b12 for high affinity binding to gp120. We report the crystal structure of the D7 VHH at 1.5 Å resolution, which reveals the molecular details of the complementarity determining regions (CDR) and substantial flexibility of CDR3 that could facilitate an induced fit interaction with gp120. Structural comparison of CDRs from other CD4 binding site antibodies suggests diverse modes of interaction. Mutational analysis identified CDR3 as a key component of gp120 interaction as determined by surface plasmon resonance. A decrease in affinity is directly coupled to the neutralization efficiency since mutations that decrease gp120 interaction increase the IC50 required for HIV-1 IIIB neutralization. Thus the structural study identifies the long CDR3 of D7 as the key determinant of interaction and HIV-1 neutralization. Furthermore, our data confirm that the structural plasticity of gp120 can accommodate multiple modes of antibody binding within the CD4 binding site.

Introduction

The envelope glycoprotein (Env) from the human immunodeficiency virus type 1 (HIV-1) forms a heterotrimer composed of the receptor binding subunit gp120 and the membrane anchored fusion protein subunit gp41. Entry into host cells is mediated by gp120 interaction with CD4 that triggers a conformational change allowing subsequent interaction with cellular coreceptors such as CCR5 or CXCR4 [1–4]. Together these events trigger a refolding of gp41 that leads to the fusion of virus and host cell membranes [5–7]. Env is the target for entry inhibitors [8] and neutralizing antibodies directed against gp120 and gp41 [9]. A main problem in HIV-1 vaccine research is the generation of broadly neutralizing monoclonal antibodies upon immunization of animals with Env antigens [33,34] except for the breakthrough has yet been reported regarding the efficient immunization of animals with Env antigens [33,34] except for the trimeric Env conformation [27].

The crystal structure of gp120 in complex with b12 revealed the molecular details including a substantial conserved gp120 surface overlapping between both the CD4- and b12-bound states [14]. The similarities of both interactions is highlighted by the fact that b12 employs Tyr53 to fill the hydrophobic pocket in gp120 that is otherwise occupied by CD4 Phe43 [14]. MAbs 2F5, 4E10 and Z13 recognize epitopes within the membrane proximal region of gp41 [18–21], mAb 2G12 recognizes a carbohydrate motif [22,23], b12 interacts within the CD4 binding site [24,25], HJ16 overlaps with the CD4 binding site [26] and antibodies PG9 and PG16 are specific for the trimeric Env conformation [27].
generation of camelid antibodies. Three heavy chain only camelid specific antibody domains D7, A12 and C8, termed V\textsubscript{HH}, have been isolated after immunization with gp120. These antibodies compete with CD4 and b12 for gp120 interaction and exert neutralizing activity against primary isolates of subtypes B and C [35].

Here we describe the crystal structure of the camelid V\textsubscript{HH} D7 and determine the molecular determinants for HIV-1 Env gp120 interaction. Mutagenesis of selected CDR residues abrogate or determine the molecular determinants for HIV-1 Env gp120 neutralizing activity against primary isolates of subtypes B and C compete with CD4 and b12 for gp120 interaction and exert been isolated after immunization with gp120. These antibodies long CDR3 typical for llama V\textsubscript{HH} with a non-canonical (Figure 1A). It contains two canonical (CDR1 and CDR2) and a CDR3 conformation [38]. CDR3 is composed of 18 residues (Lys 95 – Tyr 102) (Figure 2). The base of CDR3 is well defined and stabilized by multiple main chain and side chain interactions including hydrogen bonds and salt bridges with CDR1 (Ser\textsuperscript{81}, Arg\textsuperscript{97}, Asp\textsuperscript{33}, Lys\textsuperscript{95}, Asp\textsuperscript{33}, Arg\textsuperscript{97} and Asp\textsuperscript{33}, Ser\textsuperscript{100b}) and CDR2 (Ser\textsuperscript{81}, Asp\textsuperscript{33} and Thr\textsuperscript{96}Asp\textsuperscript{100c}) (Figure 1B). The extensive inter CDR stabilization suggests a potentially lower flexibility of the CDRs upon binding to gp120. The CD4 binding site antibody b12 employs only one polar (Ser\textsuperscript{52}, Tyr\textsuperscript{53}) and few hydrophobic inter heavy chain CDR contacts [14,39]. However, the tip of the D7 CDR3 (Arg\textsuperscript{100} - Ser\textsuperscript{100b}) is highly mobile evidenced by the lack of continuous main chain density for three residues, including Tyr\textsuperscript{100a} positioned at the apex of CDR3, indicating that their conformational flexibility might be important for gp120 recognition.

### Results and Discussion

#### Structure of the V\textsubscript{HH} D7

The crystal structure of the llama heavy chain antibody fragment V\textsubscript{HH} D7 was solved by molecular replacement and refined to a resolution of 1.5 Å with an R factor of 16.6% and an R\textsubscript{free} of 19.4% (table 1). D7 folds into a typical immunoglobulin domain closely resembling known llama V\textsubscript{HH} structures [36] (Figure 1A). It contains two canonical (CDR1 and CDR2) and a long CDR3 typical for llama V\textsubscript{HH} with a non-canonical CDR conformation [38]. CDR3 is composed of 18 residues (Lys\textsuperscript{95} – Tyr\textsuperscript{102}) (Figure 2). The base of CDR3 is well defined and

#### Table 1. X-ray data collection and refinement statistics.

| Unit cell dimensions |   |
|----------------------|---|
| a (Å)                | 37.37 |
| b (Å)                | 62.18 |
| c (Å)                | 62.74 |
| Space group          | P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} |
| Wavelength (Å)       | 0.974 |
| Resolution (Å)       | 44.0-1.5 |
| Completeness (%)     | 94.1 (69.7) |
| Total reflections    | 150510 |
| Unique reflections   | 22695 |
| R\textsubscript{merge} | 0.05 (0.20) |
| σ                    | 23 (6.5) |

#### Refinement statistics

| Resolution range (Å) | 44.2-1.5 |
| Reflections/test set | 22363/1135 |
| R-factor             | 0.1657 (0.1819) |
| R\textsubscript{free} | 0.1935 (0.2294) |
| No. of residues      | 127 |
| No. of water molecules | 189 |
| No. of ligand atoms  | 4 sulfate ions |
| Average B-factor (Å\textsuperscript{2}) | 13.39 |
| r.m.s.d. from ideal  | 0.006 |
| Bond angles (deg.)   | 1.070 |
| Ramachandran statistics |   |
| Most favoured (%)    | 93.5 |
| Additionally allowed regions (%) | 6.5 |

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Here we focus our analysis on the CDR3 region as a potential gp120 interaction site. Part of this loop region is highly mobile in the absence of ligand and thus suitable for an induced fit conformation. CDR3 of D7 is tilted 40° towards CDR2 compared to the orientation of the CDR H2/H3 of the other antibodies, whereas the apex of CDR3 is also built by an aromatic residue (Tyr\textsuperscript{100}) like in b12 (Trp\textsuperscript{53}), F105 (Phe\textsuperscript{53}) and Tyr\textsuperscript{100b}, b13
Figure 1. Structure of the llama heavy chain antibody fragment V_{H} D7. (A) Ribbon representation of D7; the complementarity determining regions (CDR) are highlighted in yellow (CDR1), orange (CDR2) and salmon (CDR3). The first and last residue of each CDR is shown together with the side chain of Trp^96 critical for gp120 interaction and neutralization. The dotted line indicates CDR3 residues lacking continuous main chain density for residues Arg^{100} to Ser^{100B}. (B) A close-up of the CDR interaction network reveals multiple polar interactions between CDR1 and CDR3 as well as CDR2 and CDR3.

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Figure 2. Structure based sequence alignment of D7 with V_{H} A12 and C8 as well as with the V_{H} domains from the neutralizing antibodies b12, b13, F105 and m18. The residue numbering is according to Chothia [38] and the CDRs are indicated by coloured bars.

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(Tyr$^{100C}$) and m18 (Phe$^{99}$). Notably, b12 employs a number of CDR H3 residues to contact gp120 including Trp 100 (Figure 3B). However, despite the fact of aromatic residues at the tip of all CDR3 loops, no significant structural homology between the CDR loops can be observed underlining the differences in mode of gp120 interaction.

**Mutational analysis of D7 binding to gp120**

We performed mutational analysis within CDR3 to substantiate the role of CDR3 in gp120 interaction by determining the affinity of the D7 mutants in comparison to the wild type using surface plasmon resonance. Mutagenesis of solvent exposed CDR3 residues Lys95Ser, Trp96Ala and Leu99Ala (Figures 2 and 3A; table 2) decreased the affinity of D7 to gp120 (IIIB) significantly to 377, 27 and 69 nM, respectively, compared to 2.9 nM of wild type D7 (table 3). Note that the affinity of D7 to gp120 determined here is 30 times lower than the previously reported $K_D$ of 0.097 nM [35]. In contrast, mutagenesis of solvent exposed residues Asp$^{100C}$, Asn$^{100D}$ and the double mutant Asn$^{101}$Tyr$^{102}$Asp (table 2) enhanced the affinity to 0.04, 0.51 and 1.15 nM (table 3). Notably, the double mutation at position 101 and 102 to Tyr and Asp restores the CDR3 sequence of V$_{HH}$ A12 (Figure 2), which has broader neutralization potency than D7 [35]. Mutagenesis of Tyr$^{100A}$ located at the tip of CDR3 (Figure 3A) has no significant effect on binding (table 3).

The moderate increase of affinity by mutagenesis of Asp$^{100C}$ and Asn$^{100D}$ might reflect a decrease of the rigidity of the loop since loop stabilizing interactions are affected (table 3 and Figure 1B). This could lead to an improved induced fit upon gp120 binding facilitated by an increased CDR flexibility. The positive effect of the double mutation of Asn$^{101}$Tyr$^{102}$ on interaction together with their close location next to Trp 96 and Lys 95 suggest that they affect binding directly. Finally, the strong decrease in affinity upon mutagenesis of residues Lys$^{95}$, Trp$^{96}$ and Leu$^{99}$ indicate that these residues make

![Figure 3. Comparison of CDR2 and CDR3 from D7, b12, b13, F105 and m18 indicates different modes of gp120 interaction.](image)

**Table 2.** Solvent accessible areas of CDR3 D7 residues.

| residue   | accessible surface area (Å$^2$) |
|-----------|---------------------------------|
| Lys$^{95}$ | 36.56                           |
| Trp$^{96}$ | 136.34                          |
| Leu$^{99}$ | 179.13                          |
| Tyr$^{100A}$ | 220.36                         |
| Asp$^{100C}$ | 47.52                          |
| Asn$^{100D}$ | 80.47                          |
| Asn$^{101}$ | 80.56                           |
| Tyr$^{102}$ | 52.65                           |

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**Table 3.** Binding affinities of D7 wild type and D7 mutants to gp120 (IIIb).

| mutation                  | $k_a$ ($10^5$ M$^{-1}$ s$^{-1}$) | $k_d$ ($10^{-4}$ s$^{-1}$) | $K_D$ (nM)  |
|---------------------------|---------------------------------|-----------------------------|-------------|
| wildtype                  | 1.55 ± 0.15                     | 5.51 ± 1.47                 | 2.91 ± 0.87 |
| Lys$^{95}$Ser             | 0.13 ± 0.01                     | 23.6 ± 4.90                 | 377 ± 276   |
| Trp$^{96}$Ala             | 1.46 ± 0.46                     | 31.3 ± 17.1                 | 27.9 ± 20.5 |
| Leu$^{99}$Ala             | 0.14 ± 0.12                     | 50.0 ± 29.1                 | 69.9 ± 14.2 |
| Tyr$^{100A}$Ala           | 2.33 ± 0.15                     | 3.59 ± 0.08                 | 1.55 ± 0.07 |
| Asp$^{100C}$Ala           | 2.36 ± 0.13                     | 1.99 ± 0.38                 | 0.84 ± 0.12 |
| Asn$^{100D}$Ala           | 2.52 ± 0.06                     | 1.28 ± 0.03                 | 0.51 ± 0.02 |
| Asn$^{101}$Tyr/Tyr$^{102}$Asp | 1.87 ± 0.08                   | 0.33 ± 0.27                 | 0.18 ± 0.15 |

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crucial contributions to gp120 interaction. The decrease in affinity is mainly due to an increase of the dissociation rate of the D7 mutants (table 3), which suggests a change of interaction between the CDR3 loop and gp120 but no major change of loop conformation. Together, these data implicate that VHH D7 interacts via CDR3 and identify Lys95, Trp96 and Leu99 as key residues for this interaction. Both Lys95 and Trp96 at the beginning of CDR3 are solvent exposed while Leu99 is part of the disordered tip of CDR3, which adds to the conformational freedom of D7 interaction with gp120 (Figure 3A). All three residues are conserved in VHH A12 suggesting that it will utilize its CDR3 to contact gp120 in a similar way (Figure 2). A12 differs only at positions 100D (Tyr instead of Asn) as well as 101 and 102 (Asn, Tyr instead of Tyr, Asp). Further sequence differences that may account for the higher neutralization potency of A12 include CDR1 residues (Thr28 is changed to Ile28 and His32 to Tyr32 in A12) and CDR2 residue Asn50 (Asp50 in A12).

In order to confirm the role of CDR3 and the correlation of gp120 interaction and neutralization, wild type D7 and mutants were tested in a TZM-b1 neutralization assay against HIV-1 IIIB. Comparison of the IC50 values reinforces the importance of CDR3. Mutations that lead to decreased gp120 interaction, namely Ala mutations of Lys95 and Trp96, showed a 100-fold increase in IC50, and Leu99 revealed a 10-fold increase in IC50 as compared to D7 wild type (table 4). In contrast, mutation of Tyr100A and Asp100C show a modest decrease in the IC50 (table 4) consisting with a slightly increased affinity for gp120 (table 3). The largest positive effect was observed for the double mutant Asn101Tyr102, which shows a ~10-fold increase in affinity (table 3) and a reduction of the IC50 value by a factor of 5 (table 4). This underlines the important role of CDR3 (Figure 4A) for neutralization of HIV-1. Since the CD4 binding site on gp120 is negatively charged [13] CDR3 could provide some complementary basic charge for interaction (Figure 4B). Together these findings indicate that the differences related to CDR3 constitute an important factor accounting for the broader neutralization profile of A12 compared to D7 [35] since both CDR1 and CDR2 are almost identical (Figure 2). It is thus possible that C8 employs different structural principles for gp120 interaction and neutralization [35].

Conclusions

The conformation of the primary receptor binding site of HIV-1 gp120 reveals a hydrophobic pocket which is the target for Phe43 of the natural receptor CD4 [13] and overlaps with the binding sites of neutralizing antibodies b12 [14], b13 and F105 [15]. The structural and mutational data presented here show that D7 does not expose an aromatic residue at its CDR2 and that Tyr100A at the apex of CDR3 does not play a key role in gp120 interaction and HIV-1 IIIB neutralization. Thus D7 might employ different structural principles than b12 to interact with gp120. This is further supported by preliminary results on the D7 interaction with HIV-1 envelope proteins gp140CN54, gp140UG37, gp120IIIB, gp120YU2 and its modified variant gp120Ds2, in which an additional S-S (109-428) bridge was introduced, thus closing the cavity below the bridging sheet [14]. VHH D7 binds to gp120IIIB, gp140UG37 and gp120YU2 but is unable to interact with gp120Ds2 strongly indicating that D7 does not bind to the outer domain, as b12 does (A. Szymol personal communication). This is further supported by antibody recognition of a gp120 escape mutant. Whereas gp120 mutation G366E results in impaired binding of sCD4 and b12, the binding of D7 was not effected (A. McKnight, personal communication), further corroborating differences in

Table 4. IC50 values of VHH D7 against HIV-1 IIIb in TZM-bl cells.

| Mutation       | IC50 (µg/ml) | IC50 (µg/ml) | IC50 (µg/ml) |
|----------------|-------------|-------------|-------------|
|                | Exp1*       | Exp2*       | Average**   |
| Wildtype       | 0.066       | 0.041       | 0.054       |
| Lys95→Ser      | 3.426       | 2.641       | 3.0         |
| Trp96→Ala      | 2.717       | 2.428       | 2.6         |
| Leu99→Ala      | 0.817       | 0.560       | 0.69        |
| Tyr100A→Ala    | 0.028       | 0.018       | 0.023       |
| Asp100C→Ala    | 0.40        | 0.014       | 0.027       |
| Asn100D→Ala    | ND          | ND          | ND          |
| Asn101→Tyr102→Asp | 0.017     | 0.005       | 0.011       |

* Experiments were carried out in duplicate wells.

Figure 4. Surface representation of the CDRs. (A) Surface representation of D7 revealing the gp120 docking interface based on gp120 binding and HIV-1 neutralization results. The CDRs are coloured as in figure 1. CDR3 residues affecting gp120 interaction and HIV-1 IIIB neutralization are indicated in white and residue differences between D7 and A12 are labelled in black. (B) Electrostatic potential map of the surface generated by the CDRs.
gp120 interaction of b12 and D7. Although we identified Trp as a key residue for gp120 interaction and neutralization its position and limited extension form the core structure albeit its solvent exposure (table 4) does not conclusively indicate how it could reach into the hydrophobic CD4 binding pocket on gp120. Thus CD4 binding site antibodies might be broadly neutralizing without closely mimicking important molecular details of the CD4-gp120 interaction. Although the structure establishes a firm role for CDR3 and its flexible anchoring in interaction and neutralization activity, structural analysis of gp120 in complex with D7 is required to fully understand the conformational flexibility of both gp120 and D7 in order to exploit this knowledge for a rational vaccine design.

Materials and Methods

D7 purification, crystallization and structure solution

S. cerevisiae strain VWk18gal1 (CEN-PK102-3A, MATa, leu2-3, ura3, gal1:URA3, MAL-8, MAL3, SUC3) was used for the fermentative production of D7. The VHH D7 gene contains the following amino acid substitutions compared to wild type D7 [35] in framework residues Val5Gln, Ala11Val, Ala61Val, Ala68Asp and Ser79Tyr, which naturally in other known VHH structures [36]. The gene was integrated into the S. cerevisiae genome in the rDNA locus [46] and grown as described [47]. VHH D7 was purified from the supernatant using Ni²⁺-affinity chromatography in PBS. A final size exclusion chromatography was performed on a Superdex 200 (GE Healthcare) in a buffer containing 20 mM HEPES pH 7, 0.1 M NaCl. V HH D7 was performed on a Superdex 200 (GE Healthcare) in a buffer containing 20 mM HEPES pH 7.5, 100 mM NaCl, 0.05% P20 at a flow rate of 30 ml/min. The association of 90 μl D7 or D7 mutants at concentrations of 25 and 100 nM was recorded for 3 min followed by a dissociation of 15 min. The CM5 chip was regenerated with a 20 μl pulse of 0.1 M glycine pH 2 at a flow rate of 50 μl/min. Data were evaluated with the BiaEvaluation software (GE Healthcare) using simultaneous fit of association and dissociation.

HIV-1 neutralization in TZM-bl cells

HIV-1 neutralization in TZM-bl cells was evaluated using an assay developed previously [56,57,58]. Three-fold serial dilutions of VHH (starting at 20 μg/ml) were prepared in growth medium (DMEM containing 10% FCS) in duplicate wells of opaque 96-well cell culture plates, in a total volume of 50 μl per well. Approximately 200 TCID50 of virus, in 50 μl of growth medium, was added to each well, and the plates were subsequently incubated at 37°C. After 1 hour of incubation, 1 x 10⁴ newly transpinnized TZM-bl cells in 100 μl of growth medium containing 30 μg/ml of DEAE-dextran (Sigma-Aldrich) were added to each well. For each plate, six wells containing cells and growth medium only, and six wells containing virus and cells only, were included. The neutralization activity of each VHH was assayed in duplicate. The plates were then incubated at 37°C for 48 hours and detection of infection of TZM-bl cells was assayed by measuring luminescence production according to the manufacturer’s instructions (Promega). Lysis of cells was allowed to occur for 2 minutes and luminescence (in relative light units; RLU) was then detected using a GloMax 96 Luminometer (Promega). Neutralization was measured as the reduction in RLU in test wells compared to virus control wells after subtraction of background luminescence. The lowest VHH or antibody concentration required to give 50% reduction in RLU (IC₅₀) was determined by fitting the data to a sigmoidal equation using the XLFit 4 software (IDBS).

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Author Contributions

Conceived and designed the experiments: RAW TCV WW. Performed the experiments: AH DLH AF WWLK. Analyzed the data: HB WW. Contributed reagents/materials/analysis tools: AG HdH. Wrote the paper: RAW TCV WW.

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