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# Stable exposure of the coreceptor-binding site in a CD4-independent HIV-1 envelope protein

TREVOR L. HOFFMAN*, CELIA C. LABRANCHE†, WENTAO ZHANG‡, GABRIELLA CANZIANI‡, JAMES ROBINSON§, IRWIN CHAIKEN‡, JAMES A. HOXIE¶, AND ROBERT W. DOMS*‖

*Department of Pathology and Laboratory Medicine, ‡Rheumatology Division, Department of Medicine, and ¶Hematology–Oncology Division, Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104; †Department of Surgery, Duke University Medical Center, Durham, NC 27710; and §Department of Pediatrics, Tulane University Medical Center, New Orleans, LA 70112

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**ABSTRACT** **We recently derived a CD4-independent virus from HIV-1/IIIB, termed IIIBx, which interacts directly with the chemokine receptor CXCR4 to infect cells. To address the underlying mechanism, a cloned Env from the IIIBx swarm (8x) was used to produce soluble gp120. 8x gp120 bound directly to cells expressing only CXCR4, whereas binding of IIIB gp120 required soluble CD4. Using an optical biosensor, we found that CD4-induced (CD4i) epitopes recognized by mAbs 17b and 48d were more exposed on 8x than on IIIB gp120. The ability of 8x gp120 to bind directly to CXCR4 and to react with mAbs 17b and 48d in the absence of CD4 indicated that this gp120 exists in a partially triggered but stable state in which the conserved coreceptor-binding site in gp120, which overlaps with the 17b epitope, is exposed. Substitution of the 8x V3 loop with that from the R5 virus strain BaL resulted in an Env (8x-V3BaL) that mediated CD4-independent CCR5-dependent virus infection and a gp120 that bound to CCR5 in the absence of CD4. Thus, in a partially triggered Env protein, the V3 loop can change the specificity of coreceptor use but does not alter CD4 independence, indicating that these properties are dissociable. Finally, IIIBx was more sensitive to neutralization by HIV-positive human sera, a variety of anti-IIIB gp120 rabbit sera, and CD4i mAbs than was IIIB. The sensitivity of this virus to neutralization and the stable exposure of a highly conserved region of gp120 suggest new strategies for the development of antibodies and small molecule inhibitors to this functionally important domain.**

Binding of the HIV-1 envelope protein (Env) to CD4 induces structural alterations in the gp120 subunit that enable it to interact with an appropriate coreceptor (1–4). The major HIV-1 coreceptors are CCR5 and CXCR4, because all HIV-1 strains identified to date require one of these molecules for viral entry (5). The impressive resistance of individuals who lack CCR5 to virus infection demonstrates the importance of this receptor *in vivo* and indicates that viruses that use CCR5 (R5 virus strains) are largely responsible for transmission (6–9). Accrual of mutations in Env impart the ability to use other coreceptors, such as CXCR4. Sequences in the V3 loop of gp120 play a major role in governing coreceptor choice, though the V1/V2 region also influences coreceptor use (10–15). The recently solved structure of a trimolecular complex consisting of a HIV-1 gp120 core fragment bound to soluble CD4 and the Fab derived from the CD4-induced (CD4i) mAb 17b has led to the identification of a highly conserved region in gp120 implicated in coreceptor binding (16–18). Because binding of antibodies to this region (including 17b) is markedly enhanced by CD4 binding as is the affinity of gp120 for CCR5 (1–3, 19, 20), it is likely that the coreceptor-binding site is at least partially sequestered in the native state. CD4 binding may lead to exposure and/or formation of this region with subsequent coreceptor binding. Ultimately, conformational changes in Env that result from these binding events lead to fusion between the viral and cellular membranes.

The entry pathway for HIV-1 described above can be subverted in part by simian immunodeficiency virus (SIV), HIV-2, and HIV-1 strains that use coreceptors in the absence of CD4 for viral entry (21–24). Potentially, CD4-independence may enable virus strains to infect CD4-negative coreceptor positive cells *in vivo*. Indeed, SIVmac17E/Fr, a neuropathogenic SIV strain, infects primary rhesus macaque brain capillary endothelial cells in a CD4-independent CCR5-dependent manner (21). The ability to use coreceptors independently of CD4 may also enable virus strains to infect cells more efficiently when CD4 expression is limiting. In addition, direct binding of CD4-independent gp120 molecules to coreceptors may induce signaling and even apoptosis (25–27).

In this manuscript, we describe the mechanisms underlying the ability of a recently derived CD4-independent variant of HIV-1/IIIB (termed IIIBx) to interact directly with the chemokine receptor CXCR4 to mediate viral entry [C.C.L., T.L.H., J. Romano, B. S. Haggarty, T. J. Matthews, R.W.D. and J.A.H. (unpublished work)]. The gp120 derived from a functional *env* clone of this virus (8x) bound directly to CXCR4 in a CD4-independent manner. In contrast, binding of IIIB gp120 to CXCR4 was strictly CD4-dependent. Using an optical biosensor, we found that mAbs to CD4i epitopes, including 17b, bound efficiently to 8x gp120 in the absence of CD4, whereas efficient binding of these mAbs to the parental IIIB gp120 required CD4. As the epitope recognized by 17b overlaps with that of the conserved coreceptor-binding site (16–18), these results indicate that the IIIBx Env protein exists in a partially triggered state in which the conserved coreceptor-binding site is exposed and functional. Exposure of this region had additional consequences in that IIIBx was more sensitive to antibody-mediated neutralization than was HIV-1/IIIB. Interestingly, substitution of the 8x V3 loop with that of an R5 virus strain resulted in a gp120 that could bind directly to CCR5 in the absence of CD4. Thus, in a partially triggered Env protein, the V3 loop can change coreceptor choice but does not impact CD4-independence, indicating that these properties are dissociable. The stable exposure of the conserved coreceptor-binding site has implications both for the development of small molecule inhibitors of Env–coreceptor interactions

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Abbreviations: Env, HIV-1 envelope protein; CD4i, CD4-induced; sCD4, soluble CD4; SIV, simian immunodeficiency virus.
‖To whom reprint requests should be addressed at: Department of Pathology and Laboratory Medicine, University of Pennsylvania, 806 Abramson, 34th and Civic Center Boulevard, Philadelphia, PA 19104. e-mail: doms@mail.med.upenn.edu.

and for vaccine development, since this region may, if properly presented, elicit broadly crossreactive neutralizing antibodies.

## MATERIALS AND METHODS

**Plasmids.** Human CCR5, CXCR4, and CD4 were expressed by using the pCDNA3 vector (Invitrogen). The luciferase gene was expressed under control of the T7 promoter in the pGEM2 vector (Promega). The *envs* from the HXBc2 clone of IIIB and 8x were both expressed in the pSP73 vector (Promega). To generate *env* constructs containing the V3 loop of the R5 HIV-1 strain BaL, the *Kpn*I-*Bam*HI fragment in *env* from the full-length proviral clone pIIIB-V3Bal (28) was cloned into pSP73-IIIB. To produce a version of 8x containing the V3 loop of BaL, the *Stu*I-*Bsu*36I *env* fragment of pIIIB-V3BaL was cloned into pSP73–8x. Stop codons were inserted into each *env* plasmid at the gp120/gp41 junction by using the Quickchange Site-Directed Mutagenesis Kit (Stratagene) to make constructs for gp120 production. The identity of all mutants and clones was confirmed by DNA sequencing.

**Cell–Cell Fusion and Luciferase Reporter Virus Assays.** These assays have been described in more detail elsewhere (29–31). For cell–cell fusion assays, effector QT6 cells in T25 flasks were infected with recombinant vaccinia virus expressing T7 polymerase (vTF1.1) and transfected with 30 $\mu$g of *env* constructs driven by the T7 promoter. Target QT6 cells were plated in 24-well plates, and each well of cells was transfected with 0.5 $\mu$g CD4 and 1.0 $\mu$g coreceptor plasmids under the control of the CMV promoter and 1.5 $\mu$g of the luciferase reporter plasmid under the control of the T7 promoter. After overnight expression, the effector cells were added to target cells, and luciferase activity was quantified in cell lysates 7.5 hr after mixing. For virus infection assays, 15 $\mu$g *env* and 5 $\mu$g NL-luc-E$^-$R$^-$ constructs were transfected into 293T cells plated in T25 flasks. Pseudotyped virions were harvested from the media 2 days after transfection and used to infect 293T cells in 24-well plates transfected with 1.5 $\mu$g each CD4 and coreceptor plasmid. Cell lysates were measured for luciferase activity 2 days after infection.

**Protein Production and Purification.** 293T cells were infected in T225 flasks with vTF1.1 and transfected with 200 $\mu$g of gp120 plasmid. Four hours after transfection, the cells were washed with PBS and placed in serum-free media for 24 hr. Media were collected, clarified by centrifugation and 0.2 $\mu$M filtration before addition of 0.1% TX-100. Protein in the supernatant was bound to a Galanthis Navalis column (Vector Laboratories), washed with Mes buffer (20 mM Mes pH 7.0/0.13M NaCl/10 mM $CaCl_2$), and eluted in Mes buffer containing 0.5M $\alpha$-methyl mannoside. The eluate was subjected to additional purification, washing, and concentration by using an Amicon ultrafiltration system with a 50-kDa cutoff. HPLC analysis determined that Env prepared in this fashion was highly pure, and accurate protein concentrations were determined by amino acid analysis and bicinchoninic acid assay (Pierce).

**Cell-Surface Binding Assay.** Binding of gp120 to coreceptors was determined as previously described (32). Approximately 5 $\mu$g of each gp120 was iodinated by using Iodogen (Pierce) to specific activities of 12.3 $\mu$Ci/$\mu$g, 7.15 $\mu$Ci/$\mu$g, 47.3 $\mu$Ci/$\mu$g, 22.0 $\mu$Ci/$\mu$g for IIIB, 8x, IIIB-V3Bal, and 8x-V3BaL, respectively. gp120 (100,000 counts per minute) was added in a total volume of 100 $\mu$l binding buffer (50 mM Hepes, pH 7.4/5 mM $MgCl_2$/1 mM $CaCl_2$/5% BSA) to 0.5–1.0 $\times$ $10^6$ 293T cells that had been transfected the previous day with 14 $\mu$g DNA. Soluble CD4 (sCD4) was added when indicated at 100 nM. Cells were incubated 1 hr at 25°C. Unbound radioactivity was removed by filtering cells through Whatman GF/C filters presoaked in 0.3% polyethylenimine and washing twice with 4 ml wash buffer (50 mM Hepes, pH 7.4/5 mM $MgCl_2$/1 mM $CaCl_2$/500 mM NaCl). The amount of radioactivity that bound nonspecifically to the filters in the absence of cells was subtracted from all data points.

**Biosensor Experiments.** All experiments were performed with a BIACORE 2000 (Biacore, Piscataway, NJ) optical biosensor at 25°C using methods described more extensively elsewhere [W. Zhang, G. Canziani, R. Wyatt, J. Sodroski, R. Sweet, P. Kwong, W. Hendrickson and I.C. (unpublished work)]. Briefly, 200 resonance units of sCD4 and the full-length human mAbs 17b and 48d were attached by amine coupling to a research grade CM5 chip. A naked sensor surface without antibody or sCD4 served as a negative control for each binding interaction. Env that had been serially diluted was run across each sensor surface at six different concentrations in a running buffer of PBS + 0.005% Tween-20 (11nM to 585nM for gp120, 5nM to 91nM for gp120 + sCD4). sCD4 was added at an 8-fold molar excess to Env at least 30 min before measuring binding, and this treatment was found to completely eliminate binding of Env to a sensor surface to which sCD4 had been attached. Binding and dissociation were measured for 300 sec each under conditions yielding a flow-independent binding on-rate (30 $\mu$l/min). The sensor surface was regenerated between each binding reaction by using two washes of 10 mM HCl for 15 sec at 100 $\mu$l/min, which completely returned the signal to baseline without decreasing the binding capacity of the immobilized surface. Each binding curve was corrected for nonspecific binding by subtraction of the signal obtained from the negative control flow cell. Kinetic constants for association and dissociation were derived from linear transformations of the exported binding data of at least five concentrations of analyte. Kinetic parameters obtained were compared with those estimated by fitting the data to the simple 1:1 Langmuir interaction model by using the BIA EVALUATION 3.1 software.

**Neutralization Assays.** Neutralization of virus by antisera or mAbs was performed by using a modification of the previously described MAGI assay (33) or luciferase reporter virus system (31). Briefly, 2.5 $\times$ $10^4$ MAGI cells were plated in a 48-well plate, allowed to adhere, and infected with virus that had been preincubated with serial dilutions of antibody for 1 hr at 37°C. The amount of virus used was the amount previously determined with the MAGI assay to contain 600–1,000 infectious units. Twenty-four hours after infection, the DP178 inhibitory peptide was added at a final concentration of 5 $\mu$g/ml to prevent the formation of syncytia. Cultures were incubated another 48 hr, fixed, and stained with 5-bromo-4-chloro-3-indolyl $\beta$-D-galactopyranoside. Blue nuclei were quantified by using an AlphaImager 2000 (Alpha Innotech, San Leandro, CA). For luciferase reporter virus infections, equal amounts of virus, as judged by relative light units, were also incubated with serial dilutions of antibody for 1 hr at 37°C. Virus was added to GHOST-CCR5 cells in 96-well plates, and cell lysates were measured for luciferase activity 2 days after infection.

## RESULTS

**Direct Binding of 8x gp120 to CXCR4.** Binding of CD4 to HIV-1 Env induces conformational changes required for subsequent Env–coreceptor interactions. These changes are likely to include increased exposure of an exceptionally well-conserved domain in gp120 that has been implicated in coreceptor binding (ref. 17 and Fig. 3). Many SIV and HIV-2 strains can short-circuit this normal entry process by using coreceptors for virus entry in a CD4-independent manner, although their efficiency is typically enhanced when CD4 is present (21–24). Recently, we derived a CD4-independent HIV-1 strain through repeated passaging of HIV-1 IIIB on CD4-negative CXCR4-positive cells [C.C.L.B., T.L.H., J. Romano, B. S. Haggarty, T. J. Matthews, R.W.D. and J.A.H. (unpublished work)]. The resulting virus strain (HIV-1 IIIBx) can use CXCR4 in the absence of CD4 to infect a wide variety

of cell types [C.C.L.B, T.L.H., J. Romano, B. S. Haggarty, T. J. Matthews, R.W.D. and J.A.H. (unpublished work)]. An *env* clone derived from cells chronically infected with IIIBx, termed 8x, maintains this phenotype. Thus, cells expressing 8x Env mediate fusion with CD4-negative CXCR4-positive cells. In the presence of CD4, fusion efficiency is enhanced approximately 3-fold [C.C.L., T.L.H., J. Romano, B. S. Haggarty, T. J. Matthews, R.W.D. and J.A.H. (unpublished work); Fig. 1*A*]. Because CD4 is known to induce conformational changes in Env that enable it to interact with coreceptors (1–4), and because the 8x Env can mediate CXCR4-dependent fusion in the absence of CD4, we hypothesized that the 8x Env protein exists in a partially triggered state, enabling it to bind CXCR4 in a CD4-independent manner.

To evaluate conformational differences in the 8x gp120, we first tested the ability of purified 8x gp120 to bind directly to CXCR4. To do this, we used a cell-surface binding assay in which iodinated gp120, with or without prior incubation with sCD4, is added to cells expressing the desired coreceptor (32). The cells are washed and the amount of bound gp120 measured. By using this assay, IIIB gp120 complexed with sCD4 bound to cells expressing CXCR4 but not to cells expressing other coreceptors (ref. 32 and Fig. 2). In contrast, the 8x gp120 bound to CXCR4-expressing cells equally well in the presence or absence of sCD4 (Fig. 2). Binding to cells expressing vector alone or CCR5 was not observed. These results indicate that, unlike the parental IIIB Env, the 8x gp120 exists in a stable conformation that enables it to interact directly with CXCR4 in the absence of CD4.

**Exposure of the Coreceptor-Binding Site.** The ability of 8x gp120 to bind to CXCR4 could be the result of increased exposure of sites on gp120 that interact with CXCR4. To address the exposure of the coreceptor-binding site, we examined the abilities of IIIB and 8x gp120 to interact with the CD4i mAbs 17b and 48d. A Fab fragment of 17b was cocrystallized with gp120 and sCD4 (16), and many of the contact residues involved in 17b–gp120 interactions are also important for coreceptor binding (ref. 17 and Fig. 3). Thus, 17b can be used as an immunological surrogate to measure exposure of the conserved coreceptor-binding site defined by Rizzuto *et al.* (17).

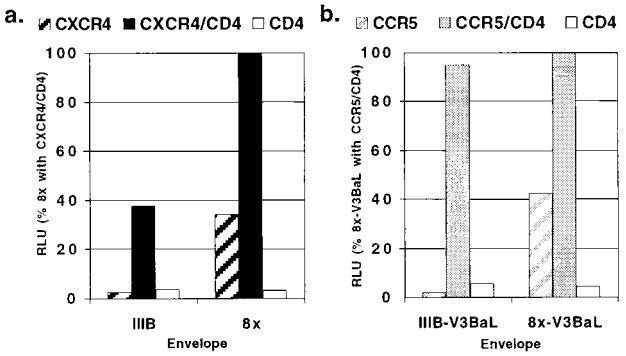


FIG. 1. CD4-independent cell–cell fusion and virus infection. (*a*) QT6 effector cells expressing the indicated Env and T7 polymerase were mixed with QT6 target cells expressing chemokine receptor, CD4, and the luciferase gene under control of the T7 promoter. Luciferase is produced in this assay only if Env mediates fusion between effector and target cells. Results for each Env are expressed in relative light units and are normalized to the amount of fusion obtained with 8x Env effector and CXCR4/CD4 target cells. Results from a typical experiment are shown. (*b*) Luciferase reporter viruses bearing the indicated Env proteins were used to infect 293T cells expressing CCR5, CD4, or both receptors, and the amount of luciferase activity determined 2 days after infection. Results for each Env are expressed in relative light units and are normalized to the results obtained with virions bearing the 8x-V3BaL Env and CCR5/CD4 target cells. A representative experiment is shown.

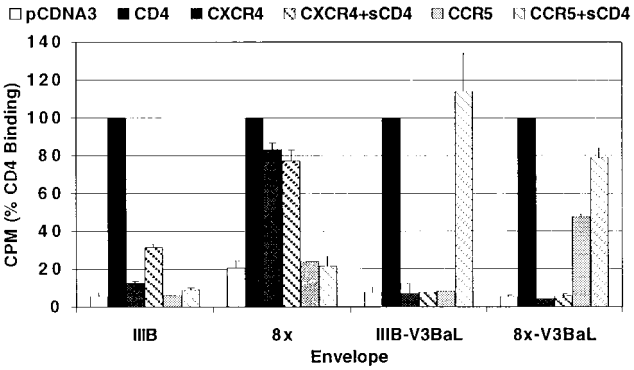


FIG. 2. Cell-surface gp120 binding. Radioiodinated gp120s were incubated with 293T cells transiently transfected with coreceptor or CD4 plasmids. sCD4 was added to the binding reaction where indicated. The amount of specific radioactivity bound to the cells is presented and is normalized for each gp120 such that binding to CD4 represents 100%. Each value represents the average of at least three independent experiments and error bars represent SEMs.

Binding of IIIB and 8x gp120, with or without prior incubation with sCD4, to the CD4i mAbs 17b and 48d was measured by using an optical biosensor. This approach makes it possible to measure protein–protein interactions in real time and can be used to derive on- and off-rates as well as affinity constants. The desired mAb was covalently coupled to the sensor surface, after which gp120 or gp120-sCD4 complexes were applied. A typical sensorgram is shown in Fig. 4. As expected, the rate at which HIV-1/IIIB gp120 bound to mAb 17b was markedly increased by prior incubation of Env with sCD4. Once bound to 17b, both IIIB gp120 and gp120-sCD4 complexes exhibited negligible off-rates. In contrast to IIIB

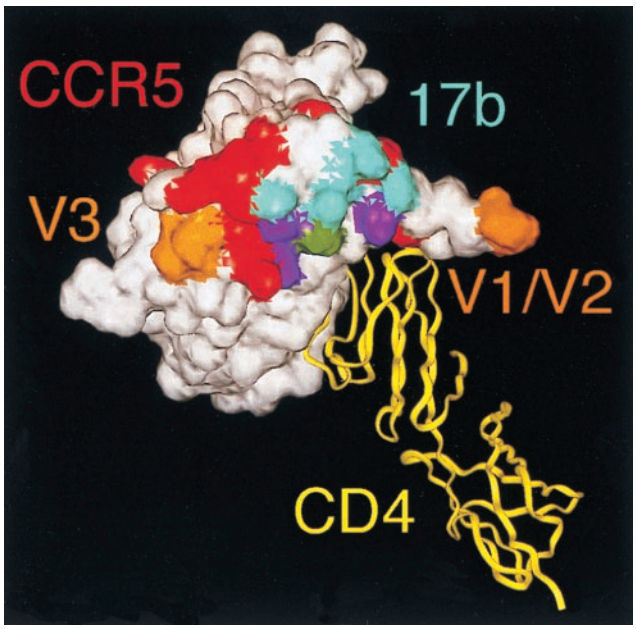


FIG. 3. Overlap between the coreceptor-binding site and the 17b epitope. A space-filling model of gp120 bound to sCD4 is depicted. Residues shown by Rizzuto *et al.*(17) to decrease CCR5 binding by >50% when mutated while reducing CD4 binding by <50% are colored red (17), contact residues for 17b are shown in light blue (16), and residues involved in both CCR5 and 17b binding are shown in purple. Although 8x contains numerous mutations in gp120 that contribute to the CD4-independent phenotype [C.C.L., T.L.H., J. Romano, B. S. Haggarty, T. J. Matthews, R.W.D. and J.A.H (unpublished work)], only one mutation in 8x affects a contact site for 17b (I423V, shown in green). The stems of the V1/V2 and V3 loops are colored orange.

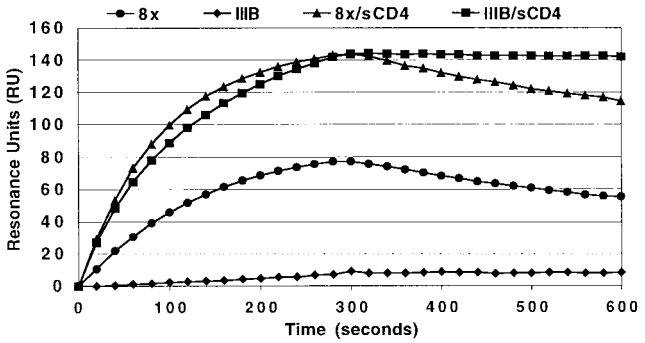


FIG. 4. Sensorgrams for gp120 binding to the CD4i mAb 17b. mAb 17b was attached to the sensor surface, after which the indicated gp120 molecule (at equal concentrations), with or without prior incubation with saturating levels of sCD4, was applied to the flow cell. A 300-sec association was followed by a wash with running buffer for an additional 300 sec, during which dissociation was measured. Kinetic constants derived from linear transformations of the data are presented in Table 1.

gp120, 8x gp120 bound efficiently to 17b without sCD4, exhibiting an on-rate that was an order of magnitude greater than that of IIIB gp120 (Table 1). Addition of sCD4 enhanced this on-rate 2-fold. When complexed with sCD4, both the 8x and IIIB gp120s exhibited identical on-rates. Interestingly, once bound to 17b, the 8x gp120 exhibited a greater off-rate than did IIIB gp120. This effect may be due to the Ile to Val mutation in the 8x gp120 at amino acid position 423 [Fig. 3; C.C.L., T.L.H., J. Romano, B. S. Haggarty, T. J. Matthews, R.W.D. and J.A.H. (unpublished work)], a residue previously shown to be a contact site for 17b (16). Other 17b contact residues are conserved between IIIB and 8x. Analysis of a different CD4i mAb, 48d, yielded results that were similar to 17b (Table 1). Finally, we found that IIIB and 8x gp120 molecules interacted with SCD4 attached to the sensor surface in an identical fashion (data not shown). Thus, the mutations in 8x that render it CD4-independent did not affect CD4 binding to an appreciable degree, but did result in greater exposure of the 17b epitope, which overlaps with the conserved coreceptor-binding site.

**Dissociation of Coreceptor Choice and CD4-Independence.** The conserved coreceptor-binding site, as well as the V1/V2 and V3 loops of gp120, play an important role in Env–coreceptor interactions. Available evidence indicates that the V3 loop and, to a lesser extent, the V1/V2 region, govern the number and types of coreceptors used by a given Env (10–15). In contrast, mutations in the conserved coreceptor-binding site can affect Env–coreceptor binding (17), but it is not clear whether this region also plays a role in coreceptor specificity. The ability of 8x gp120 to interact directly with CXCR4 provided an opportunity to determine whether coreceptor choice and changes in Env that expose the coreceptor-binding site are dissociable. Previous work by Ross *et al.* demonstrated that the introduction of an R5 V3 loop (from HIV-1 BaL) into an HIV-1 IIIB background resulted in an Env protein (IIIB-V3BaL) that used CCR5, but not CXCR4, for virus infection (11, 34). To determine whether insertion of an R5 V3 loop into 8x could create a virus that was CD4-independent but CCR5 specific, we generated luciferase reporter viruses (31) bearing either IIIB-V3BaL or 8x-V3BaL Env proteins. Virions bearing 8x-V3BaL Env mediated CD4-independent CCR5-dependent virus infection (Fig. 1*B*). As was observed with 8x Env in fusion assays, the presence of CD4 increased the efficiency of virus entry. Neither IIIB-V3BaL nor 8x-V3BaL used CXCR4 in the presence or absence of CD4 (data not shown). We also purified recombinant IIIB-V3BaL and 8x-V3BaL gp120 proteins and found that IIIB-V3BaL gp120 bound to CCR5 in a CD4-dependent manner, whereas 8x-V3BaL bound to CCR5 independently of CD4 (Fig. 2). Neither protein bound to CXCR4 under any conditions examined. Thus, changes in the V3 loop affected which coreceptor was used but did not impact CD4-independence.

Table 1. Apparent kinetic and equilibrium constants derived from binding of gp120 to CD4i antibodies in biosensor experiments

| Antibody | $k_{on}$, 1/Ms | $k_{off}$, 1/s | $K_d$, nM |
|---|---|---|---|
| Binding to 17B | | | |
| 8x | $1 \times 10^5$ | $2 \times 10^{-3}$ | 15.0 |
| 8x/CD4 | $2 \times 10^5$ | $9 \times 10^{-4}$ | 4.5 |
| IIIB | $8 \times 10^3$ | $2 \times 10^{-5}$ | 2.5 |
| IIIB/CD4 | $3 \times 10^5$ | $5 \times 10^{-5}$ | 0.2 |
| Binding to 48d | | | |
| 8x | $2 \times 10^5$ | $1 \times 10^{-3}$ | 6.0 |
| 8x/CD4 | $3 \times 10^5$ | $6 \times 10^{-4}$ | 2.0 |
| IIIB | $1 \times 10^4$ | $5 \times 10^{-5}$ | 5.0 |
| IIIB/CD4 | $5 \times 10^5$ | $3 \times 10^{-5}$ | 0.1 |

The CD4i antibodies 17b and 48d were attached to the biosensor surface, and serial dilutions of 8x and IIIB gp120 were measured for both binding and dissociation. Similar measurements were also performed with serial dilutions of Env that had been premixed with a saturating amount of sCD4. All binding reactions were performed at 25°C. A sample sensogram is shown in Fig. 4. The best fitted values for the slopes of the linearized plots of the data ($r^2 \geq 0.98$) are reported. The parameters estimated by fitting the simple 1:1 Langmuir interaction model globally were within 15% of the reported values. The off rates for IIIB and IIIB/sCD4 for both antibodies were so slow that they were at the limits of detection for the biosensor, making the affinity constants derived from these values less accurate.

**Neutralization of IIIBx.** Antibodies that block Env–coreceptor interactions can neutralize HIV-1 (1, 2). The exposed nature of the coreceptor-binding site in HIV-1 IIIBx gp120 might therefore be expected to make this virus more sensitive to antibody-mediated neutralization. Several SIV and HIV-1 strains with modifications in the V1/V2 region have been shown to be neutralization sensitive, presumably because

Table 2. Virus neutralization by sera and mAbs

| Serum or mAb | Immunogen | HIV-1/IIIB | | HIV-1/IIIBx | |
|---|---|---|---|---|---|
| | | 50% | 90% | 50% | 90% |
| Serum | | | | | |
| Rabbit 1169 | IIIB | 1,934 | 132 | 3,081 | 287 |
| Rabbit 1170 | IIIB | 1,869 | 109 | 93,756 | 5,616 |
| Rabbit 1171 | 8x | >10,240 | 1,005 | >163,840 | 13,748 |
| Rabbit 1172 | 8x | 1,552 | 94 | 64,295 | 4,426 |
| Human ZT02575 | | 1,616 | 46 | 20,894 | 4,195 |
| Human JT02140 | | 155 | 11 | 892 | 187 |
| | | IIIB-V3BaL | | 8x-V3BaL | |
| | | 50% | 90% | 50% | 90% |
| mAb | | | | | |
| 17b | | 14 | 90 | 2 | 15 |
| 48d | | >5,000 | >5,000 | 3 | >200* |
| 50.1 | | 95 | 375 | 45 | 610 |

For serum neutralizations, equivalent amounts of HIV-1 IIIB and IIIBx were used to infect MAGI cells. Infection was determined 72 hr after infection as previously described (33). For mAbs 17b, 48d, and 50.1, equivalent amounts of luciferase reporter viruses bearing the IIIB-V3BaL or 8x-V3BaL Envs were used to infect GHOST-CCR5 cells that were lysed 48 hr after infection. For all infections, virus and serial dilutions of human sera, rabbit sera, or different concentrations of mAbs were mixed for 1 hr before addition to target cells. The dilution of sera or the concentration of mAb (ng/ml) required to neutralize 50% and 90% of input virus is indicated. mAb 48d did not neutralize IIIB-V3BaL under any condition tested, and only 88% neutralization of 8x-V3BaL was achieved by this mAb at a concentration of 200 ng/ml (indicated by * in table). mAb 50.1 is directed against the BaL V3-loop (43).

of increased exposure of conserved determinants (35, 36). We therefore compared the relative sensitivities of HIV-1/IIIB and IIIBx to neutralization by HIV-positive human sera and to sera from rabbits immunized with either IIIB or 8x gp120. We found that IIIBx was uniformly more sensitive to neutralization than the parental HIV-1 IIIB, in many cases by one log or more (Table 2). In addition, we found that virions containing 8x-V3BaL Env were much more sensitive to neutralization than were virions with IIIB-V3BaL Env by the CD4i mAbs 17b and 48d but not by an antibody recognizing the V3 loop, which was identical between these two viruses (Table 2). Thus, the increased exposure of the coreceptor-binding site as well as of CD4i epitopes is likely to account for the increased sensitivity of IIIBx to antibody-induced neutralization.

## DISCUSSION

A number of HIV-1, HIV-2, and SIV virus strains have been described that short-circuit the normal viral entry process by interacting directly with CCR5 or CXCR4 to infect cells (21, 22). Although CD4-independent viruses may impact viral tropism and pathogenesis, they also serve as useful tools for dissecting the virus entry pathway. Two lines of evidence indicate that the CD4-independent HIV-1 Env studied here exists in a stable, partially triggered state in which the conserved coreceptor-binding site is well exposed. First, 8x gp120 bound directly to CXCR4, while the parental IIIB gp120 bound CXCR4 in a CD4-dependent manner. Second, 8x gp120 bound much more rapidly to two CD4i mAbs than did the parental CD4-dependent protein. However, as a consequence of a faster off-rate, the overall affinity of 8x gp120 for CD4i mAbs was similar to that of HIV-1 IIIB gp120, providing a striking example of how important differences in protein–protein interactions can be revealed by the real-time analysis afforded by the use of an optical biosensor. The faster off-rate exhibited by 8x relative to IIIB could be caused by mutation of residue 423 in Env, which serves as a contact site for 17b (16) in the 8x protein (Fig. 3).

HIV-1 tropism is governed in large part by coreceptor choice. The ability of a virus to use CCR5, CXCR4, or both largely dictates the type of CD4-positive cells it can enter. The V3 loop in gp120 plays a critical role in coreceptor choice, with the V1/2 region playing a more subsidiary role (10–15). We have shown that the determinants underlying coreceptor choice and CD4-independence in 8x Env are dissociable [C.C.L., T.L.H., J. Romano, B. S. Haggarty, T. J. Matthews, R.W.D. and J.A.H. (unpublished work)]. Thus, 8x Env containing a V3 loop from an R5 Env maintains its CD4-independent phenotype but now uses CCR5 rather than CXCR4 for cell–cell fusion and virus infection. We also found that 8x-V3BaL gp120 is able to bind to CCR5-expressing cells in the absence of CD4. These findings clearly demonstrate that coreceptor choice and CD4-independent use of a chemokine receptor are dissociable in the context of both virus infection and gp120 binding [C.C.L., T.L.H., J. Romano, B. S. Haggarty, T. J. Matthews, R.W.D. and J.A.H. (unpublished work)]. This finding also supports the proposal that the coreceptor-binding region can interact with both CXCR4 and CCR5, depending on the nature of the associated V3 loop (17). Thus, in the context studied here, the V3 loop can affect coreceptor choice in a partially triggered Env as well. It will be important to clarify the respective roles the variable regions and the conserved binding site play in coreceptor interactions and to identify the domains in CCR5 and CXCR4 with which each interacts.

A particularly striking feature of HIV-1 IIIBx was its sensitivity to neutralization. IIIBx was approximately 10-fold more sensitive to neutralization than IIIB by HIV-positive human sera as well as to rabbit sera generated against either IIIB gp120 or 8x gp120. The sensitivity of IIIBx to antibody-mediated neutralization suggests that one or more neutralization determinants in this partially triggered Env are more accessible to antibodies than in the parental CD4-dependent Env. To determine whether increased exposure of the V3 loop was responsible for this phenotype, we generated IIIB-V3BaL and 8x-V3BaL pseudotyped virions and examined their sensitivity to neutralization by mAb 50.1. We could not directly compare the sensitivities of IIIB and IIIBx to neutralization by V3-loop antibodies because of differences in their V3-loop sequences. mAb 50.1 neutralized IIIB-V3BaL and 8x-V3BaL equally well (Table 2). By contrast, CD4i mAbs neutralized 8x-V3BaL Env-pseudotyped virions more effectively than IIIB-V3BaL virions, suggesting that the conserved coreceptor-binding site is a likely target that may account for the neutralization-sensitive phenotype of IIIBx. In addition, the 8x Env also exhibits a remarkable loss of five glycosylation sites relative to parental IIIB, raising the possibility that the loss of carbohydrates could play a role in exposing this region [C.C.L., T.L.H., J. Romano, B. S. Haggarty, T. J. Matthews, R.W.D. and J.A.H. (unpublished work)]. Several other neutralization-sensitive viruses have been described recently, including a SIVmac239 lacking glycosylation sites in the V1/V2 region (36), a HIV-1 SF162 strain containing a deletion in V1 (35), and an HXBc2 Env containing a V1/V2 deletion that was shown to be more sensitive to neutralization by 17b as well as V3-loop antibodies (37). It will be interesting to determine whether the coreceptor-binding site that adjoins the V1/2 stem is exposed in these viruses as well, enabling these Envs to bind directly to coreceptors.

Numerous studies have shown that immunization with recombinant gp120 typically fails to generate broadly cross-reactive neutralizing antibodies, yet it is clear that such antibodies are generated in some individuals as a consequence of virus infection (reviewed by ref. 38). It is possible that only strain-specific neutralization has been observed after immunization by gp120, because conserved regions of Env are sequestered in CD4-dependent gp120s before CD4 binding. Our results show that premature exposure of the conserved coreceptor-binding site results in a neutralization-sensitive phenotype, suggesting that the humoral immune response may exert considerable selective pressure against exposure of this region, perhaps accounting for the fact that this site either is not exposed or is not generated until CD4 binding. Increased neutralization of CD4-independent viruses may explain the lack of CD4-independence among primary isolates of HIV-1. We have found that many primary SIV strains can use rhesus CCR5 to infect cells in the absence of CD4, at least to some degree, suggesting that CCR5 was the primordial SIV receptor (21). Acquisition of the ability to use CD4 may provide the means to sequester the conserved face in gp120 that mediates CCR5 binding, thus providing a mechanism to escape neutralization by antibodies to this region.

If exposure of a highly conserved region such as the coreceptor-binding site renders virus more sensitive to neutralization, might immunization with Envs that are partially triggered result in more efficient generation of broadly cross-reactive neutralizing antibodies directed against this region? For antibodies against this site to be effective, they must be able to access the coreceptor-binding site in native CD4-dependent Env proteins, perhaps after CD4 binding induces a triggered conformation in which access to this region is enhanced. Identifying determinants that render Envs CD4 independent and that influence exposure of this region will make it possible to systematically address the potential of this site to elicit neutralizing antibodies. Indeed, CD4- and coreceptor-triggered forms of Env were recently used to generate an impressive humoral immune response in mice against diverse primary virus isolates (39). Although the immunological targets in Env that underlie this interesting finding have not yet

been identified, antibodies directed to the coreceptor-binding site in gp120 or determinants in gp41 are obvious candidates.

That the coreceptor-binding site can be stably exposed may have implications for viral entry as well. Conceivably, exposure and/or formation of the coreceptor-binding site subsequent to CD4 binding could result in a conformation of Env that is relatively unstable, requiring interactions with a coreceptor within a short period of time. For example, triggering the conformational change in the Semliki Forest virus spike glycoprotein by acid pH leads to rapid inactivation of the protein's membrane fusion potential, unless it can interact with its lipid coreceptors within several minutes (40). However, the ability of the 8x Env protein to exist in a partially triggered but stable state and bind CXCR4 suggests that exposure of the coreceptor-binding site is compatible with a long-lived Env conformation. Thus, coreceptor binding could occur long after the conformational changes induced by CD4 that make this event possible, perhaps accounting for the ability of HIV-1 to infect cells that express very low levels of coreceptor when adequate levels of CD4 are present (41, 42).

In conclusion, we have found that the CD4-independent phenotype of the 8x Env protein is associated with stable exposure of the coreceptor-binding site as well as a neutralization-sensitive phenotype. Thus, this protein likely represents a structural intermediate of the normal fusion process and can be used to investigate the structural parameters that influence the conformational changes that lead to membrane fusion. Importantly, the highly conserved nature of this stably exposed domain and the fact that the neutralizing antibodies can be directed against it raise the possibility that this domain, if properly presented, can be used to elicit broadly cross-reactive neutralizing antibodies.

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