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ORIGINAL ARTICLE

Structural analysis of a novel rabbit monoclonal antibody R53 targeting an epitope in HIV-1 gp120 C4 region critical for receptor and co-receptor binding

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The fourth conserved region (C4) in the HIV-1 envelope glycoprotein (Env) gp120 is a structural element that is important for its function, as it binds to both the receptor CD4 and the co-receptor CCR5/CXCR4. It has long been known that this region is highly immunogenic and that it harbors B-cell as well as T-cell epitopes. It is the target of a number of antibodies in animal studies, which are called CD4-blockers. However, the mechanism by which the virus shields itself from such antibody responses is not known. Here, we determined the crystal structure of R53 in complex with its epitope peptide using a novel anti-C4 rabbit monoclonal antibody R53. Our data show that although the epitope of R53 covers a highly conserved sequence 433AMYAPPI439, it is not available in the gp120 trimer and in the CD4-bound conformation. Our results suggest a masking mechanism to explain how HIV-1 protects this critical region from the human immune system.

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Keywords: C4; CD4; Env; HIV-1; monoclonal antibody

INTRODUCTION

The HIV-1 envelope glycoprotein (Env) gp120 initiates viral entry into host cells by binding to its receptor CD4 and to its co-receptor CCR5/CXCR4, and it is the major target for acquired immune deficiency syndrome vaccine development. However, gp120 uses many decoys to evade immune surveillance in humans, rendering the development of a protective vaccine very challenging. Conformational masking, by evading immune responses reactive with a C4 peptide, named T1 (a 16-mer containing the region of residues 428-443). The C4 region can also induce humoral immune responses. In fact, the CD4 binding region of gp120 was first identified by an anti-C4 mAb, 5C2E5, which is comprised of various conserved regions including the fourth conserved region (C4). The C4 region of gp120, which consists of residues 416-459 (HxB2 numbering), has many important functional roles. For example, it is directly involved in receptor binding, co-receptor binding and co-receptor selection (tropism). Crystal structures of gp120 complexes have revealed that residues 425 (Asn), 426 (Met), and 427 (Trp) in the C4 region have direct contact with CD4. The C4 region, together with the third variable loop (V3), is also involved in co-receptor binding. Early mutagenesis studies indicated that residues 438 (Pro) and 441 (Gly) in the C4 region are important for CCR5 binding. Structural studies of gp120 in complex with CD4 and monoclonal antibody (mAb) 412d showed that residues 439 (Ile), 440 (Arg), and 441 (Gly) in the C4 region are involved in binding with the N-terminus of CCR5. A slight conformational change in the C4 region can influence the structure of V3, and even a single amino acid mutation in the C4 region can increase the neutralization sensitivities of anti-V3 antibodies. The C4 region is also involved in co-receptor selection, and mutations of residue 440 in the C4 region can alter co-receptor specificity.

The C4 region is highly immunogenic. It can induce cell-mediated immune responses in HIV-1 infected patients and in immunized animals. For example, monomeric gp120 can elicit mouse helper T-cell immune responses reactive with a C4 peptide, named T1 (a 16-mer containing the region of residues 428-443). The C4 region can also induce humoral immune responses. In fact, the CD4 binding region of gp120 was first identified by an anti-C4 mAb, 5C2E5, which was raised by immunizing mice with a recombinant gp120,23,24 and rat mAbs ICR 38.8f and ICR38.1a that were raised with the recombinant BH10 gp120,25 mouse mAbs G3-42, G3-299, G3-508, and G3-536 that were raised with a recombinant BH10 gp120,23,24 and rat mAbs ICR 38.8f and ICR38.1a that were raised with the recombinant BH10 gp120. One of the characteristics of these antibodies is that they can block CD4 binding of gp120, and thus, they were collectively named CD4-blocking antibodies.

The C4 region was initially suggested to form amphipathic helices; however, crystal structures of CD4-bound gp120 molecules have
shown that it actually forms two beta strands, numbered 20 and 21, of the bridging sheet and loop F. \(^{13}\) Strand 20 is involved in CD4 binding, while strand 21 is involved in co-receptor binding. The structures of gp120 in complex with various mAbs showed that the C4 region could display distinct conformations among the unliganded, CD4-bound, and different antibody-bound states. \(^{7,13,27–29}\)

Here, we present functional and structural characterizations of a recently generated rabbit anti-C4 mAb, R53, which was elicited by JR-FL gp120 using a DNA prime-protein boost regimen. \(^{26}\) It can neutralize sensitive viruses quite potently but cannot neutralize tier 2 viruses. We show here that R53 has broad cross-clade binding activities against gp120 proteins from clade A, B, C, D, and AE. We have determined crystal structures of R53 Fab/epitope complex and R53 Fab alone, and have structurally defined the R53 epitope. We found that this epitope harbors a conserved motif, AMYAPPI (residues 433–439), located at the C-terminus of the bridging sheet and loop F of gp120. Our data provide a structural understanding of this immunogenic and functionally critical region and the mechanism by which it is masked.

### MATERIALS AND METHODS

#### Enzyme-linked immunosorbent assay (ELISA)

Gp120 proteins produced from 293Fc cells were coated onto 96-well microtiter plates (Sigma-Aldrich, St. Louis, MO, USA) at 1 μg/mL in 100 μL of phosphate-buffered saline (PBS) as previously described. \(^{30}\) Plates were washed five times with PBS containing 0.1% Triton-X (EWB) and blocked overnight at 4 °C in PBS containing 4% whey and 5% powdered milk. R53, biotinylated anti-rabbit secondary antibody (Vector Labs BA-1000) at 1.5 mg/mL, and 5% powdered milk. R53, biotinylated anti-rabbit secondary antibody (Vector Labs BA-1000) at 1.5 mg/mL, and a streptavidin horse-radish peroxidase construct (Vector Labs SA-5004, Burlingame, CA, USA) at 500 ng/mL were added sequentially to the wells in a volume of 100 μL. Plates were incubated for 1 h at room temperature and washed five times after each step with EWB and then developed for 3 min in 96-well format, as previously described. \(^{30}\) Protein A-coated tips (ProA rate, Koff) were measured in the kinetics buffer. The sensorgrams were corrected with the blank reference and the binding curves were generated by global and local fitting with ForteBio Data Analysis software package 7.0 using a 1:1 binding model when R² values were greater than 0.95. The affinity constant Kₚ values (in molar units) were calculated using off-rates (Kₚoff/on-rates (Kₚon)). To regenerate the Protein A sensor for reuse, the sensor was placed in wells containing regenerating buffer (500 mM phosphoric acid) as necessary.

#### Competition binding assays

The sheep gp120 C5-specific mAb, D3724 (Aalto Bio Reagents, Dublin, Ireland) was used to coat ELISA plates at 2 μg/mL. The plates were washed five times and blocked with PBS containing 4% whey and 5% powdered milk, and gp120 proteins were then added to these pre-coated plates. After the five washes, the competitor rabbit mAbs or sCD4 were diluted in the blocking buffer and added to the plates at three-fold serial dilutions, starting at 500 μg/mL. After a 30-min incubation, 10 μL of Ig-CD4 at 0.1 μg/mL was added to the wells. Bound Ig-CD4 was detected as described above in the ELISA assay.

#### Structure of mAb against HIV-1 Env C4 region

Virus capture assay

Virus capture assays were performed as previously described. \(^{31}\) Pseudovirions expressing the JR-FL Env and vesicular stomatitis virus (VSV) G protein were produced with the pSG3⁰ENV backbone in 293T cells. Plates were coated with 50 μL of mAb at 5 μg/mL for 1 h at room temperature and then blocked in PBS with 3% BSA overnight at 4 °C. Hybridoma supernatants of mAb cell cultures were incubated with pseudovirions for 1 h before the mixtures were added to the ELISA wells, and incubated for 3 h at room temperature. Plates were washed five times with sterile PBS, overlaid with 10,000 TZM-bl cells per well and incubated for 48 h at 37 °C. Luciferase activity was determined according to the manufacturer’s instructions (Promega, Madison, WI, USA). The competition percentage was reported as the reduction of the luciferase signal compared to a serum negative control.

#### Fab production and purification

Details of the production and antigenicity characterization of rabbit mAb R53 have been published. \(^{30}\) The Fab fragment of R53 was prepared by papain digestion as previously described. \(^{32}\) Briefly, IgG was mixed with papain (Worthington, Lakewood, NJ, USA) at a 20:1 ratio in 100 mM Tris (pH 6.8) containing 1 mM cysteine hydrochloride and 4 mM EDTA. The mixture was incubated for 1 h at 37 °C and the reaction was stopped by the addition of 10 mM iodoacetamide. The Fab fragment was separated from the Fc fragment and undigested IgG using a Protein A column and was further purified by size exclusion chromatography. The concentration of the Fab fragment used for crystallization was approximately 10 mg/mL.

#### Crystallization, data collection, structure determination and refinement

The C4 peptide was synthesized by Biomatik (Wilmington, DE, USA) and was dissolved in water to a concentration of 10 mg/mL.

| Table 1: The inhibition percentages of R53 to outcompete binding of mAbs to a JR-FL and VSV-G pseudotyped virus |
|---------------------------------------------------------------|
| Competing mAb | Hybridoma cell line ID |
|----------------|------------------------|
| b12           | 56%                    |
| 3074          | 21%                    |
| 2G12          | 39%                    |
| 53            | 56%                    |
| 56            | 34%                    |
| 15            | 244%                   |
Figure 1  Broad reaction of R53 with gp120s of diverse clades. (A) Competition assay of R53 with Ig-CD4. The soluble CD4 (sCD4) and anti-V3 mAb R56 were used as positive and negative controls, respectively. Results shown represent the mean levels of two independent experiments and error bars indicate the standard deviations. (B) R53 bound nine gp120s from clades A, B, C, D, and AE, as revealed by ELISA. (C) Western blot analysis showed that five selected gp120 proteins from different clades were recognized by R53. (D) A protein sequence alignment of R53 epitope region of the gp120s used in panel B and C. The R53 epitope is underlined in the JR-FL sequence.

Figure 2  Binding kinetics of R53 and gp120s. The binding kinetics of R53 to 92UG037 (clade A), JR-FL (clade B), 93MW965 (clade C), 92UG021 (clade D), and AE consensus (clade AE) were measured using ForteBio at different concentrations of the ligand. The brown, red, blue, purple, green, and orange lines represent 300 nM, 100 nM, 33.3 nM, 11.1 nM, 3.7 nM, and 1.23 nM of gp120 proteins, respectively. Dashed gray lines represent the theoretical fitting curves. The binding data are summarized in Table 2.
Preliminary crystals of Fab in complex with peptide, or Fab alone, were obtained by robotic screening using the vapor diffusion hanging drop method. Well-diffraacted crystals of R53 Fab in complex with the peptide were obtained in a well solution of 24% polyethylene glycol 6000, 0.1 M citric acid, pH 5.0, and 0.02% NaN₃. Crystals of R53 Fab alone were obtained in a well solution of 14% polyethylene glycol 8000, 0.1 M HEPES, pH 7.5, and 8% ethylene glycol. Crystals of Fab R53/epitope complex and Fab R53 alone were first soaked in the mother liquor with M HEPES, pH 7.5, and 8% ethylene glycol. Crystals of Fab R53/epitope complex and Fab R53 alone were then placed in the X-ray beam. X-ray diffraction data of the additional 20% glycerol (v/v) and 20% ethylene glycol, respectively, were collected at beam line X6A, before being placed in the X-ray beam. X-ray diffraction data of the crystals of R53 Fab alone were collected at the synchrotron National Synchrotron Light Source, Brookhaven National Laboratory.

**Table 2** R53 binding kinetics with five representative gp120 proteins

| gp120 proteins (clade) | \( K_D \) (M) | \( K_m \) (M⁻¹s⁻¹) | \( K_{on} \) (s⁻¹) |
|-----------------------|---------------|-------------------|-----------------|
| 92UG037 (A)           | 1.54E-09      | 3.41E+05          | 5.24E-04        |
| JR-FL (B)*            | 1.96E-09      | 1.57E+05          | 3.09E-04        |
| 93MW965 (C)           | 3.98E-09      | 3.04E+05          | 1.21E-03        |
| 92UG021 (D)           | 1.79E-09      | 3.56E+05          | 6.38E-04        |
| AE consensus (AE)     | 2.49E-09      | 3.91E+05          | 9.73E-04        |

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**RESULTS**

Immunological characterization of rabbit mAb R53

Rabbit mAb R53 is one of the mAbs that we recently generated from a New Zealand white rabbit that was immunized with JR-FL DNA prime, followed by a homologous protein boost, as previously reported. The location of its epitope was mapped to the C4 region of gp120 using a 15-aa overlapping peptide array, and a virus capture assay was used to identify whether the epitope of R53 overlapped functionally with those of CD4-binding sites (CD4bs) mAbs (Table 1). CD4bs mAb b12, V3-specific mAb 3074, and glycan-specific antibody 2G12 were used as competition targets. Supernatant from the R53 hybridoma was able to prevent 56% of the virus binding to mAb b12, suggesting that R53 indeed targets the CD4bs region, but not as effectively as the CD4bs mAbs. In contrast, it could not compete against either anti-V3 mAb 3074 or anti-glycan mAb 2G12, further supporting its specific competition against b12. Because previously described mAbs targeting the C4 region were found to block CD4 binding of gp120, we determined the binding specificity of R53 using a competition ELISA (Figure 1A). R53, sCD4 and R56 were used to compete with Ig-CD4 for binding to JR-FL gp120. Here, R56 is a rabbit mAb that was isolated from the same rabbit as that of R53, and recognizes the V3 crown region of gp120. As expected, R53 could inhibit Ig-CD4 binding, although it is not as effective as the positive

**Table 3** Crystallization and refinement statistics

| Data collection | Fab R53/epitope | Fab R53 |
|-----------------|-----------------|---------|
| Space group     | P2₁2₁2₁         | C2      |
| Cell dimensions |                 |         |
| a, b, c (Å)     | 72.28, 84.83, 167.24 | 105.43, 78.55, 68.48 |
| α, β, γ (°)     | 90, 90, 90       | 90, 92.25, 90 |
| Resolution (Å)  | 2.26 (2.30–2.26) | 1.63 (1.66–1.63) |
| Rmerge (%)      | 16.5 (48.3)      | 5.6 (44.0)    |
| I/σI            | 17.9 (3.7)       | 21.5 (2.3)    |
| Completeness (%)| 99.1 (97.5)      | 95.4 (79.0)   |
| Redundancy      | 6.5 (6.1)        | 3.9 (3.1)     |
| Refinement      |                 |         |
| Resolution (Å)  | 44.1–2.3 | 31.5–1.6 |
| Number of reflections | 45616 | 66081 |
| Rmerge/Rfree    | 16.7/22.3 | 19.5/22.8 |
| Number of atoms |                 |         |
| Protein         | 6632            | 3251    |
| Solvent         | 988             | 624     |
| B-factors       |                 |         |
| Protein         | 19.8            | 29.9    |
| Solvent         | 25.4            | 40.6    |
| Root-mean-square deviations | | |
| Bond lengths (Å) | 0.008 | 0.007 |
| Bond angles (°) | 1.073 | 1.096 |

Values in parentheses are for the highest resolution shell.
control sCD4. As a negative control, R56 had no impact on Ig-CD4 binding of gp120. These results confirm that R53 can block CD4 binding to gp120.

To determine the binding breadth of R53 for HIV-1 gp120 from different clades, nine representative gp120s were tested: 92UG037.8 from clade A; 92US715, BaL, and JR-FL from clade B; 96ZM651 and 93MW965 from clade C; 92US021 from clade D; and 93TH976.17 and AE consensus from clade AE. R53 was able to recognize all of the Envs that were tested in the ELISA (Figure 1B). This result was verified by a Western blot analysis, which demonstrated that the five representative gp120s from different clades were recognized by R53 under denaturing conditions (Figure 1C). Binding kinetics of R53 with five selective gp120s were further determined using a ForteBio instrument (Figure 2). R53 was shown to have high binding affinities to all five gp120s tested, with $K_D$ values ranging from 1.79 nM to 3.98 nM (Figure 2 and Table 2). A sequence alignment with the R53 epitope region revealed that it is highly conserved among clades A, B, C, D, and AE (Figure 1D).

Crystal structure of the Fab epitope complex of rabbit mAb R53

We determined the crystal structure of the R53 antigen-binding fragment (Fab) in complex with a peptide (VGKAMYAPPGRQIR, residues 430 to 444 in HXB2 numbering). The complex structure of the R53 Fab/epitope was solved by molecular replacement and refined to a resolution of 2.3 Å with an $R_{work}/R_{free}$ of 16.7%/22.3% (Figure 3 and Table 3). The crystals grew in the orthorhombic space group $P2_1 2_1 2_1$ with two Fab/epitope complexes in the asymmetric unit. The two complexes are highly similar (RMSD = 0.55 Å); we thus chose only one for description here. We numbered the residues following the Kabat et al.’s convention, with the light and heavy chains preceded by “L” and “H”, respectively, and the residues of the epitope by a “P”. Although a 15-mer peptide was used in the crystallization, only 11...
residues, i.e., GKAMYAPPIRG (residues 431 to 441), were observed in the electron density map. We also determined the structure of the Fab alone (Table 3); superposition of the two Fab structures showed that the R53 Fab appears to undergo a minimal conformational change upon epitope binding (RMSD = 0.77 Å). R53 has a typical rabbit antibody inter-domain disulfide bond between residues 80 and 170 of the light chain, which places a constraint on the variability of its elbow angle.38

The epitope of R53 (GKAMYAPPIRG441) does not form a regular secondary structure, but it is shaped like a stretched spring, lying across the top of the light and heavy chains (Figure 3). Its N-terminus sits in a groove formed by the heavy chain, while the C-terminus straddles on a saddle formed by the R53 light chain residue AspL30, on one side hanging the side chain of ArgP440 and on the other side the side chain of IleP439. Electrostatic potential analysis indicates that the antigen-binding site of R53 is negatively charged (Figure 3D), complementing the positively charged epitope that harbors two positively charged residues, LysP432 and ArgP440. All complementarity-determining regions (CDRs) are involved in antigen binding, except for the second CDR loop of the light chain (CDR L2).

Figure 5  Locations and conformations of the R53 epitope region in gp120 structures. The structure of the R53 epitope, together with Fab R53 (only the Fv region is shown), was superimposed onto the epitope region of gp120 structures with distinct C4 conformations, including the CD4-bound (A), b12-bound (B), F105-bound (C), and b13-bound (D) gp120s.11,25–27 The R53 epitope is colored magenta, while gp120 is colored gray. Clashes between R53 and gp120s are indicated. The R53 epitope is accessible (without clashes between the antibody and gp120) only in the b13-bound conformation of gp120. (E) The location of the R53 epitope in the recent published structure of BG505 SOSIP.664 trimer. The R53 epitope region, located underneath V1V2/V3, is colored magenta, while the rest of the three gp120s is colored gray, yellow, and orange, respectively.
Antigen–antibody interaction of R53
The antigen–antibody interaction of R53 involves extensive hydrophobic contacts, with ~730 Å² of buried surface area, and multiple potential hydrogen bonds between the antibody and the epitope. The epitope of R53 is comprised of six hydrophobic residues, including AlaP431, MetP432, AlaP434, ProP435, ProP436 and IleP439, and they are involved in several hydrophobic interactions (Figure 4). Interestingly, the second proline residue in the epitope, ProP438, has barely any contact with R53 (less than 5 Å² of calculated contact area). The epitope residue TyrF435 has the largest contact with the antibody; its side chain stacks in parallel with the backbone of SerF395 of CDR L3. There are also hydrogen bonds between the epitope backbone and the antibody: for example, TyrL32 and TyrL92 of the light chain with the backbone of the epitope residues ProP437 and IleP439, respectively; the backbone amide of AlaP433 with the side chain of heavy chain residue AspL518; the backbone of MetL34 with the amide of TyrL197; and the OH group of the epitope residue TyrP435 with the side chain of the heavy chain GluH55 (Figure 4).

The epitope of R53 contains no acidic residues but has two basic residues, LysP432 and ArgP440. The side chain of LysP432 is located in an acidic environment that is formed by AspH511 and GluH55, while the side chain of ArgP440 forms salt bridges with two heavy chain residues, AspL30 and GluL31, of R53 (Figure 4). The R53–contacting residues, AM/IYAPPI, are highly conserved among clades A, B, C, D, and AE (Figure 1D).

Spatial location of the R53 epitope in gp120 structures
After an examination of all currently available gp120 structures in the RCSB PDB, we found that the region of the R53 epitope can form four distinct conformations (Figure 5): (i) the CD4-bound (represented by the first gp120 core structure with PDB ID 1GCI), the majority of the gp120 structures, including the unbound core structures, belong to this group), (ii) the mAb b12-bound (PDB ID 2NY7), (iii) the mAb F105-bound (PDB ID 3H11), and (iv) the mAb b13-bound conformations (PDB ID 3IDX). In the CD4-bound conformations, this region extends from beta strand 21 of the bridging sheet to loop F13. In the b12-bound gp120, the conformation of this region is similar to that of the CD4-bound gp120. However, the bridging sheet is not completely formed due to the dislocation of the two strands (strands 2 and 3) from the inner domain. In the b13-bound and F105-bound gp120s, this region, as well as the region of strand 20, forms a coil. Superimposing the R53 epitope with the C4 region in these gp120 structures showed that the epitope is only available in the b13-bound conformation, i.e., R53 binding of the epitope does not clash with the rest of the gp120 molecule. In a recently reported work, structures of the stabilized BG505 SOSIP.664 trimer, through conformational masking of receptor-binding sites.

DISCUSSION
We have structurally defined a linear C4 epitope in complex with a broadly reactive gp120-specific mAb to understand the antigen–antibody interactions in a region of gp120 that serves as a target for many previously reported CD4 blocking mAbs, for which structural information was previously unavailable. The epitope is located at the C-terminus of the bridging sheet and loop F. This region plays critical roles in gp120 functions and thus is highly conserved. Residues TyrF435, ProP436, IleP439, ArgP440, and GlyP442 in the R53 epitope region are critical for co-receptor binding. This region is also highly immunogenic, as indicated by several mAbs that were reported in the literature. However, most of the antibodies that target this region are not neutralizing or have only weak neutralization activities, suggesting that this region is masked in the gp120 trimer.

Our data provide a mechanistic understanding of how this region is masked. In the pre-fusion gp120/gp41 complex trimer, this region is buried by V1V2 and V3 loops that lie on top of it; thus, this region is sequestered from interactions with antibodies. In the CD4-bound state, this region is located on the surface of the gp120 monomer (not shown). However, in this conformation the epitope of R53 is not available for mAb binding (Figure 5A), as the side chains of amino acids, such as TyrP435 that binds R53, are buried and face the core of the molecule. Thus, the high immunogenicity of this region may be derived from the side chains that are not accessible in the CD4-bound state. Therefore, our structure can explain the mechanism by which this family of mAbs blocks the binding of CD4, i.e., as illustrated in Figure 5A, the CD4-bound conformation of gp120 is not compatible with R53 binding. In other words, the binding of the CD4-blocking mAbs will prevent the correct formation of the CD4 binding site, thus preventing CD4 binding to gp120.

RCSB PDB accession numbers for the crystal structures
Atomic coordinates for the R53 Fab epitope complex and the Fab alone have been deposited in the RCSB PDB with the accession codes 4ZTO and 4ZTP, respectively.

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