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Fusion proteins of HIV-1 envelope glycoprotein gp120 with CD4-induced antibodies showed enhanced binding to CD4 and CD4 binding site antibodies

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ABSTRACT

Development of successful AIDS vaccine immunogens continues to be a major challenge. One of the mechanisms by which HIV-1 evades antibody-mediated neutralizing responses is the remarkable conformational flexibility of its envelope glycoprotein (Env) gp120. Some recombinant gp120s do not preserve their conformations on gp140s and functional viral spikes, and exhibit decreased recognition by CD4 and neutralizing antibodies. CD4 binding induces conformational changes in gp120 leading to exposure of the coreceptor-binding site (CoRbs). In this study, we test our hypothesis that CD4-induced (CD4i) antibodies, which target the CoRbs, could also induce conformational changes in gp120 leading to better exposed conserved neutralizing antibody epitopes including the CD4-binding site (CD4bs). We found that a mixture of CD4i antibodies with gp120 only weakly enhanced CD4 binding. However, such interactions in single-chain fusion proteins resulted in gp120 conformations which bound to CD4 and CD4bs antibodies better than the original or mutagenically stabilized gp120s. Moreover, the two molecules in the fusion proteins synergized with each other in neutralizing HIV-1. Therefore, fusion proteins of gp120 with CD4i antibodies could have potential as components of HIV-1 vaccines and inhibitors of HIV-1 entry, and could be used as reagents to explore the conformational flexibility of gp120 and mechanisms of entry and immune evasion.

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1. Introduction

HIV-1 entry is initiated by binding of its envelope glycoprotein (Env) gp120 to the primary receptor CD4 on the target cell surface [1]. The binding induces extensive conformational changes in gp120, resulting in formation of the coreceptor-binding site and enabling binding of gp120 to a coreceptor, either CCR5 or CXCR4. Therefore, the CD4-binding site (CD4bs) and coreceptor-binding site (CoRbs) on gp120 are two conserved structures that can be targeted by antibody-mediated neutralizing responses. The recent identification of a panel of exceptionally potent, broadly neutralizing gp120-specific monoclonal antibodies (mAbs) validates the potential of gp120 as vaccine immunogens. These antibodies include VRC01 [2] and HJ16 [3], which target the CD4bs, PG9 and PG16 [4], which are directed against the conserved regions of variable loops of gp120 preferentially expressed on trimeric Envs, and the series of PGT antibodies [5], which bind to various novel epitopes on gp120. However, previous attempts to elicit broadly neutralizing antibodies using recombinant gp120 have met with limited success likely partially because the highly flexible gp120 may present numerous conformations to the humoral immune system that are not found on the native viral spike and therefore, elicit antibodies that bind to recombinant gp120 but do not neutralize genetically diverse viruses [6].

The requirement of CD4 for HIV-1 entry makes it reasonable to hypothesize that the conformations of gp120 in CD4-bound states would be more likely to exist on the functional viral spike. In line with this hypothesis is the finding that the complexes of gp120 with soluble CD4 (sCD4) or CD4 mimetics (miniCD4) were better recognized by the HIV-1 coreceptor CCR5 and antibodies directed against the CoRbs, so called CD4-induced (CD4i) antibodies [7,8]. To present both CD4bs and CoRbs to the immune system and to reduce gp120 flexibility, structural and mutagenic approaches have been used to conformationally fix gp120 in CD4-bound states in the absence of CD4. These approaches include deletion of variable loops, introduction of inter-domain disulfide bonds, and substitution of the Phe43 cavity in the CD4bs [6,9–11]. The stabilized gp120s generally had a significant increase in binding to sCD4 and CD4i antibodies compared with wild-type gp120s. However, immunization with the stabilized gp120s showed only a trend of improvement in eliciting cross-reactive neutralizing antibodies relative to their wild-type counterparts [10–12]. One possible explanation is that the structural modifications hide or remove gp120 conformations recognized by some neutralizing antibodies. This

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line of reasoning is supported by the findings that interactions of some cross-reactive neutralizing antibodies against the CD4bs with the stabilized gp120s were abrogated [6,9–11] and that a number of recently identified broadly neutralizing mAbs (bnmAbs) targeted the conserved components of variable loops [4,5], which were partially or completely deleted in the stabilized gp120s.

In this study, we describe supporting evidence for our hypothesis that CD4i antibodies could induce conformational changes in gp120 leading to better exposed conserved neutralizing antibody epitopes including the CD4bs.

2. Materials and methods

2.1. Cells, viruses, plasmids, HIV-1 Envs, and antibodies

We purchased the 293T cells from ATCC and the 293 free style cells from Invitrogen. $Gp140_{SC}$, $gp140_{MS}$, $gp120_{SC}$, sCD4, sCD4Fc, m36.4, m36.4h1Fc, m9, m14, and m18 were produced in our laboratory as described previously [13–17]. Antibodies VRC01, b12 and 17b, other cell lines, and plasmids used for expression of various HIV-1 Envs were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (ARRRP). Horseradish peroxidase (HRP)-conjugated mouse anti-FLAG tag antibody and HRP-conjugated goat anti-human IgG (Fc-specific) antibody were products of Sigma–Aldrich.

2.2. Cloning of gp120, gp120 mutants, and CD4i antibody-Env fusion proteins

The following primers were used: MSF, 5′-GACGCGGCCCAGCCGGCCGAGATGGACCTGGAGAAC-3′ (sense); SCR1, 5′-CGGGTTTAAACTCAGTGGTGGTGGTGGTGCTCGATCTTCACCACCTT-3′ (antisense); SCF, 5′-TGACGCGGCCCAGCCGGCCGAGGTGGTGCTGGGCAAC-3′ (sense); TSF, 5′-GTGAGCTCCCAGCTGCTGCTGAAC-3′ (sense); TSR, 5′-CTGGGAGCTCACCACTGG-3′ (antisense); SWF, 5′-GTGATGCACTGGTTCAACTGCGGC-3′ (sense); SWR, 5′-GAACCAGTGCATCACGATCTC-3′ (antisense); m36F, 5′-TGGTTTCGCTACCGTGGCCCAGCCGGCCCAGGTGCAGCTGGTG-3′ (sense); m36.4R2, 5′-TGAACCGCCTCCACCGCTCCCTCCACCGCCACTTCCCCCGCCACCGCTGCCACCCCCTCCTGAGGAGACGGTGAC-3′ (antisense); SCF1, 5′-AGCGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCAGAGGTGGTGCTGGGCAAC-3′ (sense); M9F, 5′-TGACGGGCCCAGCCGGCCGTGTTGACACAGTCTCCA-3′ (sense); M9R, 5′-TGAACCGCCTCCACCGCTCCCTCCACCGCCACTTCCCCCGCCACCGCTGCCACCCCCTCCAGAGGAGACGTTGACCAGGGTTCC-3′ (antisense); SCF2, 5′-AGCGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCAGAGATGGACCTGGAGAAC-3′ (sense); m36.4F1, 5′-ACGAAGCTTCAGGTGCAGCTGGTGCAG-3′ (sense); 140F, 5′-AGCGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCAAAGCTGTGGGTGACCGTG-3′ (sense); and 140R, 5′-CGGGGCCCGCTGTCCATGTGCTGCCG-3′ (antisense).

For cloning of $gp120_{MS}$, the gene fragment was PCR amplified with primers MSF and SCR1. To generate $gp120_{SC}$m, three gene fragments were amplified with primer pairs SCF/TSR, TSF/SWR, and SWF/SCR1, respectively. Full-length $gp120_{SC}$m gene was assembled by overlapping PCR with the three gene fragments as templates (in the same molarity) and primers SCF and SCR1. To construct m36.4–$gp120_{SC}$, m36.4 and $gp120_{SC}$ gene fragments were PCR amplified with primer pairs m36F/m36.4R2 and SCF1/SCR1, respectively. $Gp120_{SC}$ was joined to the 3′ end of m36.4 by overlapping PCR using primers m36F and SCR1. m9–$gp120_{SC}$ and m36.4–$gp120_{MS}$ were generated in the same way, with the substitution of primer pairs M9F/M9R and SCF2/SCR1 for amplification of scFv m9 and $gp120_{MS}$ gene fragments, respectively, and M9F/SCR1 and m36F/SCR1 for overlapping PCR, respectively. All final PCR products were digested with SfiI and PmeI, and cloned into pSecTagB. To generate m36.4–$gp140_{SC}$, m36.4 and $gp140_{SC}$ gene fragments were amplified by PCR with primers m36.4F1/m36.4R2 and 140F/140R, respectively. $Gp140_{SC}$ was linked to m36.4 by overlapping PCR using primers m36.4F1 and 140R. The resultant product was digested with HindIII and ApaI, and cloned into pSecTagB.

2.3. Protein expression and purification

Gp120s, gp140s, and their fusion proteins with CD4i antibodies were expressed and purified from 293 free style cell culture supernatants, as described previously [18].

2.4. Size-exclusion chromatography

A Superdex75 10/300 GL column (GE Healthcare, Piscataway, NJ) was calibrated with protein molecular mass standards of 75 kDa Conalbumin, 158 kDa aldolase, 440 kDa ferritin and 669 kDa thyroglobulin. Purified proteins in PBS (pH 7.4) were loaded onto the pre-equilibrated column and eluted with PBS at 0.5 ml/min.

2.5. ELISA

ELISA was performed as described [18]. The half-maximal binding (EC_{50}) was calculated by fitting the data to the Langmuir adsorption isotherm.

2.6. Surface plasmon resonance (SPR)

The binding kinetics of sCD4 with HIV-1 Envs and their fusion proteins were assessed by SPR analysis on Biacore X100 (GE Healthcare) using a single-cycle approach as described previously [13].

2.7. Pseudovirus neutralization assay

HIV-1 pseudoviruses were generated and the neutralization assay was performed as described previously [18] except that the target cells HOS-CD4-CCR5 were first incubated with the Envs for 30 min before viruses were added.

3. Results and discussion

3.1. Generation of truncated HIV-1 gp120s and their fusion proteins with CD4i antibodies

Truncated HIV-1 gp120s with an N-terminal deletion up to residue 82, a C-terminal deletion down to residue 493, and variable loop deletions were previously described and widely used for crystallization and other experimental analysis [1,19]. Such gp120 derivatives, also termed gp120 cores, could be further conformationally stabilized by structure-guided mutagenesis and have been evaluated for their ability to elicit broadly neutralizing antibodies *in vivo* [6,9–11]. In this study, we generated two truncated gp120s, one ($gp120_{SC}$) from a clade-B and the other ($gp120_{MS}$) from a clade-A virus isolated from chronically HIV-1-infected patients. For consistency with previous studies [1,19], both gp120 sequences ranged from amino acid residues 83 to 492 but they contained intact variable loops (Fig. 1A). Their corresponding gp140s, $gp140_{SC}$ and $gp140_{MS}$, were described previously [13,14]. To generate single-chain fusion proteins, the gp120s and gp140s were fused to the C terminus of CD4i antibodies, m36.4 or m9, via a polypeptide linker composed of six repeats of G_4S motif (Fig. 1A). m36.4 is an affinity-matured version of the first reported engineered human

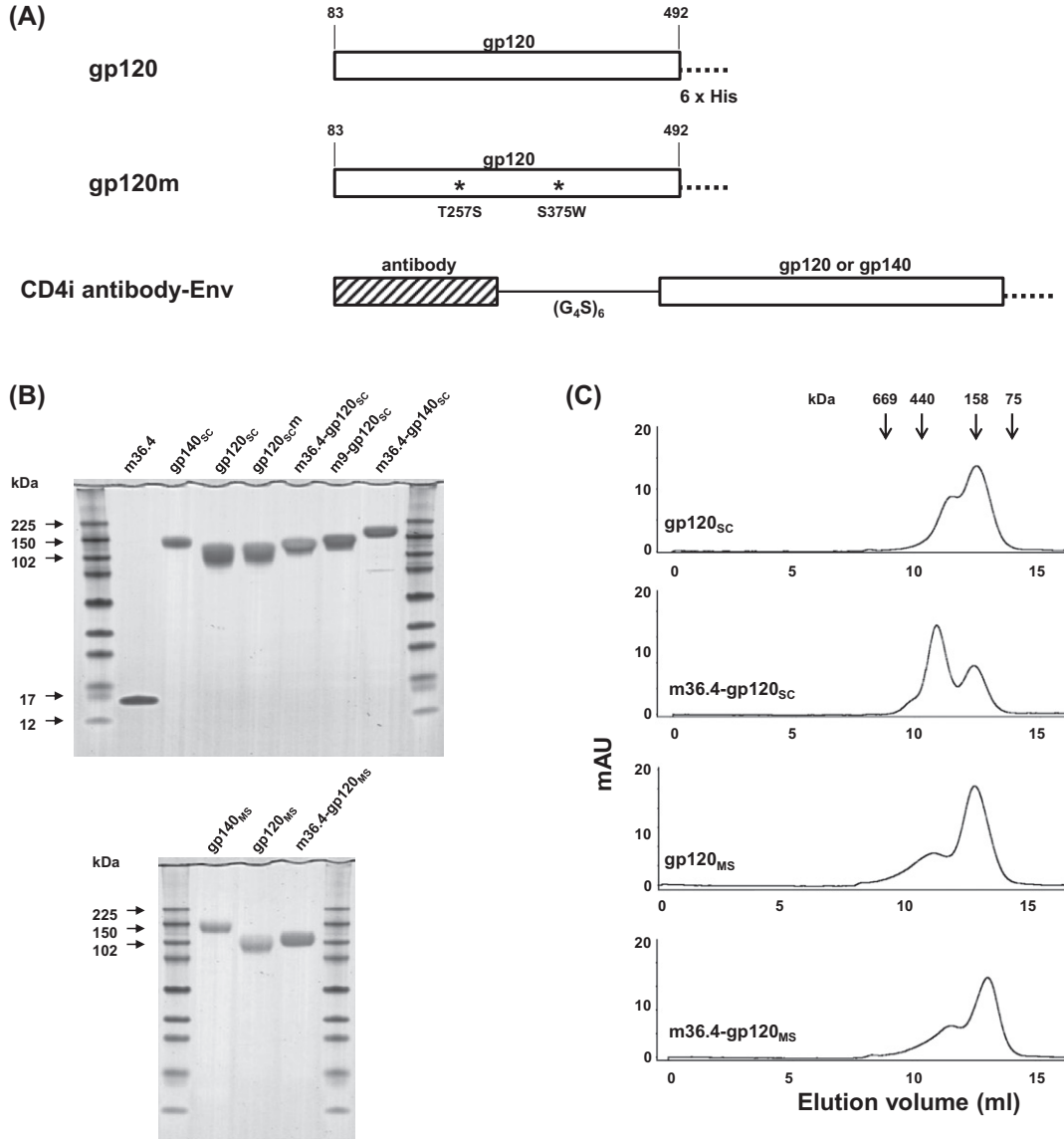

Fig. 1. Schematic representation of HIV-1 Envs and biophysical characterization of the purified proteins. (A) Design of truncated gp120s and CD4i antibody-Env fusion proteins. The gp120 sequences range from amino acid residues 83 to 492. The 6 × His denotes a hexahistidine tag and $(G_4S)_6$ represents a polypeptide linker composed of six repeats of the G_4S motif. The stars indicate the T257S+S375W mutations in gp120. (B) Reducing SDS–PAGE of the purified proteins. (C) Size-exclusion chromatography. The bold arrows shown at the top indicate the elution volumes of the molecular mass standards in PBS: Conalbumin (75 kDa), Aldolase (158 kDa), Ferritin (440 kDa), and Thyroglobulin (669 kDa).

antibody heavy chain variable domain (VH) m36, which targets a CD4i epitope [18]. m9 is an affinity-matured version of the CD4i antibody X5 [15] and is in the format of single-chain variable fragment (scFv). A stabilized $gp120_{SC}$, designated $gp120_{SC}$m, was generated by introducing two amino acid mutations (T257S+S375W) including a substitution of the Phe43 cavity in the CD4bs, according to a previously reported approach [11].

All HIV-1 Envs and their fusion proteins were expressed and purified from 293 free style cell culture supernatants with favorable yields (5–10 mg l^{-1}). They ran on reducing SDS–PAGE with apparent molecular weights larger than their calculated molecular weights due to heavy glycosylation (Fig. 1B). On a size-exclusion chromatography, the gp120s eluted as a mixture of monomer and dimer (Fig. 1C), in agreement with a previous study [20]. The m36.4–gp120 fusion proteins eluted similarly although a larger quantity of m36.4–$gp120_{SC}$ was in a dimeric state, and no higher-order oligomers were observed.

3.2. $Gp120_{SC}$ showed largely diminished binding to CD4 and CD4i antibodies weakly enhanced the interaction

It has been previously observed that truncated HIV-1 gp120 lacking hypervariable loops and segments of its N and C termini could have reduced (up to 10 folds) affinity for CD4 compared with full-length gp120 and gp140 [21]. To assess how well the truncated gp120s we generated could bind to CD4, we performed ELISA and SPR analysis. In SPR analysis, we used two-domain human sCD4, which is in a monomeric state according to our previous study [13], while in ELISA assays, an Fc-fusion protein of sCD4 (sCD4Fc) [13] was used for consistency of valency with several mAbs tested (mostly in IgG1 format). In an ELISA assay, $gp140_{SC}$ strongly bound to sCD4Fc with an EC_{50} of approximately 2 nM (Fig. S1A). However, $gp120_{SC}$ showed a great decrease in binding (EC_{50}, >100 nM). In a SPR analysis, $gp120_{SC}$ had an affinity for sCD4 (K_D = 104 nM) about 80-fold lower than that (K_D = 1.3 nM) of $gp140_{SC}$ (Table 1). These

Table 1
Binding kinetics of sCD4 with HIV-1 Envs measured by SPR.

HIV-1 Envs	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (nM)
$gp140_{SC}$	2.4×10^5	3.0×10^{-4}	1.3
$gp120_{SC}$	8.1×10^4	8.4×10^{-3}	104
$gp120_{SC}$m	1.0×10^5	6.1×10^{-3}	59
m36.4–$gp120_{SC}$	7.7×10^4	5.6×10^{-5}	0.73
m9–$gp120_{SC}$	1.6×10^5	2.9×10^{-4}	1.8
m36.4–$gp140_{SC}$	2.2×10^6	4.2×10^{-3}	1.9
$gp140_{MS}$	3.9×10^5	1.4×10^{-4}	0.36
$gp120_{MS}$	2.3×10^6	4.8×10^{-4}	0.21
m36.4–$gp120_{MS}$	2.5×10^6	3.9×10^{-4}	0.15

K_a, association constant.
K_d, dissociation constant.
K_D, equilibrium dissociation constant.

results suggest an alteration of the CD4bs conformation in the context of $gp120_{SC}$. CD4 induces conformational changes in gp120 leading to the exposure/formation of CD4i epitopes. To find out whether CD4i antibodies could do likewise, we measured the binding of sCD4Fc to $gp120_{SC}$ in the absence or presence of m36.4 or m9. The results showed that m36.4 and m9 at a constant concentration of 3.5 μM weakly enhanced sCD4Fc binding to $gp120_{SC}$ but not to $gp140_{SC}$ (Fig. S1). The enhancement effects on $gp120_{SC}$ are minor likely due to the relatively low local antibody concentrations and generally very weak binding of CD4i antibodies to independently folded gp120 in the absence of CD4.

3.3. CD4i antibody-$gp120_{SC}$ fusion proteins exhibited much better binding to CD4 and significantly better binding to CD4bs antibodies than $gp120_{SC}$

To test whether further increasing the local concentration of CD4i antibodies could have more pronounced enhancement effects, we generated fusion proteins of the Envs with CD4i antibodies. In our previous study [14], we found that fusing m36 to the C terminus of a cargo protein via a polypeptide linker resulted in largely decreased binding of m36 to HIV-1 gp120 likely because the linker, which is in close proximity to the antigen-binding site of m36, interrupts the interaction. Therefore, we decided to join m36.4 and m9 to the N terminus of the Envs. In an ELISA assay (Fig. 2), sCD4Fc bound to m36.4–$gp120_{SC}$ coated on 96-well plates with an EC_{50} (2 nM) much lower than that (>100 nM) of $gp120_{SC}$. Interaction of sCD4Fc with m36.4 was not detected suggesting that the enhanced binding of sCD4Fc to m36.4–$gp120_{SC}$ was

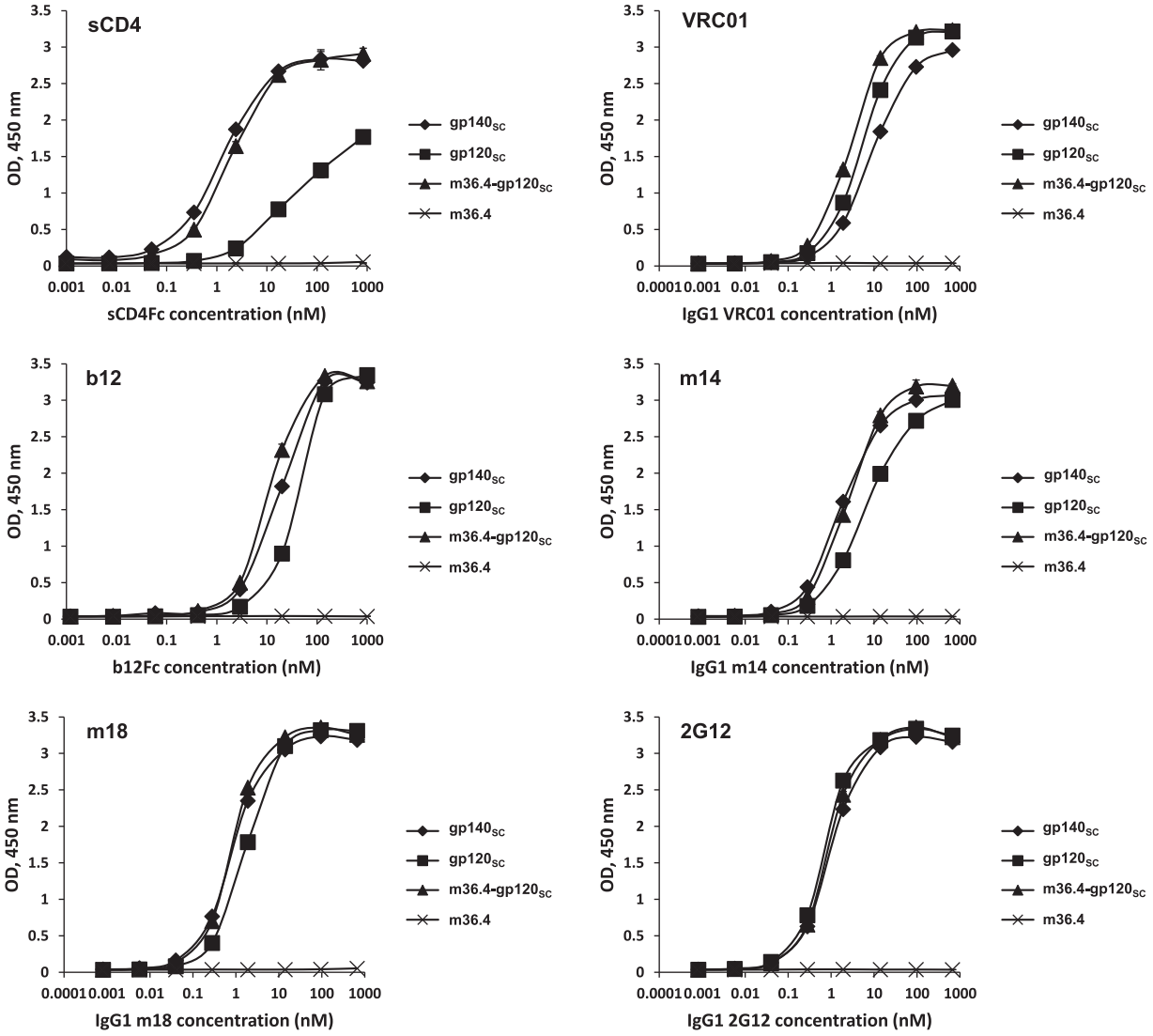

Fig. 2. ELISA binding of sCD4Fc and mAbs to m36.4–$gp120_{SC}$. m36.4–$gp120_{SC}$ and the control antigens were coated on 96-well plates. All mAbs except b12, which is an Fc-fusion protein of scFv, are in IgG1 format. Bound sCD4Fc and mAbs were detected by HRP-conjugated goat anti-human IgG (Fc-specific) antibody.

gp120-specific. Compared to $gp120_{SC}$, m36.4–$gp120_{SC}$ also showed a considerable increase in binding to the well-characterized CD4bs mAbs, b12 [22] and m18 [17], while the increase in binding to VRC01 [2] and m14 [16] was less significant. VRC01 was the only antibody that bound to $gp120_{SC}$ better than to $gp140_{SC}$. When 2G12 [23], a bnmAb targeting a conserved cluster of oligomannose glycans on gp120, was tested, m36.4–$gp120_{SC}$ exhibited a binding strength comparable with that of $gp120_{SC}$, suggesting that fusion to m36.4 did not alter the glycosylation of gp120 with respect to 2G12 recognition. In all cases, m36.4–$gp120_{SC}$ showed comparable or even better binding activities than $gp140_{SC}$. Enhanced recognition by sCD4Fc was also observed when $gp120_{SC}$ was fused to m9 (Fig. S2A). In the SPR-based analysis (Table 1), the affinities (K_D = 0.73 and 1.8 nM, respectively) of m36.4–$gp120_{SC}$ and m9–$gp120_{SC}$ for sCD4 were comparable with that (K_D = 1.3 nM) of $gp140_{SC}$ but much higher than that (K_D = 104 nM) of $gp120_{SC}$, in agreement with the results from ELISA. Notably, the changes in affinity were mostly caused by the altered dissociation constants although the association constants also contributed.

To test whether the enhancement could be achieved with a gp140 or a gp120 that does not show largely decreased binding compared to the corresponding gp140, we fused m36.4 to $gp140_{SC}$ and $gp120_{MS}$ (Fig. 1). In contrast to the effects on $gp120_{SC}$, m36.4 was ineffective in enhancing sCD4 interaction with $gp140_{SC}$ and $gp120_{MS}$, which alone had an affinity comparable with that of $gp140_{MS}$ (Table 1). In an ELISA assay, however, m36.4–$gp120_{MS}$ and m36.4–$gp140_{SC}$ bound to sCD4Fc about threefold better than $gp120_{MS}$ and $gp140_{SC}$, respectively (Fig. S2B and C). These results suggest that although fusion of CD4i antibodies to gp140s or gp120s with well-preserved CD4bs may not increase their affinity for CD4, it could still have an impact on gp120 conformation leading to higher avidity binding to CD4.

3.4. m36.4–$gp120_{SC}$ bound to CD4 and antibodies better than mutagenically stabilized $gp120_{SC}$

To compare our approach to a previously reported structural and mutagenic strategy for gp120 stabilization, we generated $gp120_{SC}$m, which carried T257S+S375W double mutations (Fig. 1A). In previous studies [9,11], gp120 derivatives with the same two mutations demonstrated a significant increase in gp120 affinity for sCD4 and CD4i antibodies. $Gp120_{SC}$m was tested for ELISA binding to sCD4Fc, CD4bs antibodies b12, VRC01 and m14, and CD4i antibody 17b [24]. As shown in Fig. 3, $gp120_{SC}$m

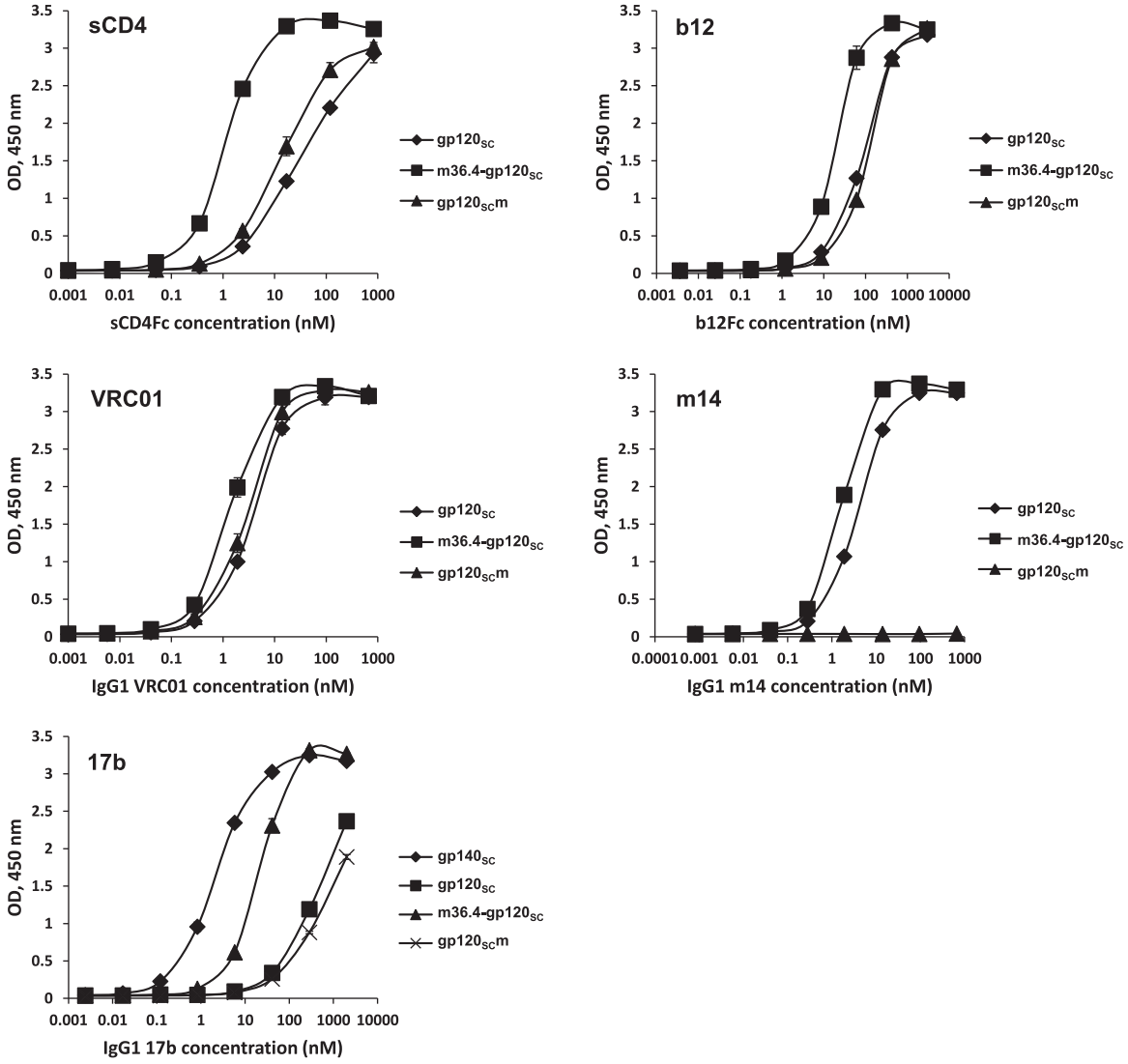

Fig. 3. ELISA binding of sCD4Fc and mAbs to $gp120_{SC}$m. The gp120 proteins and control antigens were coated on 96-well plates. Bound sCD4Fc and mAbs were detected by HRP-conjugated goat anti-human IgG (Fc-specific) antibody.

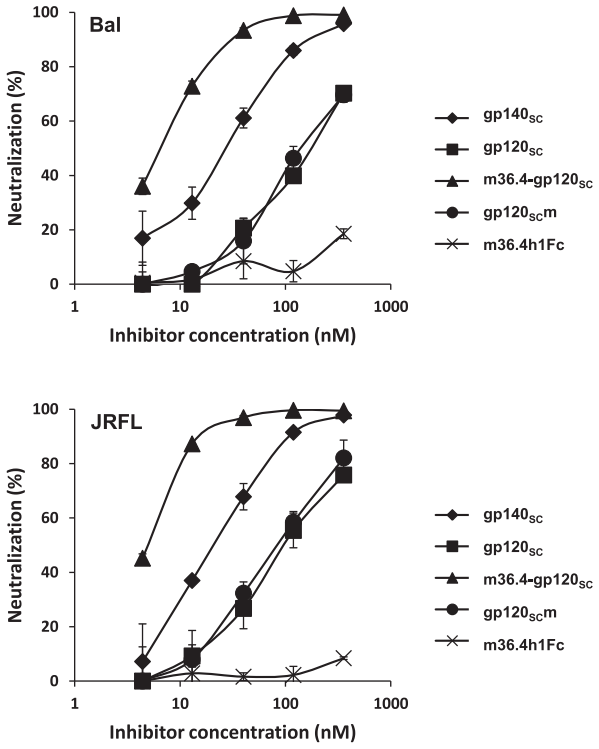

Fig. 4. Neutralization of HIV-1 by the Envs and fusion proteins. The pseudotyped viruses were generated from 293T cells and the assays were performed on HOS-CD4-CCR5 cells, as described in materials and methods.

bound to sCD4Fc slightly better than $gp120_{SC}$. However, it showed comparable (b12 and VRC01), decreased (17b) or no binding (m14) to the antibodies tested. In all cases, m36.4–$gp120_{SC}$ exhibited stronger interactions than both $gp120_{SC}$ and $gp120_{SC}$m. 17b bound to $gp140_{SC}$ about fivefold better than to m36.4–$gp120_{SC}$. In agreement with the ELISA data, the results from SPR analysis showed that $gp120_{SC}$m had an affinity for sCD4 of 59 nM, which was about twofold higher than that (104 nM) of $gp120_{SC}$ but still much lower than those (0.73 and 1.8 nM, respectively) of m36.4–$gp120_{SC}$ and m9–$gp120_{SC}$ (Table 1).

The T257S+S375W double-mutation strategy was developed and evaluated mainly using conserved gp120 cores with shortened or deleted variable loops [9,11]. It is therefore likely that the gp120 variable loops, which remained intact in the gp120s we generated, may impair the stabilization effects of the T257S+S375W mutations. Surprisingly, the mutagenic strategy appeared to disrupt the gp120 conformations required for interaction with some CD4bs antibodies [9], which was confirmed in our study by using m14 (Fig. 3). In contrast, our approach led to an increase in binding to all CD4bs antibodies tested (Figs. 2 and 3). This can be particularly important for the use of gp120 as vaccine immunogens because extensive exposure of the conserved epitopes for neutralizing antibodies could be critical for elicitation of broadly neutralizing responses. The breadth of serum neutralization has been mapped to multiple epitope specificities [25]. Moreover, fusion to a CD4i antibody could partially shield the CoRbs from the immune system. CD4i antibodies are abundant in patients with HIV-1 infection [26] but generally do not or only weakly neutralize the virus as full-size antibody molecules due to steric occlusion of their epitopes [18,24]. Therefore, sequential immunization with gp120 fused to different CD4i antibodies might partially avoid or minimize elicitation of such antibodies while diverting the immune responses to other conserved structures on gp120 such as the CD4bs.

3.5. Potent neutralizing activity of m36.4–$gp120_{SC}$

Recombinant Envs are potent HIV-1 entry inhibitors because they block the attachment of the virus to the cell membrane-associated CD4. To assess how well m36.4–$gp120_{SC}$ could prevent HIV-1 infection, we conducted a pseudovirus neutralization assay. The results showed that $gp120_{SC}$ and $gp120_{SC}$m neutralized two primary isolates from clade B, Bal and JRFL, much less potently than $gp140_{SC}$ (Fig. 4), in correlation with their relative binding strengths in ELISA and SPR. m36.4 as an Fc-fusion protein, m36.4h1Fc, did not or only very weakly neutralized the viruses due to an increase in molecular size, in agreement with our previous study [14]. Surprisingly, m36.4–$gp120_{SC}$ displayed neutralizing activities (IC_{50}s, 5 nM) against both isolates fourfold higher than those (IC_{50}s, 20 nM) of $gp140_{SC}$ despite their comparable binding to sCD4, suggesting synergistic effects of the antibody-gp120 fusion on neutralizing the viruses.

Note added in proof

A recent paper (PLoS Pathogens, 8:e1002797, 2012) reported that CD4i antibody 17b was able to induce conformational changes in a trimeric Env leading to the same open state of the Env with CD4.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.013.

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