Paring Down HIV Env: Design and Crystal Structure of a Stabilized Inner Domain of HIV-1 gp120 Displaying a Major ADCC Target of the A32 Region

Highlights

- ID2 consists of the gp120 inner domain expressed independently of the outer domain
- ID2 displays the A32 ADCC epitope within minimal structural unit of the HIV-1 Env
- ID2 is stabilized in the CD4-bound conformation by an interlayer disulfide bond
- The A32 epitope maps to layers 1 and 2 of the C1-C2 region of gp120

Accession Numbers

5FCU
4YBL
4YC2

In Brief

Tolbert at al. describe a novel construct, ID2, consisting of inner domain of gp120 expressed independently of outer domain. ID2 expresses the A32 ADCC epitope within a minimal structural unit of the HIV-1 Env, thus it is a novel probe for the analysis and/or selective induction of A32-like antibody responses.

Correspondence

mpazgier@ihv.umaryland.edu

Authors

William D. Tolbert, Neelakshi Gohain, Maxime Veillette, ..., Andrés Finzi, George K. Lewis, Marzena Pazgier

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SUMMARY

Evidence supports a role of antibody-dependent cellular cytotoxicity (ADCC) toward transitional epitopes in the first and second constant (C1-C2) regions of gp120 (A32-like epitopes) in preventing HIV-1 infection and in vaccine-induced protection. Here, we describe the first successful attempt at isolating the inner domain (ID) of gp120 as an independent molecule that encapsulates the A32-like region within a minimal structural unit of the HIV-1 Env. Through structure-based design, we developed ID2, which consists of the ID expressed independently of the outer domain and stabilized in the CD4-bound conformation by an inter-layer disulfide bond. ID2 expresses C1-C2 epitopes in the context of CD4-triggered full-length gp120 but without any known neutralizing epitope present. Thus, ID2 represents a novel probe for the analysis and/or selective induction of antibody responses to the A32 epitope region. We also present the crystal structure of ID2 complexed with mAb A32, which defines its epitope.

INTRODUCTION

Numerous studies have indicated a relevant role for Fc receptor (FcR)-effector functions including antibody-dependent cellular cytotoxicity (ADCC) in protective immunity, as these responses have been shown to correlate with slower progression of HIV disease (Jia et al., 2013; Wren et al., 2013) or decreased virus replication. Studies of SIV or SHIV-infected non-human primates, HIV-1-infected humanized mouse models, and the RV144 clinical trial have linked ADCC with post-infection control of viremia and/or the blocking of HIV-1 acquisition, often in the absence of neutralization (Chung et al., 2014; Fouts et al., 2015; Haynes et al., 2012; Rerks-Ngarm et al., 2009; Yates et al., 2014). In particular, the immune correlates analyses of the RV144 trial associated high ADCC activity (together with low immunoglobulin A levels) and polyfunctional immunoglobulin G3 (IgG3) antibodies (Abs) with a reduced risk of infection, thus potentially contributing to vaccine efficacy (Chung et al., 2014; Tomaras et al., 2013; Yates et al., 2014).

Considerable evidence suggests that potent ADCC responses in humans are directed against A32-like epitopes of the Cluster A region (Guan et al., 2013) which map to the first and second constant (C1-C2) region of gp120 (reviewed in Lewis et al., 2014; Pollara et al., 2013; Veillette et al., 2014b). These epitopes are classified based on whether they are extant on target cells during viral entry, prior to productive infection of a target cell (entry targets) or exposed on an infected cell producing virions (release targets) (Lewis et al., 2014; Pollara et al., 2013). The A32-like entry targets become exposed during the initial steps of viral entry after Env trimers engage the host CD4 receptor and, possibly, the co-receptor. They persist on newly infected cell surfaces for extended periods of time (Finnegan et al., 2001, 2002; Mengistu et al., 2015) where they have the capacity to mediate Fc-effector functions in vitro (Guan et al., 2001, 2002; Mengistu et al., 2015) where they have the capacity to mediate Fc-effector functions in vitro (Guan et al., 2013; reviewed in Lewis et al., 2014; Pollara et al., 2013; Veillette et al., 2016). In the context of release targets these epitopes are expressed on infected/budding cells upon triggering of Env trimer with the cell surface CD4. Recent studies confirm that monoclonal Abs (mAbs) recognizing A32-like release targets exhibit considerable ADCC potency and are thus capable of eliminating infected cells, including cells budding HIV-1 (Ferrari et al., 2011; Veillette et al., 2014b; reviewed in Kramski et al., 2015; Lewis et al., 2014; Pollara et al., 2013; Veillette et al., 2016). The picture is even stronger for vaccine-induced immunity in humans, as the A32-like
epitopes have been linked to protective ADCC responses in the RV144 vaccine trial (Bonsignori et al., 2012; Haynes et al., 2012). Moreover, the A32-like Abs synergized with V2-specific Abs for infectious virus capture and ADCC of both tier-1 and -2 viruses, suggesting that crosstalk between these two specificities contributed to vaccine efficacy due to FcR-effector functions (Pollara et al., 2014).

While A32 region epitopes were shown to be highly immune-
genic during HIV-1 infection as they become exposed during transitional rearrangements of the Env trimer bound to CD4 on nascently or persistently infected cells, efforts to selectively construct these transitional and conformational targets as stable protein molecules have been difficult for three reasons. First, this region is buried inside the Env trimer where it is not accessible (or poorly accessible) for Ab recognition in the ligand-free closed state (Kwon et al., 2015; Ray et al., 2014; Sanders et al., 2013). Second, although the A32 region is exposed on monomeric gp120, its exposure is stabilized only upon binding soluble CD4. gp120 is highly dynamic, undergoing large conformational changes in solution (Gutman et al., 2012), and CD4 triggering is required for stable formation of these targets in monomeric gp120 (Fouts et al., 2000). Third, the Cluster A epitope region is localized exclusively within the inner domain (ID), which is known to be conformationally more mobile (Finzi et al., 2010; Gutman et al., 2012; Pancera et al., 2010) than other portions of the HIV-1 Env (such as the outer domain [OD], which has successfully been grafted and expressed independently of the ID) (Joyce et al., 2013; Yang et al., 2004). This makes efforts toward isolating this region into a minimal structural unit highly challenging.

Here, we describe the first successful attempt at isolating the ID of HIV-1 gp120 as an independent protein molecule that efficiently encapsulates conformational A32-like epitopes within a minimal structural unit of Env without the complication of other known epitope specificities. The ID design was guided by our atomic-level description of the A32 epitope region gained from the crystal structures of several A32-like Abs in complexes with CD4-triggered gp120 (Acharya et al., 2014; Gohain et al., 2015). Through two phases of structure-based design we developed ID2, which consists of the ID of the gp120 core stabilized in the CD4-bound conformation and expressed independently of the OD. ID2 stably expresses the transitional C1-C2 epitopes involved in potent FcR-effector responses to HIV-1 as indicated by physicochemical, antigenicity, and functional testing. The crystal structure of ID2 in complex with the A32 Fab confirms its proper folding and represents the first co-crystal structure of mAb A32, the canonical Ab of the ADCC Cluster A region, complexed with its cognate Env antigen.

RESULTS

Design and Purification of an Independent Inner Domain Molecule, ID1

Our previous structural analysis of complexes formed between A32-like mAbs and the gp120 antigen indicated that A32-like mAbs invariably recognize the epitope surface around the βT, βZ strands and the α0 and α1 helices of layers 1 and 2 of the ID of gp120 in its CD4-bound conformation (Acharya et al., 2014; Gohain et al., 2015). Residues of the variable loops and the OD are not involved in binding. Based on this information, we hypothesized that an independent ID molecule, with the V1V2 loop deleted and preferentially stabilized in the CD4-bound conformation, would be a suitable minimal structure for presenting A32-like epitopes with the same antigenic features as seen in gp120-CD4 complexes. Our initial design was based on an observation that the unliganded extended gp120 core (coreex) tends to spontaneously adopt the CD4-bound conformation (Dey et al., 2009; Kwon et al., 2012). We developed a stable ID construct of clade A/E isolate 93TH057, which is composed of a gp120 coreex sequence with the OD sequence (residues 258–472, HxBc2 numbering) replaced within ID layer 3 by a simple -GG- dipeptide linker (Figure 1). The first design of the independent ID molecule is referred to as ID1. A synthetic ID1 gene was fused to the signal peptide sequence, placed into the pCMV6-A-puro expression vector, expressed by transient transfection of 293T cells, and purified as described in Experimental Procedures and Figure S1.

mAb A32 is capable of binding unliganded gp120, but CD4 triggering significantly enhances its exposure and stability in the context of the single-chain protein (Coutu and Finzi, 2015; Guan et al., 2013). As a prelude to testing ID1, we tested binding of Cluster A mAbs including mAb A32, and the anti-Cluster A mAbs N5-i5, N60-i3, 2.2c, and JR4 (Guan et al., 2013) to monomeric full-length Bal gp120 (gp120bal) and to the single-chain gp120bal-sCD4 complex (full-length single-chain [FLSC]) (Fouts et al., 2000) by surface plasmon resonance (Table 1). All mAbs tested showed increased affinities for the gp120-CD4 complex as shown by an 8- to 32-fold lower affinity constant (K0) for FLSC compared with unliganded gp120. In all cases, the increased affinity for the gp120bal-sCD4 complex resulted from the faster association rates and reduced dissociation rates, confirming that the epitopes in the C1-C2 region are indeed inducible and stabilized in the context of gp120 triggered by CD4. Next we tested ID1 with the same Ab set to test how the C1-C2 region epitopes are preserved within the ID1 construct. As shown in Table 1, ID1 bound effectively to each of five mAbs tested, with a K0 value in a range of 1.1–9.3 nM, thus with affinities comparable with or slightly improved over that of gp120 (K0 value in a range of 2.0–12.0 nM). This indicates that ID1 preserves the antigenic properties of gp120 and is folded to display A32-like epitopes. To our knowledge, ID1 represents a first stable construct of the gp120 ID expressed independently of the OD.

Structural Characterization of ID1 in Complex with Anti-Cluster A mAb JR4

To assess how ID1 is folded, we determined the crystal structure of ID1 in complex with the Fab of JR4, a C1-C2-specific mAb recognizing the A32-C11 mixed epitope within the Cluster A region (Gohain et al., 2015). The structure of Fab JR4-ID1 complex was solved at 1.85 Å with one copy of complex present in the asymmetric unit of the crystal. The calculated electron density maps showed clearly defined density for the whole JR4 Fab and for 104 of 169 residues of ID1 (Table 2).

The overall structure of JR4 Fab-ID1 complex is shown in Figure 2. ID1 preserves well the overall fold of the ID as in the gp120 coreex. The seven-stranded β sandwich is fully folded, the βZ and β4 strands, and three (C54-C74, C218-C237, C228-C248) out of four disulfide bonds are defined and formed as in the gp120
coree. On the other hand, large portions (38.5%) of the ID1 molecule are disordered in the complex and not resolved in the structure. These include sequences forming the $\alpha_0$ helix in the gp120 core (residues A58KAHETEVHNVWA70), the first and second -GG- linker (I109SLWDQSLQPCVKLT-GG-SVIKQACPISFD211 and P253VVSS-GG-GNIKDNW479, including the fourth $C_{119-C_{205}}$ disulfide of the ID, respectively) and the C-terminal residues (E492PLGI496) of ID1 (Figures 1B and 2A). The JR4 Fab anchors ID1 mainly through its heavy chain (85.0% of the Fab buried surface area [BSA] of the complex) and by contacting the residues of layer 1 (75.2% of the ID1 BSA, Table S1) (Figure 2A).

To determine whether the JR4 mixed A32-C11 epitope is preserved within the ID1 molecule, we compared the JR4 Fab-ID1 complex with the previously determined crystal structure of the ternary complex formed by the JR4 Fab, the gp120 core and the CD4 mimetic M48 (Gohain et al., 2015) (Figures 2B and 2C). As shown in Figure 2B, large portions of layers 1 and 2 of the ID, forming the structural framework for the JR4 epitope within the CD4 mimic-triggered gp120 core, are disordered in the JR4 Fab-ID1 complex. These include the parts of the $\beta_2$-$\alpha_0$-connecting coil (residues A58KAH) and $\alpha_0$ helix (residues E64VHNVWA) of layer 1 and $\alpha_1$ helix (residues N98NMVEQMQEDV) of layer 1. In addition, the $\beta_1$ strand (residues A74CV) of layer 1, which in the gp120 core is adjacent to the $\alpha_0$ helix, is not formed in the ID1 molecule. Overall, the interactive surface that becomes buried due to the JR4 Fab-ID1 interaction encompasses a total of 1,930 Å$^2$ (953 Å$^2$ contributed by ID1 and 977 Å$^2$ by Fab); this is 410 Å$^2$ smaller than the interactive surface buried at JR4 Fab-gp120 complex interface (BSA of 2,340 Å$^2$ with 1,195 Å$^2$ buried by gp120 core and 1,145 Å$^2$ by Fab). Several layer-1 contacts are missing in the JR4-ID1 complex compared with JR4-gp120 complex, specifically the JR4 heavy chain contacts to layer 1 (Figures 2C and S2). The missing contacts can be attributed to disorder in the ID1 structure. The similarity in the A32-like Ab affinities for ID1 and gp120 suggest that the disordered residues in ID1, specifically the $\alpha_0$ helix and parts of the $\alpha_1$ helix, might mirror disorder in full-length gp120 in the absence of CD4.

**Structure-Based Design of ID2 and Evaluation of Its Antigenic Properties**

Structural analysis of the Fab JR4-ID1 complex revealed that the conformational CD4i epitopes of the A32-like region are not fully...
formed within ID1, with regions around the V1V2 stem and \( \alpha_0 \) helix flexible and not contributing to anti-Cluster A mAb binding. We hypothesized that adding a disulfide bond at the bases of \( \alpha_0 \) and \( \alpha_1 \) helices could restore and preserve CD4i epitopes in this region (Figure 1). Accordingly, we mutated V\(_{65} \) and S\(_{115} \) to cysteines to form a single disulfide bond at the \( \alpha_0- \) and \( \alpha_1 \)-helix junction. Similar mutations have been shown to increase CD4i epitope exposure in the context of full-length gp120 (Kassa et al., 2013). This revised second independent ID molecule, with the V1V2 stem removed and stabilized by a C\(_{65}-\)C\(_{115} \) disulfide bond, was designated ID2.

We tested the affinity of ID2 for a panel of anti-Cluster A mAbs using surface plasmon resonance (Figure 3 and Table S2). ID2 bound the panel of mAbs tested with an average of 17-fold tighter affinities than ID1, and similar or higher than those of FLSC (Figure 3A). The increase in affinity was largely attributable to an increase in \( k_a \) for the complex and to a lesser extent a decrease in \( k_d \), suggesting that ID2 more closely resembles the CD4-bound conformation of gp120 recognized by these mAbs. To further determine that ID2 is folded to stably present the A32-like epitopes in solution, we tested the binding of mAb A32 to ID2 by isothermal titration calorimetry (ITC) and compared it with the mAb A32 interaction with FLSC (Figure 3B). These data clearly indicate that ID2 adopts a CD4-bound conformation in solution that closely resembles the presentation of the A32-epitope in CD4-triggered gp120. The binding kinetics of mAb A32 to ID2 and FLSC are very similar, with \( K_d \) values of 9.0 and 11.5 nM and \( \Delta H \) of \(-2.553 \) (±0.18) and \(-3.15 \) (±0.69) kcal/mol for the mAb A32-ID2 and mAb A32-FLSC interactions, respectively. Finally, we also tested whether ID2 is recognized by A32-like mAbs isolated from RV144 vaccinees (Bonsignori et al., 2012) and mouse mAbs specific for C1-linear epitopes poorly exposed within the properly folded gp120 preparations (Abaciglu et al., 1994; Moore et al., 1994b) (Table S3). Whereas RV144 mAbs bound efficiently to ID2 with affinities comparable with anti-Cluster A mAb JR4, the peptide specific mAbs B18 and C4 failed to recognize ID2. Altogether, these data confirm that ID2 stably presents the desired conformational epitopes within the C1-C2 gp120 region and shows the antigenicity profile of the CD4-triggered gp120.

### Structural Characterization of ID2 in Complex with mAb A32

To confirm whether ID2 preserves the Cluster A epitopes of CD4-triggered gp120 better than ID1, we determined its crystal structure in complex with mAb A32 Fab (Experimental Procedures; Table 2; Figure 4 and S3). The structural characterization of the ID2 complex fully validated its design. As shown in Figure 4A, ID2 fully preserves the fold of the ID of the gp120 core\(_e \) in its CD4-bound conformation with the seven-stranded \( \beta \) sandwich and all the secondary structural elements of layers 1 and 2 (including \( \alpha_0 \) and \( \alpha_1 \) helices) present and arranged to form the C1-C2 epitope. The CD4-bound conformation of ID2 is defined in the complex (85% of the molecule, respectively. Finally, we also tested whether ID2 is recognized by A32-like mAbs isolated from RV144 vaccinees (Bonsignori et al., 2012) and mouse mAbs specific for C1-linear epitopes poorly exposed within the properly folded gp120 preparations (Abaciglu et al., 1994; Moore et al., 1994b) (Table S3). Whereas RV144 mAbs bound efficiently to ID2 with affinities comparable with anti-Cluster A mAb JR4, the peptide specific mAbs B18 and C4 failed to recognize ID2. Altogether, these data confirm that ID2 stably presents the desired conformational epitopes within the C1-C2 gp120 region and shows the antigenicity profile of the CD4-triggered gp120.

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**Table 1. Binding Kinetics of mAb A32, N5-i5, N60-i3, JR4, and 2.2c to the FLSC, gp120, and ID1 as Measured by Surface Plasmon Resonance**

| mAb    | gp120\(_{basal} \) | FLSC | Fold Difference | ID1 | Fold Difference\(^{a}\) | Fold Difference\(^{b}\) |
|--------|---------------------|------|----------------|-----|------------------------|-----------------------|
| mAb A32 |                     |      |                |     |                        |                       |
| \( K_d \) (M) \( \times 10^{-9} \) | 3.2 ± 0.07 | 0.23 ± 0.01 | 13.9 | 9.30 ± 1.10 | 2.9 | 40.4 |
| \( k_a \) (1/M s) \( \times 10^{5} \) | 1.39 ± 0.02 | 4.43 ± 0.01 | 3.2 | 0.85 ± 0.04 | 1.6 | 5.2 |
| \( k_d \) (s) \( \times 10^{-5} \) | 43.7 ± 0.99 | 10.2 ± 0.09 | 4.3 | 78.50 ± 5.20 | 1.8 | 7.7 |
| mAb N5-i5 |                     |      |                |     |                        |                       |
| \( K_d \) (M) \( \times 10^{-9} \) | 5.03 ± 0.03 | 0.29 ± 0.14 | 17.3 | 1.10 ± 0.16 | 4.6 | 3.8 |
| \( k_a \) (1/M s) \( \times 10^{5} \) | 0.72 ± 0.01 | 8.25 ± 2.50 | 11.5 | 4.50 ± 1.00 | 6.3 | 1.8 |
| \( k_d \) (s) \( \times 10^{-5} \) | 36.0 ± 0.28 | 23.8 ± 13.80 | 1.5 | 48.30 ± 4.30 | 1.3 | 2.0 |
| mAb N60-i3 |                     |      |                |     |                        |                       |
| \( K_d \) (M) \( \times 10^{-9} \) | 2.03 ± 0.07 | 0.25 ± 0.01 | 8.0 | 3.50 ± 0.13 | 1.7 | 13.8 |
| \( k_a \) (1/M s) \( \times 10^{5} \) | 2.53 ± 0.06 | 12.4 ± 0.00 | 4.9 | 4.30 ± 0.25 | 1.7 | 2.9 |
| \( k_d \) (s) \( \times 10^{-5} \) | 51.2 ± 0.50 | 31.4 ± 0.71 | 1.6 | 148 ± 2.70 | 2.9 | 4.7 |
| mAb JR4 |                     |      |                |     |                        |                       |
| \( K_d \) (M) \( \times 10^{-9} \) | 2.34 ± 0.16 | 0.18 ± 0.01 | 13.0 | 6.00 ± 0.33 | 2.6 | 33.3 |
| \( k_a \) (1/M s) \( \times 10^{5} \) | 2.62 ± 0.09 | 6.43 ± 0.06 | 2.5 | 1.60 ± 0.10 | 1.6 | 4 |
| \( k_d \) (s) \( \times 10^{-5} \) | 61.1 ± 1.80 | 11.6 ± 0.28 | 5.3 | 94.40 ± 0.60 | 1.5 | 8.1 |
| mAb 2.2c |                     |      |                |     |                        |                       |
| \( K_d \) (M) \( \times 10^{-9} \) | 12.00 ± 0.07 | 0.50 ± 0.06 | 24.0 | 3.00 ± 0.30 | 4 | 6.0 |
| \( k_a \) (1/M s) \( \times 10^{5} \) | 0.73 ± 0.01 | 11.5 ± 1.40 | 15.7 | 2.70 ± 0.16 | 3.7 | 4.3 |
| \( k_d \) (s) \( \times 10^{-5} \) | 87.5 ± 1.10 | 55.6 ± 14.40 | 1.6 | 80.30 ± 2.40 | 1.1 | 1.4 |

\(^{a}\)Fold difference relative to gp120.

\(^{b}\)Fold difference relative to FLSC.
Overall

B

Ligand/ion 32 0 0
Water 376 0 4

No. of atoms

R

I

Refinement Statistics

Redundancy 5.8 (5.7) 8.3 (8.3) 5.8 (5.8)
Completeness (%) 94.9 (91.4) 93.2 (96.8) 87.1 (86.7)
Outliers (%) 0.5 3.1 3.6
Allowed (%) 8.9 9.4 9.4
Favored (%) 8.9 87.5 87.0

Complexes (a.u.) 1 2 2
Resolution (Å) 50–1.85 (1.88–1.85) 50–3.10 (3.15–3.10) 50.0–3.00 (3.05–3.00)

No. of reflections

Total 299,100 165,660 119,799
Unique 51,569 19,959 20,655

Rmerge (%) 10.7 (100) 18.6 (95.5) 16.3 (88.0)

I/σRmerge (%) 15.0 (1.1) 14.6 (1.3) 9.5 (1.2)

Completeness (%) 94.9 (91.4) 93.2 (96.8) 87.1 (86.7)

Redundancy 5.8 (5.7) 8.9 (8.3) 5.8 (5.8)

Refinement Statistics

Resolution (Å) 50–1.85 50–3.10 50.0–3.00
R1 (%) 18.6 23.2 22.7
Rfree (%) 22.3 28.9 28.8

No. of atoms

Protein 4,034 8,470 8,600
Water 376 0 4
Ligand/ion 32 0 0

Overall B value (Å²)

Protein 44.7 95.8 90.2
Water 42.4 – 61.7
Ligand/ion 47.5 – –

RMSD

Bond lengths (Å) 0.018 0.010 0.008
Bond angles (°) 1.79 1.53 1.45

Ramachandran°

Favored (%) 8.9 87.5 87.0
Allowed (%) 8.9 9.4 9.4
Outliers (%) 0.5 3.1 3.6

PDB 5FCU 4YBL 4YC2

117–118, 206–209, 254–256, and 472–474). Each of the four disulfide bonds (including the newly introduced C65-C115 disulfide bond) are defined and maintained as predicted. The structural alignment of ID2 from the Fab A32-ID2 complex with the gp120 coreα (from Fab N5-i5-gp120N60-i3 HEK Cells; Acharya et al., 2014) and with ID1 resulted in a root-mean-square deviation (RMSD) between main-chain atoms of 0.76 Å (523 atoms) and 1.36 Å² (416 atoms), respectively, indicating a closer similarity of the overall structure of ID2 to the ID in the CD4-triggered gp120 coreα bound to A32-like mAbs, than to ID1.

The A32 Fab-ID2 complex also represents the first co-crystall structure of mAb A32 with its cognate gp120 antigen. mAb A32, similar to A32-like mAbs N5-i5, JR4, and N60-i3 described previously in complexes with gp120 coreα, binds exclusively within the C1-C2 regions of gp120 (Figure 4B). Its epitope is formed by bridging mobile layers 1 and 2 with anchoring points centered on the β2 strand, the α0 helix, and the β1 strand, and the α1 helix (residues 51–54, 60–61, 69, and 71–78 [layer 1] and 103, 106–107, 110, 113–114, 217, and 220–221 [layer 2]) (Figures 4B and 4C). The interactive surface that becomes buried at the Fab A32-ID2 interface is 1,701 Å² (856 Å² contributed by Fab and 845 Å² by ID2, Table S1). Most of the Fab contacts are contributed by its heavy chain, with complementarity-determining region (CDR) H3 making contacts to the β2 strand of gp120 (residues T52–L55) and CDR H1–3 contacting the residues of the α0 helix and the β1 strand (e.g., W62THACVP motif). In contrast, contacts to the α1 helix of layer 2 are almost exclusively contributed by two arginines of CDR H3 (R97 and R100), with some minor contributions from the CDR L1, CDR H1, and the FWR1 (Figures 4B and 4C). The A32 paratope is electropositive largely due to three sequence identity in their heavy- and light-chain variable (V) domains, respectively (70.1% identity for the CDRs alone) and recognize gp120 with striking similarities (Figure 5). They approach the antigen through the same angle and utilize the same CDR contacts to interact with their cognate epitopes. The close structural similarity of the Fab-antigen interfaces is reflected in a relatively low RMSD value of 1.80 Å² calculated for the C1-C2 regions. The binding analysis indicates that anti-Cluster A Abs bind to ID2 with affinities comparable with that of the gp120-CD4 complex; thus the desired epitopes are preserved within the ID2 design (Figure 3). Additionally, to test whether ID2 engrafts epitopes involved in Fc-effector functions directed to the C1-C2 region of HIV-1 Env, we performed a competition assay ID2 Fully Preserves Functional Epitopes of the C1-C2 Region of gp120

ID2 represents the first stable design of the gp120 ID expressed independently of the OD and stabilized in a CD4-bound confirmation to exclusively express the non-neutralizing, ADCC epitopes of the C1-C2 region. The binding analysis indicates that anti-Cluster A mAbs bind to ID2 with affinities comparable with that of the gp120-CD4 complex; thus the desired epitopes are preserved within the ID2 design (Figure 3). Additionally, to test whether ID2 engrafts epitopes involved in Fc-effector functions directed to the C1-C2 region of HIV-1 Env, we performed a competition assay.
in which effector functions of anti-Cluster A mAbs were tested at their effective concentrations in the presence of increasing concentrations of ID2. The assay was performed using two complementary methods: the rapid fluorimetric (RF)-ADCC assay (Gomez-Roman et al., 2006), which measures trogocytosis (e.g., Fc-effector function mediated by CD14+ monocytes) rather than ADCC (Kramski et al., 2012), and the flow cytometry (fluorescence-activated cell sorting [FACS])-based ADCC assay, which allows the quantification of cell killing by ADCC (e.g., mediated by natural killer [NK] cells) (Richard et al., 2014). As shown in Figure 6A, ID2 completely inhibited all of the A32-like mAbs tested in the (RF)-ADCC assay and as well as the mixed A32-C11 mAb JR4 at a concentration range of 0.1–10 μg/ml. The Fc-effector activity of mAb C11, which recognizes an epitope involving residues of N and C termini and seven-stranded β sandwich of gp120 (Gohain et al., 2015) not present within the ID design, was minimally affected. The same pattern of inhibition of mAb A32 and C11 was observed in the FACS-based ADCC assay (Figure S4). Together, these data confirm that ID2 is folded to stably display functional A32-like and mixed A32-C11-like epitopes within the ADCC Cluster A region.

Next, we tested how efficiently ID2 can adsorb Abs present in HIV+ sera and compete with their ADCC activities. It was shown previously that infected individuals frequently elicit Abs specific for A32-like epitopes and that the predominant fraction of the ADCC response in sera from HIV-1-infected individuals is directed at epitopes exposed on the CD4-triggered gp120 core including the A32 region (e.g., according to Ferrari et al., [2011], pre-incubation of target cells with Fabs of A32 and 17b blocked about 50% of the ADCC activity in 7 of 14 HIV-1-infected individuals). As shown in Figure 6B, we pre-incubated HIV+ sera (20 samples of sera derived from HIV-1-infected individuals including both progressors and long-term non-progressors as characterized in Table S4) with either a D368R gp120 dV1V2V3V5 mutant or ID2 protein before being assayed for their ability to bind to CEM.NKr cells infected with a Nef/C0Vpu/C0 virus (Figure 6B, left) and to mediate ADCC (Figure 6B, right). We used viruses lacking Nef and Vpu (i.e., presenting high levels of CD4 and Env at the cell surface) to ensure effective exposure of A32-like targets on the infected cell surface as described by Ferrari et al. (2011) and Veillette et al. (2015). The D368R gp120 dV1V2V3V5 mutant or ID2 protein before being assayed for their ability to bind to CEM.NKr cells infected with a Nef/Vpu virus (Figure 6B, left) and to mediate ADCC (Figure 6B, right). We used viruses lacking Nef and Vpu (i.e., presenting high levels of CD4 and Env at the cell surface) to ensure effective exposure of A32-like targets on the infected cell surface as described by Ferrari et al. (2011) and Veillette et al. (2015). The D368R gp120 dV1V2V3V5 mutant, bearing CD4i epitopes of gp120 core, was previously shown to almost completely abrogate cell-surface staining and ADCC of Nef/Vpu-infected cells (Ding et al., 2015; Veillette et al., 2015). ID2 specifically bound Abs present in HIV+ sera and adsorbed approximately 30% of total
ADCC activity. The levels of observed ADCC competition are with agreement with previous observations indicating that A32 epitopes constitute a large fraction but not the only fraction of ADCC targets in sera of infected individuals (Ferrari et al., 2011; Veillette et al., 2015). Altogether these data indicate that ID2 stably engrafts the epitopes recognized by A32-like Abs involved in ADCC from the sera of HIV-1-infected individuals.

DISCUSSION

The A32-like epitopes of the Cluster A region map exclusively within the inner domain of the gp120 (Acharya et al., 2014; Gohain et al., 2015), on the gp120 face that interacts with the gp41 ectodomain in the trimeric structure of the HIV-1 Env (Pancera et al., 2014; Yang et al., 2003; York and Nunberg, 2004). On the native trimer of most HIV-1 isolates, this region is buried at the trimer interface and is inaccessible for Ab recognition until the interaction with the target cell receptor and, possibly, the co-receptor (reviewed in Lewis et al., 2015; Veillette et al., 2016). Our recent studies indicate that substantial structural rearrangements of the ID are required to convert the conformation recognized by A32-like Abs from the Ab-inaccessible native, untriggered trimeric Env (Acharya et al., 2014). Moreover, accumulating evidence suggests that exposure of these epitopes on the virion spike strictly depends on binding cell surface anchored CD4, since triggering with soluble CD4 does not lead to exposure of these epitope targets on virion-associated HIV-1 trimers (Acharya et al., 2014; Mengistu et al., 2015; Ray et al., 2014).

In this report we describe a new molecule, ID2, which consists of the ID of HIV-1 gp120 expressed independently of the OD and stabilized to effectively express the conformational C1-C2 region epitopes. To our knowledge, we are the first to successfully isolate the ID of gp120 as an independent and fully stable molecule. In the past, the isolation of this gp120 region into a minimal structural unit was thought to be highly challenging or impossible, mostly due to the fact that the ID is highly mobile and undergoes pronounced conformational rearrangements during the structural transitions of the HIV-1 Env trimer in the viral entry process (Pancera et al., 2014; Yang et al., 2003). A stable ID construct of HIV-1 gp120 was developed, expressed in both mammalian and bacterial systems, and characterized by structural and functional means. Through two phases of rational structure-based design, we obtained the ID2 molecule, which displays the A32-like epitopes within a minimal structural unit of gp120 consisting of 167 residues and constrained by the engineered C66–C115 bond to adopt a CD4-bound state. ID2 stably expresses the C1-C2 epitopes as indicated by significantly increased or comparable affinities for anti-Cluster A mAbs compared with unliganded gp120 or gp120-CD4 complexes, respectively. Structural analysis of ID complexes with A32 and JR4 mAbs fully validated the ID design. ID1, the first ID design, consisting of ID as in gp120 core (Dey et al., 2009; Kwon et al., 2012), failed to adopt the CD4-bound conformation required for formation of CD4i epitopes of A32-like region. ID1 displayed antigenic properties of full-length untriggered gp120 and the epitope of anti-Cluster A mAb JR4 was only partially preserved within its design, as indicated by large portions of the JR4 binding surface being disordered in the Fab JR4-ID1 complex. Regions of the ID1 molecule found to be disordered in the Fab JR4-ID1 complex were previously shown to undergo a significant conformational change upon CD4 binding, evident in the comparisons of CD4-bound gp120 core structures (Acharya et al., 2014; Kwon et al., 2012) with the recent gp140 SOSIP trimers (Julien et al., 2013; Lyumkis et al., 2013; Pancera et al., 2014). We were able to eliminate the ID1 disorder and fully induce the CD4i A32-like region in the ID2 design that incorporates truncations of the V1V2 stem region and a new C65-C115 disulfide bond. The C65–C115 bond links the z0 (layer 1) and z1 (layer 2) helices and thus stabilizes the ID in the CD4-bound conformation. We show here the ID2 construct, which is based on the clade A/E 93TH057 virus sequence, but we were also able to obtain stable ID2 preparations using sequences of other clades including clade B strain YU2. Both of these preparations preserve antigenic properties of ID2 of clade A/E 93TH057 (Table S5), confirming that the ID2 design is a durable structural
template and can be used to generate other independent ID molecules based on sequences of any HIV-1 strain.

ID2 abrogates entirely Fc-mediated effector function of A32 and A32-like mAbs and specifically adsorbs ADCC activities against target CEM.NKr T cells infected with Nef/C0 Vpu/C0 NL4.3 virus from the sera of HIV-1-infected individuals. It was shown previously that CD4i Env epitopes, including targets within the A32 region, are preferentially recognized by Abs capable of potent ADCC present in sera (Ferrari et al., 2011; Richard et al., 2014; Veillette et al., 2014a, 2014b, 2015) or cervicovaginal lavages (Batraville et al., 2014) of HIV-1-infected individuals. ID2 is therefore folded to stably display the functional epitopes of the C1-C2 region. It is designed to engraft non-neutralizing A32-like epitopes selectively without any other known epitopes, especially those involved in neutralizing Ab response. By design ID2 consists of two faces: a face harboring the C1-C2 epitopes and a face exposed by the removal of the OD that does not harbor any known epitope targets (Figure 1).

As a minimal structural unit of gp120 effectively expressing the A32-like region, ID2 has significant translational value because it can be employed as a specific probe for the analysis of Ab responses to these non-neutralizing epitope targets (e.g., in immune correlates analysis of future vaccine trials or epitope mapping). It also constitutes a novel immunogen candidate,

Figure 4. Crystal Structure of A32 Fab-ID2 Complex
(A) A32-ID2 complex (left) and its 180° view about a vertical axis are shown in ribbon diagram with molecular surface displayed over the residues of the Fab involved in ID2 binding (right). See also Figure S3 and Table S1.
(B) Details of the A32 Fab-ID2 interface. A32 Fab contacts on ID2 and ID2 contacts on the A32 Fab are shown as balls displayed over the ID2/A32 Fab ribbon diagram and highlighted in black over the ID2/A32 Fab electrostatic potential surface. The A32 Fab contacts are shown in dark purple, light purple, and purple for heavy, light, and both chain(s) contacts, respectively. The ID2 contacts through residues of layer 1, layer 2, and both are shown in yellow, cyan, and gray, respectively.
(C) A32 Fab and ID2 contact residues on the primary sequence of ID2 and A32 Fab, respectively. Residues contributing to the A32 Fab-ID2 interface as defined by PISA (Krissinel and Henrick, 2007) are highlighted, and contacts as defined by a 5-Å cutoff are marked above the sequence. Side-chain (+) and main-chain (−) contacts are colored based on contact type; hydrophobic in green, hydrophilic in blue, or both in black.
selectively and stably presenting the ADCC A32-like epitopes that could be used in challenge studies to address the exclusive role of non-neutralizing Abs in vaccine protection. We previously showed that many of the residues forming the Cluster A epitope are highly conserved among HIV isolates (Acharya et al., 2014; Gohain et al., 2015; Guan et al., 2013), indicating that ADCC responses specific for this region will be cross-reactive and undergo limited immune escape. As such, they represent an important vaccine target. Anti-Cluster A mAbs also share similar characteristics in their V domains, with moderate length CDR H3 loops and a low degree of V_{H} chain maturation (Acharya et al., 2014; Gohain et al., 2015; Guan et al., 2013). Thus, ID2 can potentially be used to elicit anti-Cluster A Abs in vivo without intensive immunization regimens and without immunogens designed and optimized to recognize an affinity-matured Ab variant. Studies are under way to evaluate ID2 as an immunogen capable of selective induction of the A32-like responses in animal hosts.

Finally, our studies are the first to define the structure of the epitope of mAb A32, the canonical mAb of the Cluster A region. The previous reports were based solely on binding studies with Env mutants associated the A32 epitope primarily with the C1 region of gp120 (Finzi et al., 2010; Moore et al., 1994a; Pancera et al., 2010). Our Fab A32-ID2 complex represents the first co-crystal structure of mAb A32 with its gp120 antigen and permits the precise description of the gp120-binding footprint of mAb A32. Accordingly, mAb A32 recognizes a conformational epitope formed by the β^{2} strand, the α_{0} helix and β^{1} strand, and the α_{1} helix of the gp120 ID. mAb A32 binds to gp120, almost entirely overlapping the footprint and mimicking the binding mode of the mAb N60-i3, a human Ab isolated from an HIV-1-infected individual of our natural virus suppressor cohort (Gohain et al., 2015). Thus, it recognizes the same discontinuous site within the C1-C2 region as other anti-Cluster A mAbs described previously to be capable of potent FcR-effector function against gp120-coated and virus-bound target cells (Acharya et al., 2014). The A32 epitope, like epitopes of other anti-Cluster A mAbs (Acharya et al., 2014; Gohain et al., 2015), is buried inside the HIV-1 Env trimer where it is not accessible (or poorly accessible) for Ab recognition in the ligand-free closed state (Mengistu et al., 2015; Ray et al., 2014; Sanders et al., 2002, 2013). Thus, our work provides a structural understanding of why mAb A32 does not efficiently recognize and inactivate (through an ADCC mechanism) target cells infected with wild-type HIV-1 viruses.
The HIV-1 accessory viral protein U (Vpu) and negative regulatory factor (Nef) protein, two well-established regulators of cell-surface CD4 expression, reduce the levels of CD4 on the surface of the infected target (reviewed in Veillette et al., 2016). In the absence of CD4, the HIV-1 Env trimers remain in the ligand-free closed state, inhibiting the effective recognition of infected cells by mAb A32 (Richard et al., 2015; Veillette et al., 2014b, 2015). Utilization of the full potential of mAb A32 and other Abs targeting A32 region epitopes to inactivate infected cells through an FcR mechanism in vivo will be possible only if effective strategies capable of diminishing the CD4-downregulation effect are developed and implemented.

**EXPERIMENTAL PROCEDURES**

**Inner Domain Design**

ID1 consists of residues 44–123 (hBc2 numbering) and 199–256 of gp120 connected by a glycine-glycine linker that is, in turn, connected to the C terminus of gp120 (residues 472–496) by another glycine-glycine linker to remove the OD. ID2 was made based on the ID1 sequence in which the V1V2 region (residues 118–206) was replaced by a two-glycine alanine linker. ID2 was further stabilized by adding an extra disulfide by mutating V65S and S115T to cysteines. Initial clones were synthesized with optimized codons for mammalian expression using the native gp120 leader sequence. Mutations were added with the Quikchange PCR mutagenesis kit (Stratagene) as per the manufacturer’s protocol.

**Protein Expression and Purification**

IDs were expressed by transfection using the FreeStyle 293T and Gnt1: 293T Mammalian Expression System (Invitrogen) for functional and structural analysis, respectively, and according to the protocol provided by the manufacturer. IDs were purified using an N5-i5 IgG affinity column. N5-i5 IgG was chemically crosslinked to protein A resin using the Pierce Protein A IgG Plus orientation kit (Thermo Fisher Scientific). Protein bound to the N5-n5 IgG affinity column was eluted with 0.1 M glycine (pH 3.0) and immediately diluted 10:1 with 1 M Tris-HCl (pH 8.5).

ID2 for co-crystallization studies was grown in the Origami (DE) Escherichia coli strain (Novagen). ID2 sequence was cloned into the pMal-c5e expression vector (New England Biolabs) with an N-terminal maltose-binding protein (MBP)-thioredoxin tag followed by a six-histidine tag and a thrombin cleavage site. MBP-thioredoxin-ID2 was eluted with 25 mM Tris-HCl (pH 8.0) and purified further using an N5-i5 IgG affinity column as described above.

**Crystallization and Data Collection**

For structural studies the ID proteins were deglycosylated with 10 units/mg Endo Hf (NE Biolabs) for 30 min) was loaded onto a Hitrap nickel column (GE Healthcare). ID protein at a concentration of 10 mg/ml of serum, respectively, before being assayed for their ability to bind to infected cells and mediate their ADCC-dependent elimination as previously described (Richard et al., 2014; Veillette et al., 2014b, 2015).

**Surface Plasmon Resonance Analysis**

The binding affinity and kinetics of ID constructs to mAb A32, N5-i5, N60-i3, JR4, and 2.2c were assessed by surface plasmon resonance on a Biacore T-100 (GE Healthcare) at 25°C. The kinetic constants were determined using a 1:1 Langmuir model of binding with BIAevaluation software (GE Healthcare).

**ITC Analysis**

ITC was used to characterize the interaction of FLSC and ID2 with A32 IgG using an ITC200 instrument (GE Healthcare). Titrations were performed at 25°C. Heats of dilutions were measured and subtracted from each dataset. All data were analyzed using Origin 7.0 software.

**Competition of Fc-mediated Effector Function of Anti-Cluster A Abs with ID2**

Measurements were performed using the RF-ADCC assay (Gomez-Roman et al., 2006) and FACS-based assay (Richard et al., 2014). In the RF-ADCC assay, EGFP-CM-NK-CCR5-SNAP target cells were stained with SNAP-Surface Alexa Fluor 647 (NE Biosciences) with or without HIV-1 tatal gp120 (50 μg/ml). Abs tested (at fixed concentrations corresponding to their IC50 value) were mixed with ID2 (serially diluted 1:3, from 10 μg/ml to 0.5 ng/ml) and incubated with gp120-sensitized targets for 15 min at room temperature.

**Serum Adsorption and ADCC Competition Assays**

Written informed consent was obtained from all study participants (the Montreal Primary HIV Infection Cohort), and research adhered to the ethical guidelines of CRCHUM and was reviewed and approved by the CRCHUM institutional review board (ethics committee). Cells and viruses (NL4-3, ADA.GFP Nef Vpu) were used as previously described to infect CEM.NK cells (Richard et al., 2014; Veillette et al., 2014b, 2015). Sera derived from HIV-1-infected individuals, both progressors and long-term non-progressors as described and characterized in Table S4, were pre-incubated for 30 min at room temperature with either purified soluble D388R dV1V2V3V5 gp120 or ID protein at a concentration of 10 μg or 2.66 μg protein/ml of serum, respectively, before being assayed for their ability to bind to infected cells and mediate their ADCC-dependent elimination as previously described (Richard et al., 2014; Veillette et al., 2014b, 2015).
ACCESS NUMBERS
The accession numbers for the crystal structures reported in this paper are: PDB: 5FCU for Fab JR4-ID1 complex, PDB: 4YBL for Fab A32-ID2 complex, and PDB: 4YC2 for Fab A32-ID2 complex.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.str.2016.03.005.

AUTHOR CONTRIBUTIONS
W.D.T., N.G., G.L., and M.P. designed, performed research, and analyzed the data; M.V., J.-P.C., and A.F. performed serum adsorption and ADCC competition assays; C.O., M.L.V., M.E., A.L.D., and T.R.F. performed experiments related to IgD functional characterization; W.D.T., N.G., and M.P. wrote the paper with all authors providing comments or revisions.

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