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Germlining of the HIV-1 broadly neutralizing antibody domain m36

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Abstract

Engineered antibody domains (eAds) have emerged as a novel class of HIV-1 inhibitors and are currently under preclinical testing as promising drug candidates for prevention and therapy of HIV-1 infection. Reverse mutation of antibodies to germline sequences (germlining) could not only identify less mutated variants with lower probability of immunogenicity and other improved properties but also help elucidate their mechanisms of action. In this study, we sequentially reverted the framework (FRs) and complementary determining regions (CDRs) of m36, a human antibody heavy chain variable domain-based eAd targeting the coreceptor binding site of the viral envelope glycoprotein gp120, back to germline sequences. Two types of amino acid mutations and one region in the antibody V segment were identified that are critical for HIV-1 neutralization. These include four mutations to acidic acid residues distributed in the CDR1 and CDR2, two mutations to hydrophobic residues in the FR3 and CDR3, and partial FR2 and FR3 sequences flanking the CDR2 that are derived from a different gene family. An m36 variant with all five mutations in the FRs reverted back to germline showed slightly increased neutralizing activity against two HIV-1 isolates tested. Another variant with seven of twelve mutations in the V segment reverted retained potency within three-fold of that of the mature antibody. These results, together with an analysis of m36-gp120-CD4 docking structures, could have implications for the further development of m36 as candidate therapeutics and elucidation of its mechanism of potent and broad HIV-1 neutralization.

Keywords

HIV-1; antibody domain; mutation; germlining; neutralization

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Engineered antibody domains (eAds), which are about one tenth the size of naturally occurring antibodies, have recently emerged as a novel class of HIV-1 inhibitors with breadth and potency comparable to those of broadly neutralizing antibodies (bnAbs) that arise during HIV-1 infection in humans (Chen and Dimitrov, 2009; Chen et al., 2014b; Forsman et al., 2008; Matz et al., 2013; McCoy et al., 2012). Due to their small molecular size (approximately 15 kDa), eAds are capable of circumventing some viral defense mechanisms such as steric occlusion of conserved, functionally important structures of the viral envelope glycoproteins (Env) (Chen et al., 2008a; Labrijn et al., 2003). M36 is the first reported human antibody heavy chain variable domain (VH)-based HIV-1 bnAb that we identified by panning and screening a large phage-display VH library sequentially against two different Env (Chen et al., 2008a; Chen et al., 2008b). It neutralized almost all (10 of 11) genetically diverse classical HIV-1 isolates tested with 50% inhibitory concentrations (IC₅₀s) 10 µg ml⁻¹ (Chen et al., 2008a) and 80% of 46 isolates predominantly circulating in China with IC₅₀s 25 µg ml⁻¹ (He et al., unpublished). Biochemical and structural investigations indicated that m36 targets the coreceptor-binding site (CoRbs) of the Env gp120, a highly conserved sterically restricted structure induced by CD4 binding (Chen et al., 2008a; Meyerson et al., 2013). M36 is currently being developed in the form of fusion proteins with ibalizumab, a clinically tested bnAb directed against the extracellular domains of CD4 (Sun et al., 2014), or single-domain soluble CD4 (Chen et al., 2014a). The bispecific fusion proteins neutralized all isolates tested with exceptional potency compared to several representatives of the first- and second-generation HIV-1 bnAbs to the Env and the highly potent U.S. FDA-approved peptide inhibitor T20.

Reverse mutation to germline sequences (germlining) is among other strategies that biopharmaceutical industry has been using to improve drug-related properties of therapeutic antibodies such as immunogenicity, stability and aggregation propensities (Lu et al., 2012; Luo et al., 2010). Germlining could also help delineate paratopes of antibodies and elucidate their mechanisms of action (Georgiev et al., 2014; Klein et al., 2013). In this study, we sequentially reverted mutations in the framework regions (FRs) and complementarity determining regions (CDRs) of m36 back to germline sequences in order to identify mutations that contribute to the antibody's ability to neutralize HIV-1 and less mutated m36 variants with preserved HIV-1 neutralizing activity.

M36 is a chimeric human VH with the CDR2 and partial flanking FRs closest to the HV4-34 germline and the rest of antibody sequence closest to the HV3-23 germline according to the IMGT/V-QUEST (<http://www.imgt.org>) analysis (Fig. 1). To find out how mutations in FRs could affect binding and neutralizing activity, we first generated m36m1 in which all five mutations in m36 FRs were back mutated (i.e., Q1E, Q6E, I66N, T93S, and I101V) (Fig. 1). Because residue 66 of the HV4-34 germline sequence could also be Y, we generated m36m1 (I66Y) which had the I66Y instead of the I66N back mutation as in m36m1. The CDR2 of m36 and flanking FR sequences (residues 47-55 and 66-76) were grafted from an HV4-34 gene family member during library construction (Chen et al., 2008a; Chen et al., 2008b). To test whether the HV4-34-originated FRs are important for antibody functions, they were substituted with the corresponding sequences of the HV3-23 germline, resulting in another construct, m36m2.

Binding of the m36 variants to the HIV-1 Envs was analyzed by the enzyme-linked immunosorbent assay (ELISA). M36m1 and m36m1 (I66Y) bound to a gp120-CD4 fusion protein (gp120_{Bal}-CD4) with half maximal effective concentrations (EC₅₀s) (~2 nM) similar to that of m36 although at a given concentration, m36m1 showed lower binding strength than the other two (Fig. 2A). No binding was observed for m36m2 at the concentrations tested, suggesting that the CDR2 and flanking sequences could play a critical role in modulating the antigen-antibody interaction. The antigen-binding fragments (Fabs) of human antibodies capable of binding to staphylococcal protein A (SPA) are encoded by gene segments belonging to the HV3 family; thus, SPA binding has been used as a marker for proper folding of human HV3 (Chen et al., 2008b; Potter, Li, and Capra, 1996). In contrast to the Env binding activity, we found that only m36m2 interacted with SPA (Fig. 2A), suggesting that HV3-derived FR sequences flanking CDR2 are essential for proper folding of the HV3 family members.

In a pseudovirus-based neutralization assay, m36m1 and m36m1 (I66Y) neutralized the clade-B HIV-1 isolate Bal comparably with or better than m36 (Fig. 2B and Table 1). When another clade-B HIV-1 isolate (JRFL) was tested, m36m1 (I66Y) also had slightly higher neutralizing activity than m36 while m36m1 showed a two-fold decrease in potency (Fig. 2B and Table 1). Surprisingly, m36m2, which showed no binding to gp120_{Bal}-CD4 in the ELISA, still neutralized the two isolates although with a large decrease in potency, suggesting that the soluble, recombinant gp120_{Bal} protein in the CD4-bound state may not fully preserve the native conformation on the functional viral spike.

We then tested whether mutations in CDRs are important for antibody neutralizing activity. Seven m36m1 variants were generated, each containing an individual reverse mutation in the CDRs of the V segment (Fig. 1). Due to the complexity of V(D)J recombination, mutations in other regions of CDR3 remained uncertain and were left intact. The pseudovirus neutralization assay with Bal and JRFL showed that back mutation of a hydrophobic residue (I106K) at the base of the CDR3 loop resulted in a large decrease in the neutralizing activity of m36m1 (Fig. 3A and Table 1). The neutralizing activity of variants with reverse mutation of acidic acid residues (D29T, D36S, E38A and D58H) in the CDR1 and CDR2 also showed a trend of decrease against at least one isolate while substitution of the other two residues (A27G and N64S) did not lead to decreased potency when IC₅₀s were compared.

To test whether back mutation of several residues in CDRs simultaneously could have a profound effect on neutralization, we generated m36m3 which carried both the A27G and N64S mutations compared to m36m1, and m36m4 where all the four acidic acid residue mutations (D29T, D36S, E38A and D58H) were combined (Fig. 1). Interestingly, m36m3 had comparable neutralizing activity while m36m4 showed largely decreased potency against both HIV-1 isolates compared to m36m1 (Fig. 3B and Table 1), suggesting that the four acidic acid residues play a combinatorial role in the antibody-antigen interaction.

Without an existing m36-gp120-CD4 complex crystal structure, we attempted to use the information gathered from m36 germlining (this study), affinity maturation (Chen et al., 2010), and cryo-electron microscopy (cryo-EM) (Meyerson et al., 2013) to guide docking of

the antibody onto a known gp120-CD4 structure. The three-dimensional structure of m36 was created by using the SWISS-MODEL program and as a template the crystal structure of the human germline antibody 3-23/B3 (Malia et al., 2011), which has the highest sequence identity with m36. The gp120-CD4 structure used for docking was extracted from the solved crystal structure of the gp120_{JRFL}-CD4-X5 complex, where gp120 is from the same HIV-1 isolate (JRFL) tested in this study and X5 is an antibody directed against the CoRbs of gp120 (Huang et al., 2005). Docking models were generated by using the Z-DOCK program and selected based on two major criteria. One is the most compatibility to general features of antibody-antigen interactions such as hydrogen bonds, salt bridges, hydrophobic packing and other interactions at the interface without any highly unusual features and steric clashes. The other is the highest level of agreement with our findings from the biochemical and structural investigations mentioned above.

The structural topology of the finally selected docking model as depicted in Fig. 4 was highly consistent with the cryo-EM structure of the m36-gp120-CD4 complex on live viruses (Meyerson et al., 2013), showing that m36 epitope is near the base of gp120 V3 loop in a location similar to that of the CoRbs antibodies 17b (Kwong et al., 1998) and X5 (Huang et al., 2005). However, unlike 17b and X5 which long protruding heavy chain CDR3s play a critical role in their interactions with gp120 (Huang et al., 2005; Kwong et al., 1998), our structural model indicated that m36 might contact gp120 mainly through the CDR1. The three acidic acid residues (D29, D36 and E38) in m36 CDR1 could participate in forming salt bridges with three finely linearized basic residues (R419, K421 and R327) on gp120 (Fig. 4, upper right square). Another acidic residue (D58) in the CDR2 was in close proximity to the K432 residue of gp120. Hydrogen bond interaction mediated by solvent could be easily formed between the two residues. Reverse mutations of these acidic residues to germline sequences could therefore perturb the interactions leading to decreased neutralizing activity, as observed in this study (Fig. 3).

The selected model also indicated hydrophobic packing as another key element of the antibody-antigen interaction. A hydrophobic core could be formed between the bases of m36 CDR2 loop involving the I56 and I66 residues and gp120 bridging sheet involving the V120, L122 and I423 residues (Fig. 4, lower left square). This line of reasoning is supported by our finding that m36m1 (I66Y) with a hydrophobic residue (Y) in position 66 was several-fold more potent in neutralizing JRFL than m36m1 with a polar neutral residue (N) in the same position (Fig. 2B). I106 at the stalk of m36 CDR3 is surrounded by a cluster of hydrophobic amino acids from the antibody itself and is in close proximity to the highly charged regions of m36 and gp120 described above (Fig. 4, lower right square). Therefore, mutation of this residue might drastically decrease the interaction by altering the overall conformation of m36 and interrupting salt bridge formation between m36 and gp120. Substitution of the I106 residue with K resulted in a large decrease of neutralizing activity of m36m1 (Fig. 3A), indicating such a possibility.

Of the top 10 poses of m36 generated by Z-DOCK, three (poses 1, 2 and 9 which is depicted in Fig. 4) adapted a structure where the Greek key anti-parallel sheet of m36 is oriented in parallel to the neighboring gp120 V3 loop, suggesting possible interaction of m36 FRs with gp120. In agreement with this possibility is our previous finding that mutation of a residue

in the FR2 of m36 (Q44E) led to a significant increase of antibody binding and neutralizing activity (Chen et al., 2010). In our model, the Q44 residue of m36 was in proximity to the basic R315 residue stemming from gp120 V3 loop. The high conformational flexibility of the V3 loop could potentiate interaction between the two and other residues.

In summary, reverting 7 of 12 mutations in the V segment of m36 back to germline sequences as in m36m3 did not result in a substantial loss of HIV-1 neutralizing activity. M36m3 could be less capable of eliciting immune responses than m36 as predicted by the immunogenicity prediction tools of the Immune Epitope Database (IEDB) (<http://tools.immuneepitope.org>) (data not shown) and may therefore be more suitable than m36 for further development as candidate therapeutics but only human clinical trials can definitely prove it. We also identified types of mutations and regions in m36 that are critical for HIV-1 neutralization. These include four mutations to acidic acid residues (T29D, S36D, A38E and H58D) distributed in the CDR1 and CDR2, two mutations to hydrophobic residues (N66I and K106I) in the FR3 and CDR3, and partial FR2 and FR3 sequences flanking the CDR2 (residues 47-55 and 66-76) that are derived from a gene family (HV4-34) different than HV3-23. These results and others helped in the selection of a docking model that appears to be highly consistent with the cryo-EM structure of the m36-gp120-CD4 complex in topology and could therefore provide a structural basis underlying the mechanism of potent and broad HIV-1 neutralization by the antibody.

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- Amino acid mutations in the HIV-1 broadly neutralizing antibody domain m36 were reverted back to germline sequences.
- Two types of mutations and one region were identified that are critical for antibody neutralizing activity.
- About a half of the mutations do not contribute to the antibody's ability to neutralize HIV-1.
- Biochemical and structural results guided docking of m36 onto gp120 that provides insights into mechanism of neutralization.
- Our results have implications for the further development of m36 as candidate therapeutics against HIV-1.

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Fig. 1.

Design and generation of germlinized m36 variants. Antibody amino acid sequences are numbered and FRs and CDRs are defined according to the IMGT numbering system (<http://www.imgt.org>). Mutations in FRs and CDRs are highlighted with gray shading. In m36 variants, reverse mutations are indicated with single-letter amino acid code while unmutated sequences are represented by dots. The DNA fragments of m36m1 (I66Y), m36m2, m36m3 and m36m4 were synthesized commercially, digested with SfiI and cloned into pComb3X. M36m1 and its variants were generated by using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) with m36m1 (I66Y) and m36m1, respectively, as a template according to the manufacturer's instructions. All m36 variants, which contain both the hexahistidine and FLAG tags at their C termini, were expressed in *Escherichia coli* HB2151 and purified from the soluble periplasmic fraction by using the Ni-NTA resin as described previously (Chen et al., 2008a).

Fig. 2.

Binding and neutralizing activity of m36 variants with back mutated FRs. (A) ELISA binding to gp120_{Bal}-CD4 and SPA. ELISA was performed as described previously (Chen et al., 2008a). Antibodies binding to gp120_{Bal}-CD4 coated on 96-well plates at a concentration of $2 \mu\text{g ml}^{-1}$ were detected by using the horseradish peroxidase (HRP)-conjugated mouse anti-FLAG tag antibody. HRP-conjugated SPA was directly used to detect binding to antibodies coated on 96-well plates at a concentration of $2 \mu\text{g ml}^{-1}$. EC₅₀s were calculated by fitting the data to the Langmuir adsorption isotherm. (B) Pseudovirus neutralization assay. Bal and JRFL are two R5-tropic HIV-1 primary isolates from clade B. Viruses pseudotyped with HIV-1 Envs were produced in 293T cells and the assay was performed in duplicate with HOS-CD4-CCR5 cells as target cells according to previously published protocols (Chen et al., 2008a).

Fig. 3.

Neutralizing activity of m36 variants with back mutated CDRs. Bal and JRFL are two R5-tropic HIV-1 primary isolates from clade B. Viruses pseudotyped with HIV-1 Envs were produced in 293T cells and the assay was performed in duplicate with HOS-CD4-CCR5 cells as target cells according to previously published protocols (Chen et al., 2008a).

Fig. 4.

Molecular docking of m36 onto a gp120-CD4 crystal structure. The crystal structures of gp120_{JRFL} and CD4 and the model of m36 are in magenta, blue and green, respectively. The bridging sheet and V3 loop of gp120 and the CDRs of m36 are designated. Side chains of the Q44 residue in m36 FR2 and the R315 residue in gp120 V3 loop are shown as overlapping spheres. Residues predicted to be important for antibody-antigen interaction are highlighted in the squares with their side chains represented by stick and sphere models. The black dashed lines in the upper right square indicate possible formation of salt bridges or hydrogen bonds between the residues. The Z-DOCK program implanted into the Accelrys Discovery studio (<http://accelrys.com>) was used to dock m36 onto the surface of gp120_{JRFL}-CD4. Before docking, the m36 and gp120_{JRFL}-CD4 structures were processed as the following: Water molecules were deleted and hydrogen atoms were added at pH7.4 and an ionic strength of 0.145, and in a dielectric environment of 10; energy was minimized based on CHARMM with a cutoff of 0.9; loop regions were rebuilt according to SEQRES data; energy was used to judge the efficacy of the geometry optimization; and cysteine bridges in these proteins were defined as blocked regions. Initially produced docked poses of m36 were filtered and re-ranked to obtain top 200 poses based on ZRank scores using electrostatic and desolvation energy and non-deterministic FFT optimization, the latter were then visually scrutinized.

Table 1

HIV-1 neutralizing activity of m36 variants

m36 variant	IC ₅₀ (nM) ^a	
	Bal	JRFL
Back mutation of FRs		
m36	22 ± 5.7	33 ± 7.1
m36m1	9.1 ± 7.0	71 ± 5.7
m36m1 (I66Y)	8.4 ± 3.7	19 ± 2.1
m36m2	112 ± 9.9	344 ± 44
Back mutation of single residues in CDRs		
m36m1	20 ± 0.71	81 ± 4.9
A27G	21 ± 0.71	76 ± 9.9
D29T	33 ± 0.71	102 ± 14
D36S	30 ± 3.5	77 ± 16
E38A	26 ± 2.8	143 ± 7.1
D58H	30 ± 6.4	80 ± 9.2
N64S	22 ± 1.4	90 ± 5.7
I106K	144 ± 7.1	874 ± 35
Back mutation of combined residues in CDRs		
m36m1	14 ± 1.4	65 ± 1.4
m36m3	18 ± 4.2	94 ± 16
m36m4	806 ± 351	8105 ± 1242

^aThe assay was performed in duplicate. Results are means ± standard deviations.