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# Characterization of CD4-Induced Epitopes on the HIV Type 1 gp120 Envelope Glycoprotein Recognized by Neutralizing Human Monoclonal Antibodies

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## ABSTRACT

**The entry of human immunodeficiency virus (HIV-1) into target cells typically requires the sequential binding of the viral exterior envelope glycoprotein, gp120, to CD4 and a chemokine receptor. CD4 binding exposes gp120 epitopes recognized by CD4-induced (CD4i) antibodies, which can block virus binding to the chemokine receptor. We identified three new CD4i antibodies from an HIV-1-infected individual and localized their epitopes. These epitopes include a highly conserved gp120 $\beta$-strand encompassing residues 419–424, which is also important for binding to the CCR5 chemokine receptor. All of the CD4i antibodies inhibited the binding of gp120–CD4 complexes to CCR5. CD4i antibodies and CD4 reciprocally induced each other's binding, suggesting that these ligands recognize a similar gp120 conformation. The CD4i antibodies neutralized laboratory-adapted HIV-1 isolates; primary isolates were more resistant to neutralization by these antibodies. Thus, all known CD4i antibodies recognize a common, conserved gp120 element overlapping the binding site for the CCR5 chemokine receptor.**

## INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS (HIV-1) is the major cause of acquired immunodeficiency syndrome (AIDS) in humans.[1,2] AIDS results from a progressive loss of $CD4^+$ T-lymphocytes that accompanies chronic HIV-1 infection.[3–8] The $CD4^+$ T lymphocytes represent major target cells for HIV-1, and this tropism is determined by specific interactions of the viral envelope glycoproteins and host cell receptors, CD4 and members of the chemokine receptor family.[9–11] Receptor binding is mediated by the gp120 exterior envelope glycoprotein,[9–11] which is organized into a trimeric complex along with the gp41 transmembrane envelope glycoprotein.[12,13] The binding of gp120 to CD4 induces conformational changes that allow gp120 to interact with the chemokine receptors, CCR5 or CXCR4.[10,14–18] One of these CD4-induced conformational changes is a shift in the position of the large, surface-exposed V1/V2 variable loops of gp120, which are thought to mask the chemokine receptor-binding site on gp120.[19–22] Chemokine receptor binding apparently involves a conserved structure on the gp120 surface and sequences in the third variable (V3) loop.[9–11,23–27] The conserved gp120 structures involved in receptor binding have been defined by X-ray crystallographic analysis of HIV-1 gp120 core elements, mutagenesis, and antibody competition analyses.[21,23,24,28–30] Receptor binding is believed to trigger additional conformational changes in the HIV-1 envelope glycoproteins that lead to exposure of the gp41 transmembrane envelope glycoprotein and to gp41-mediated fusion of the viral and target cell membranes.[31,32]

The persistence of HIV-1 infection and eventual disease induction in most infected hosts imply that the virus can evade and, ultimately, eliminate the immune response.[33,34] The HIV-1 envelope glycoproteins possess surface-exposed loops that exhibit considerable variation among strains. A high degree of glycosylation and conformational flexibility are other features that are thought to minimize the elicitation or effect of neutralizing antibodies.[21,35] Many antibodies elicited by the HIV-1 envelope glycoproteins do not exhibit the ability to neutral-

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ize the virus. Neutralizing antibodies elicited relatively early in the course of natural infection often inhibit the infection of a limited number of viral strains, and typically are directed against variable loops. Later-arising antibodies exhibit broader neutralizing activity against HIV-1 strains. These antibodies include CD4-binding-site (CD4BS) antibodies, which block CD4 binding, and CD4-induced (CD4i) antibodies, which recognize gp120 structures that are formed or exposed by CD4 binding. The CD4i antibodies block the binding of HIV-1 gp120 to the chemokine receptors. To date, only two HIV-1-specific CD4i antibodies, designated 17b and 48d, have been identified and studied.[22,36–38] The binding of these antibodies to HIV-1 gp120 can be disrupted by changes in the bridging sheet, a highly conserved gp120 element implicated in chemokine receptor binding. X-ray crystal structures of two HIV-1 gp120 cores complexed with CD4 and Fab fragments of the 17b antibody have been determined, allowing precise definition of the 17b epitope.[28,29] The epitopes for the 17b and 48d antibodies are thought to be partially masked by the second variable (V2) loop of gp120, and virus variants lacking the V1/V2 or V2 loops exhibit greater sensitivity to neutralization by these antibodies.[20,22,39,40] The gp120 variable loop conformations on many primary HIV-1 isolates apparently mask the 17b and 48d epitopes quite effectively, because these viruses are relatively resistant to 17b and 48d neutralization.[21,22,38,41–44] Steric factors dictate that to neutralize HIV-1 effectively, these antibodies must bind the viral envelope glycoproteins prior to the engagement of the CD4 receptor on the target cell.[20] Thus, although the conservation of these antibody epitopes is an attractive feature, HIV-1 has evolved mechanisms to diminish their accessibility to antibodies.

To obtain further understanding of gp120 epitopes induced by CD4 binding, we identified three new CD4i antibodies (23e, 21c, and 49e) from HIV-1-infected individuals. We characterized their binding to wild-type and mutant gp120 glycoproteins, the influence of CD4 binding on their interaction with gp120, and their neutralizing ability.

## MATERIALS AND METHODS

### *Cells*

293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin/streptomycin. CCR5 binding assays utilized Cf2Th-synCCR5 cells.[12] To study the entry of R5 viruses, Cf2Th cells expressing CD4 and CCR5 were used. To study the entry of X4 isolates, Ghost cells expressing CD4 and CXCR4 were used.

### *Mutant HIV-1 gp120 glycoproteins*

Single amino acid changes were introduced into the wtΔ protein from the YU2 isolate, a primary R5 HIV-1 derived directly from the brain of an infected individual. The wtΔ protein contains deletions of gp120 residues 31–81 in the N-terminus and 128–194, removing the V1 and V2 variable loops but retaining the conserved V1/V2 stem. (Numbering of gp120 amino acid residues is based on the sequence of the prototypic HXBc2 strain of HIV-1, according to current convention.[45])

### *Antibodies*

The human monoclonal antibodies (HMAbs) 17b, 23e, 49e, 21c, and 48d were derived by Epstein–Barr virus (EBV) transformation of B cells from cryopreserved peripheral blood mononuclear cells (PBMC) obtained from HIV-1-infected individuals. The 23e, 49e, and 21c monoclonal antibody-producing cell lines were obtained from an HIV-1 infected long-term nonprogressor (AD19) followed at the Aaron Diamond AIDS Research Center. EBV-inoculated PBMC cultures were plated in multiple 96-well culture plates containing irradiated, mature human macrophages as feeder cells, as previously described.[46,47] Supernatant fluids from transformed cultures were screened for antibodies binding to HIV-1 envelope glycoproteins using a "reverse capture" sandwich immunoassay, according to our published method.[46] Briefly, potential HMAbs in B cell culture fluids were first captured in wells of ELISA plates coated with 2 $\mu$g/ml goat anti-human IgG-Fc. Detergent-solubilized virus containing 10% normal human serum (Irvine Scientific) was incubated in the wells to allow binding of viral antigens to immobilized antibodies. Detection of bound viral envelope glycoproteins was accomplished by addition of a mixture of several biotinylated HMAbs (17b, A32, C11), also diluted in buffer containing 10% human serum, which recognize nonoverlapping, well-conserved sites on HIV-1 gp120. In the present experiments we screened for antibodies binding to a mixture of HIV-1 strains (IIIB and J62).[47] Antigen stocks consisted of supernatant fluids of cultures of MT4 cells chronically infected with each virus. Culture fluids were collected once weekly, clarified by centrifugation, and treated with 1% Triton-X100 to inactivate virus infectivity and solubilize the glycoproteins. We used several noncompeting, biotin-labeled HMAbs to make sure that if the captured HMAb competed for the binding site of one labeled HMAb, one of the other labeled HMAbs would still detect bound gp120. Normal human serum (10%) was added to the dilution buffer in both the virus and biotin-HMAb steps to saturate all anti-IgG-Fc binding sites, thus preventing binding of the biotinylated antibodies, which otherwise would cause unacceptably high background signals. Microwell cultures that contained antibody-producing cells were subcultured at low cell densities and rescreened for antibody production. Stable antibody-producing cell lines were finally cloned at limiting dilutions. To improve cell growth and antibody production, the cell lines producing 23e and 21c HMAbs subsequently were converted to hybridomas by fusion with HMMA cells, kindly provided by Marshall Posner. HMAbs were purified from several liters of culture supernatant using protein A affinity chromatography. Protein concentration was determined by the bicinchinonic acid (BCA) method (Pierce Chemical Co.).

### *Antibody binding competition assays*

To test the effect of soluble CD4 (sCD4) on HMAb binding, the wild-type YU2 gp120 glycoprotein was captured on an ELISA plate by the D7324 antibody (Aalto BioReagents, Dublin, Ireland) which is directed against the HIV-1 gp120 C-terminus.[30] HMAbs were added to the wells for 1 hr in the absence or presence of 10 $\mu$g/ml sCD4. After washing, the bound antibody was detected with horseradish peroxidase-conjugated anti-human IgG antibody (Sigma).

To test the ability of the HMAbs to compete with the 17b antibody for gp120 binding, the 17b HMAb was biotinylated

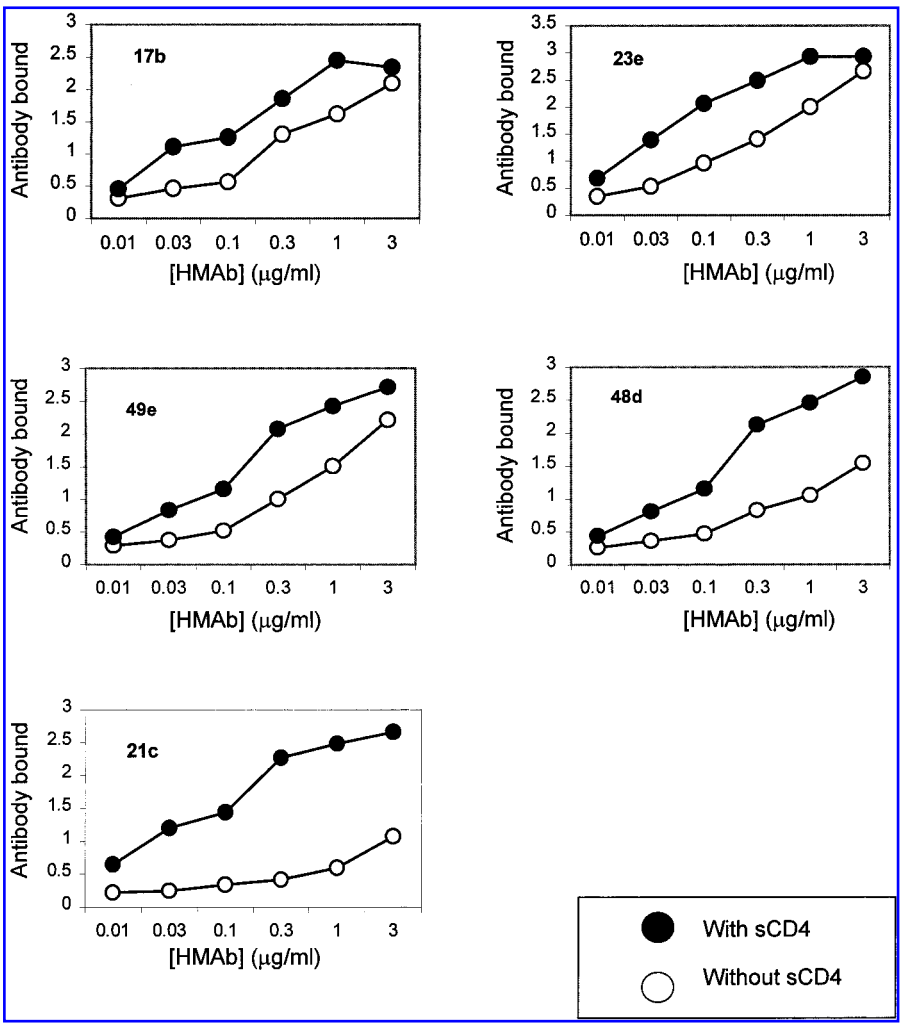


**FIG. 1.** Effects of sCD4 on HMAb binding to HIV-1 gp120. The gp120 glycoprotein from the YU2 HIV-1 isolate was captured on ELISA plates by the D7324 antibody. The captured gp120 glycoprotein was incubated without sCD4 (open circles) or with 10 $\mu$g/ml sCD4 (filled circles), together with the HMAbs 17b, 23e, 49e, 21c, and 48d. The bound antibody was detected as described under Materials and Methods. The amount of antibody bound is expressed in optical density units.

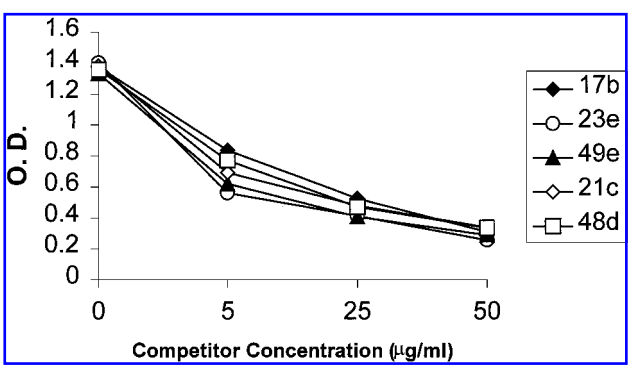


**FIG. 2.** Ability of CD4i HMAbs to compete with 17b antibody for gp120. The HIV-1 gp120 glycoprotein was captured on ELISA plates with the D7324 antibody. The binding of biotinylated 17b antibody to the gp120 glycoprotein in the presence of the indicated concentrations of competitor HMAbs was detected by avidin-peroxidase as described under Materials and Methods.

using EZ-link sulfo-NHS-LC-biotin (Pierce) according to the manufacturer's instructions. Ninety-six-well ELISA plates were coated with the sheep D7324 antibody (Aalto BioReagents, Dublin, Ireland), which is directed against the extreme C-terminus of HIV-1 gp120.[30] The wtΔ gp120 protein of the YU2 HIV-1 strain was produced by transient expression of 293T cells and added to the D7324 antibody-coated wells. A dose–response curve was generated for 17b-biotin, and a concentration of 0.5 $\mu$g/ml 17b-biotin was chosen because this concentration was not saturating, yet yielded a robust signal in the assay. For the competition assay, various concentrations of the competitor antibody were first mixed with the 17b-biotin (0.5 $\mu$g/ml) and then added to the wells containing the captured gp120 glycoproteins. The 17b antibody itself and the 2/11c antibody were used as positive and negative competitor controls, respectively. The bound 17b-biotin was detected with avidin-peroxidase conjugate (Sigma), and the assays were developed with the TMB peroxidase EIA substrate (BioRad).

*CCR5 binding assay*

To examine whether the HMAbs competed with the ability of gp120 to bind CCR5, 100 $\mu$l of $^{35}$S-labeled gp120 was incubated with approximately $5 \times 10^6$ Cf2ThsynCCR5 cells[12] and with various concentrations of HMAbs in the presence of soluble CD4 (5 $\mu$g/ml). After 1.5 hr incubation at room temperature, the cells were pelleted (3000 rpm for 2 min), washed with phosphate-bufferedsaline (PBS), and lysed in NP-40 buffer. The lysates were centrifuged at 14,000 rpm at 4°C for 5 min to remove cell debris. The supernatants containing the bound envelope glycoproteins were precipitated by a mixture of sera from HIV-1-infected individuals and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

TABLE 1. RECOGNITION OF HIV-1 gp120 MUTANTS BY LIGANDS[a]

| *gp120 region* | *Envelope protein* | *sCD4* | *Serum* | *17b* | *23e* | *49e* | *21c* | *48d* | *CCR5* |
|---|---|---|---|---|---|---|---|---|---|
| | YU2 wtΔ | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| C1 | 107 D/R | 1.02 | 0.98 | 0.97 | 0.73 | 0.94 | 0.65 | 0.85 | 1.02 |
| | 114 Q/L | 0.79 | 0.60 | 0.73 | 1.08 | 1.34 | 0.79 | 0.97 | 1.22 |
| | 117 K/D | 0.74 | 0.75 | 0.40 (+) | 0.29 (+) | 0.02 (−) | 0.30 (+) | 0.00 (+) | 0.15 |
| | 121 K/D | 0.73 | 1.15 | 0.00 (+) | 0.17 (+) | 0.00 (+) | 0.10 (+) | 0.00 (−) | 0.07 |
| | 122 L/S | 0.84 | 1.80 | 1.07 | 1.08 | 0.97 | 0.94 | 0.08 (+) | 0.98 |
| | 123 T/D | 0.99 | 0.70 | 1.06 | 0.89 | 0.56 | 1.06 | 0.00 (−) | 0.08 |
| C2 | 197 N/D | 1.34 | 1.01 | 0.80 | 0.83 | 1.40 | 0.97 | 1.75 | 1.33 |
| | 199 S/L | 1.32 | 0.95 | 0.94 | 0.44 | 0.92 | 1.40 | 2.00 | 1.50 |
| | 200 V/S | 0.91 | 0.94 | 1.05 | 1.57 | 0.96 | 0.43 | 0.00 (+) | 0.84 |
| | 201 I/A | 0.90 | 1.28 | 0.67 | 1.13 | 1.40 | 0.73 | 0.02 (+) | 0.46 |
| | 203 Q/L | 0.85 | 1.18 | 0.88 | 0.97 | 0.73 | 0.73 | 0.01 (+) | 0.68 |
| | 207 K/D | 0.85 | 1.03 | 0.10 (+) | 1.20 | 0.00 (−) | 0.68 | 0.01 (−) | 0.00 |
| | 209 S/L | 1.11 | 0.87 | 0.85 | 0.89 | 0.67 | 0.81 | 0.92 | 1.00 |
| | 210 F/S | 0.81 | 1.36 | 0.81 | 0.81 | 1.45 | 1.24 | 1.22 | 0.65 |
| | 211 E/K | 1.13 | 1.44 | 1.03 | 1.07 | 1.37 | 1.52 | 0.79 | 0.73 |
| | 257 T/D | 0.00 | 0.38 | 0.80 (+) | 0.18 (+) | 0.01 (+) | 0.05 (+) | 0.00 (+) | 0.05 |
| V3 | 295 N/E | 0.75 | 1.29 | 0.73 | 0.64 | 1.18 | 1.28 | 1.39 | 0.86 |
| | 308 N/D | 1.10 | 1.63 | 0.89 | 0.55 | 1.32 | 0.69 | 1.21 | 0.31 |
| | 311 L/S | 1.12 | 1.02 | 1.05 | 1.09 | 1.40 | 1.08 | 1.93 | 0.08 |
| | 330 H/A | 0.75 | 1.01 | 0.55 | 0.88 | 0.81 | 0.60 | 1.05 | 0.22 |
| | ΔV3 (Δ298-329) | 0.80 | 1.45 | 0.00 (+) | 0.00 (+) | 0.00 (+) | 0.00 (+) | 0.00 (+) | 0.00 |
| C3 | 370 E/Q | 0.00 | 0.66 | 1.04 | 1.54 | 1.04 | 0.83 | 0.00 (+) | 0.17 |
| | 372 V/S | 1.03 | 1.14 | 1.08 | 1.02 | 0.67 | 0.77 | 0.96 | 0.85 |
| | 373 T/D | 1.12 | 0.77 | 1.10 | 1.34 | 1.43 | 1.19 | 0.71 | 0.48 |
| | 377 N/E | 0.71 | 1.00 | 0.52 | 0.55 | 0.46 | 2.00 | 1.83 | 0.22 |
| | 381 E/R | 0.81 | 1.27 | 0.20 (+) | 1.15 | 0.09 (−) | 1.50 | 0.00 (−) | 0.07 |
| | 383 F/S | 0.00 | 0.49 | 0.02 (+) | 0.04 (+) | 0.00 (+) | 0.00 (+) | 0.00 (+) | 0.04 |
| | 386 N/D | 1.14 | 1.83 | 0.97 | 1.62 | 1.18 | 1.32 | 1.04 | 1.22 |
| C4 | 419 R/D | 0.86 | 1.12 | 0.00 (+) | 1.48 | 0.98 | 1.60 | 0.00 (−) | 0.19 |
| | 420 I/R | 0.59 | 0.92 | 0.00 (−) | 0.00 (−) | 0.00 (−) | 0.10 (−) | 0.00 (−) | 0.06 |
| | 421 K/D | 0.86 | 0.56 | 0.00 (+) | 1.41 | 0.86 | 1.00 | 0.00 (−) | 0.07 |
| | 422 Q/L | 0.53 | 0.80 | 0.00 (−) | 0.03 (+) | 0.47 (+) | 0.00 (−) | 0.00 (−) | 0.07 |
| | 423 I/S | 0.97 | 1.05 | 0.00 (−) | 1.20 | 1.11 | 0.88 | 0.00 (−) | 0.61 |
| | 424 I/S | 0.25 | 0.58 | 0.48 (+) | 0.15 (+) | 0.04 (+) | 0.25 (+) | 0.00 (+) | 0.37 |
| | 426 M/A | 0.69 | 0.85 | 0.69 | 0.74 | 0.58 | 0.30 | 0.08 (+) | 0.75 |
| | 429 E/R | 1.17 | 1.04 | 1.00 | 1.11 | 0.68 | 0.70 | 1.75 | 1.54 |
| | 432 K/A | 1.00 | 0.52 | 0.92 | 0.81 | 1.00 | 1.02 | 0.00 (+) | 0.06 |
| | 434 M/A | 0.90 | 1.31 | 0.65 | 0.77 | 0.63 | 1.00 | 0.00 (−) | 1.22 |
| | 435 Y/S | 0.33 | 0.85 | 0.00 (+) | 0.13 (+) | 0.00 (+) | 0.00 (+) | 0.00 (+) | 0.21 |
| | 436 A/S | 1.05 | 0.97 | 0.91 | 1.03 | 1.09 | 0.93 | 1.73 | 0.98 |
| | 437 P/A | 0.80 | 1.42 | 0.68 | 0.59 | 0.79 | 0.53 | 0.00 (−) | 1.79 |
| | 438 P/A | 1.18 | 0.74 | 1.00 | 1.08 | 0.79 | 0.89 | 0.10 (+) | 0.06 |
| | 439 I/A | 0.68 | 1.04 | 0.76 | 0.92 | 1.00 | 1.33 | 1.28 | 0.45 |
| | 440 R/D | 1.03 | 0.99 | 1.05 | 0.94 | 1.04 | 1.45 | 0.02 (+) | 0.09 |
| | 441 G/V | 0.67 | 1.10 | 0.70 | 0.96 | 0.77 | 1.43 | 0.02 (+) | 0.00 |
| | 442 Q/L | 1.11 | 0.74 | 0.74 | 1.03 | 1.38 | 0.70 | 1.54 | 2.00 |
| | 444 R/D | 0.79 | 1.04 | 0.67 | 1.26 | 1.18 | 0.75 | 0.40 | 0.25 |
| C5 | 474 D/R | 0.59 | 1.02 | 0.81 | 1.09 | 1.34 | 0.72 | 0.52 | 1.03 |

[a]The residue number of the mutants is based on sequence of the prototype strain HXBc2.[45] The value for ligand binding was determined by immunoprecipitation of radiolabeled protein and quantified by a Phosphorlmager. The values were normalized by the formula as follows: (mutant protein/wt protein) ligand × (wt protein/mutant protein) serum mixture. Values for sCD4, CCR5, and 17b binding are from Rizzuto *et al.*[24] + indicates that ligand binding is restored to near-wild-type levels by incubation with sCD4.

*Transient expression of HIV-1 envelope glycoprotein variants*

293T cells grown to 70% confluency in 100-mm dishes were transfected with 2 $\mu$g of a plasmid expressing the wtΔ protein or mutant derivatives thereof, and 1 $\mu$g of a plasmid expressing the HIV-1 Tat protein, using the Effectene Transfection Reagent (QIAGEN). Forty-eight hours later, the medium was removed, the cells were washed once with 10 ml PBS, and labeling medium [4.5 ml DMEM, 0.5 ml heat-inactivated, dialyzed FBS, 50 $\mu$l penicillin-streptomycin solution, and 20 $\mu$l (~230 $\mu$Ci) [$^{35}$S]cysteine] was added. The cells were incubated

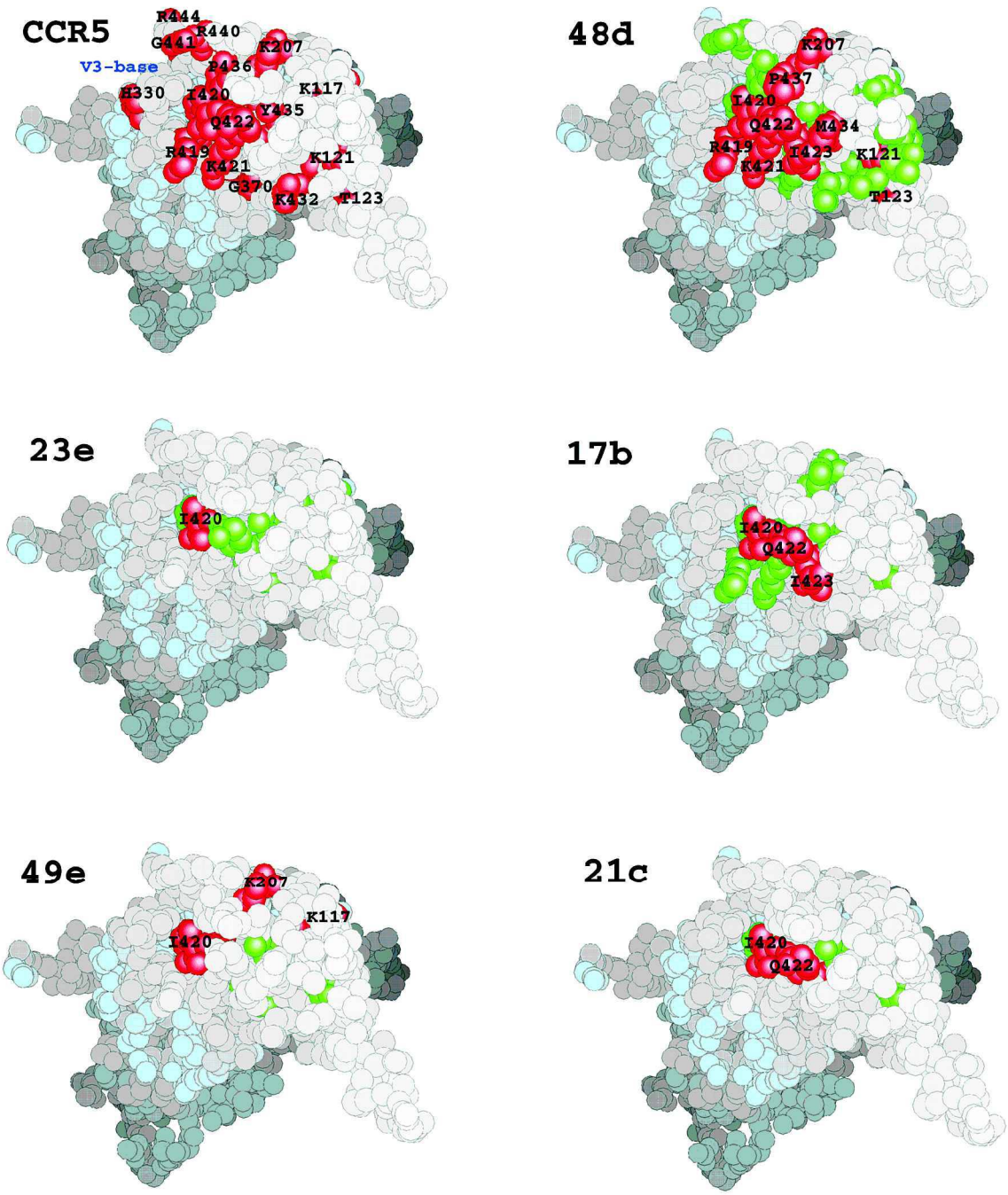


**FIG. 3.** (**A–F**) HIV-1 gp120 structures implicated in CCR5 and CD4i-HMAb binding. A CPK model of the HIV-1 YU2 gp120, derived from the ternary gp120–CD4-17b complex,[28] is shown from the perspective of the target cell. Amino acid residues in which changes reduce the binding of ligands by 70% or more are colored. The residues in green indicate that ligand binding was restored by sCD4 binding. Changes in the residues colored red resulted in decreased ligand binding that was not restored by sCD4. The red residues are labeled with the residue number corresponding to that of the prototypic HXBc2 sequence.[45] The residues important for CCR5 and 17b binding were previously defined by Rizzuto *et al.*[23,24]

at 37°C for 24 hr and the medium was collected. The medium was cleared by centrifugation and stored at 4°C.

### *Immunoprecipitation of radiolabelled envelope glycoproteins*

For precipitation of radiolabeled HIV-1 envelope glycoproteins, 400 μl medium containing the labeled proteins was mixed with 100 μl of 10% protein A-Sepharose (Pharmacia), 50 μl 4% bovine serum albumin, and either 1–2 μg HMAb or 4 μl of a mixture of HIV-1–infected sera. PBS was added to bring the total volume to 1 ml. The samples were rocked at 4°C overnight or at room temperature for 2 hr. The Sepharose beads were then washed twice with 1 ml 0.5 M NaCl in PBS and once with 1 ml PBS. The beads were mixed with 2× gel loading buffer and boiled for 3 min. Following the removal of the beads by centrifugation, the supernatants were loaded on a 10% SDS–polyacrylamide gel. The gel was enhanced with Autofluor (National Diagnostic) for 45 min before drying at 80°C for 2 hr and exposure to film. The gel was also used for PhosphorImager (Molecular Dynamics) analysis.

### *HIV-1 neutralization assay*

The HIV-1-neutralizing ability of the HMAbs was tested using a single-round virus entry assay. Recombinant HIV-1 expressing the firefly luciferase gene was produced by transfecting 293T cells with the pCMV Gag-Pol packaging construct and the pHIV-luc vector, along with a pSVIIIenv plasmid expressing the envelope glycoproteins of different HIV-1 strains.[20] Two days after transfection, the cell supernatants were harvested and frozen in aliquots.

The target cells (either Cf2Th cells expressing CD4 and CCR5 or Ghost cells expressing CD4 and CXCR4) were seeded at a density of $10^5$ cells/well in a 24-well plate and cultured overnight at 37°C. From 10,000 to 30,00 reverse transciptase units of virus was incubated with serial dilutions of antibody for 1 hr at 37°C in a 500 μl volume. The target cells were washed once with PBS and the virus–antibody mixture was added to the cells. The cells were cultured for 3 days, washed once with PBS, and lysed with 150 μl lysis buffer for the luciferase assay (Tuner 20, Promega).

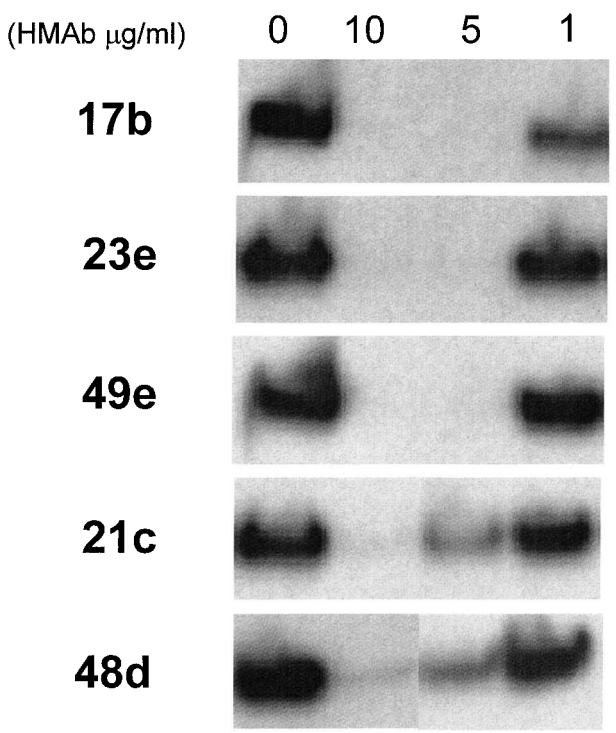


**FIG. 4.** Ability of CD4i HMAbs to inhibit gp120-CCR5 binding. Radiolabeled gp120 was mixed with sCD4 and then incubated with Cf2ThsynCCR5 cells in the presence of the indicated concentrations of CD4i HMAbs. The cells were washed and the bound gp120 was precipitated as described under Materials and Methods. The gp120 glycoprotein bound to the cells is shown.

## RESULTS

### *Effects of CD4 binding on HMAb recognition of gp120*

The effects of soluble CD4 (sCD4) binding on the recognition of the HIV-1 gp120 glycoprotein by the newly identified HMAbs 23e, 49e, and 21c were examined. The wild-type gp120 glycoprotein derived from the YU2 R5 HIV-1 strain was captured on an ELISA plate with a polyclonal serum (D7324) directed against the gp120 C-terminus. The captured gp120 was incubated with the HMAbs in the absence or presence of sCD4. Figure 1 shows that the recognition of the YU2 gp120 glycoprotein by the 23e, 49e, and 21c antibodies was increased by sCD4. Soluble CD4 also increased the binding of the previously characterized 17b and 48d CD4i antibodies to gp120, as expected.[30,38]

### *Ability of CD4i HMAbs to compete with 17b antibody for gp120*

To examine whether the newly identified CD4i HMAbs would compete with the previously characterized HMAb 17b for binding to the HIV-1 gp120 glycoprotein, increasing concentrations of the CD4i HMAbs and a control antibody 2/11C were mixed with biotinylated 17b antibody and incubated with the gp120 glycoprotein captured on an ELISA plate with the D7324 antibody.[30] After washing, the bound biotinylated 17b antibody was detected. All of the CD4i HMAbs competed with the 17b antibody for gp120 binding (Fig. 2). The control antibody, 2/11C, which recognizes a gp120 epitope that does not overlap with the CD4i epitopes,[30] did not compete with the 17b antibody (data not shown). This result suggests that all of the CD4i HMAbs recognize related gp120 regions.

### *Mapping the gp120 epitopes of the CD4i HMAbs*

To characterize the CD4i antibody epitopes, a panel of YU2 wtΔ gp120 mutants with single amino acid changes was used for precipitation by the HMAbs, in the absence of sCD4. Mutant gp120 wtΔ glycoproteins that were recognized signifi-

cantly less efficiently than the wild-type gp120 were also examined in the presence of sCD4. The wtΔ glycoprotein is identical to gp120 except for deletions of the N-terminus and V1/V2 variable loops. These deletions were previously demonstrated not to affect the binding of CD4 and CD4i antibodies.[24] The results are summarized in Table 1. Changes in amino acids located in several discontinuous gp120 regions (C1, C2, C3, and C4) resulted in decreased recognition by one or more of the HMAbs. In some cases, sCD4 binding to the mutant gp120 restored recognition by the HMAb. This subset of gp120 mutants probably exhibits local conformational changes that disrupt the particular HMAb epitope, and sCD4 binding restores the native conformation of the epitope. Some gp120 mutants that were able to bind sCD4 were not precipitated by the HMAbs in either the absence or presence of sCD4. These mutants are probably altered in amino acid residues that play key roles in antibody binding. Figure 3 illustrates the location of the amino acid changes that affected HMAb recognition, using the structure of the YU2 gp120 core complexed with two-domain CD4 and the Fab fragment of the 17b antibody.[28] The results suggest that the CD4i HMAbs recognize different gp120 epitopes that share a common element near isoleucine 420. This common element also appears to be important for CCR5 binding.[33,34] The 48d antibody most closely resembles CCR5 with respect to its sensitivity to gp120 changes. The binding of both 48d and CCR5 was disrupted by more gp120 residue changes than was the binding of the 17b, 23e, 49e, and 21c antibodies.

### *Ability of CD4i HMAbs to block CCR5 binding*

The mapping studies described above suggest that the epitopes for all of the CD4i HMAbs are proximal to conserved gp120 elements involved in CCR5 binding. To test the ability of the CD4i HMAbs to inhibit gp120–CCR5 binding, radiolabeled gp120 complexed with sCD4 was added to Cf2ThsynCCR5 cells, which express high levels of CCR5,[12] in the presence of different concentrations of HMAbs. Figure 4 shows that all of the CD4i HMAbs inhibited the binding of gp120-sCD4 complexes to CCR5-expressing cells.

### *CD4 and CD4i HMAbs recognize related gp120 conformations*

A few of the gp120 amino acid changes studied disrupted the binding of CD4 and most of the CD4i HMAbs. Examples of such mutants are 257 T/D, 383 F/S, and 435 Y/S. Previous studies[24] noted that the recognition of the 257 T/D and 383 F/S mutants by a conformation-dependent CD4BS HMAb, F105, was undetectable. The disruption of the binding of several conformation-dependent gp120 ligands and the low solvent accessibility of these residues in the available gp120 crystal structures[28,29] suggest that these changes significantly alter the conformation of the free gp120 glycoprotein. Figure 5A shows that although sCD4 and the 48d HMAb alone did not efficiently precipitate these mutant gp120 glycoproteins, a combination of sCD4 and 48d did recognize the gp120 mutants. A similar result was observed for the 257 T/D gp120 mutant with the other CD4i HMAbs (Fig. 5B). These observations suggest that CD4 and the CD4i HMAbs recognize a similar gp120 conformation and therefore can mutually cooperate to allow precipitation of a conformationally disrupted gp120 variant.

### *HIV-1-neutralizing activity of CD4i HMAbs*

To examine the ability of the CD4i HMAbs to inhibit HIV-1 infection, we employed recombinant HIV-1 encoding fire-

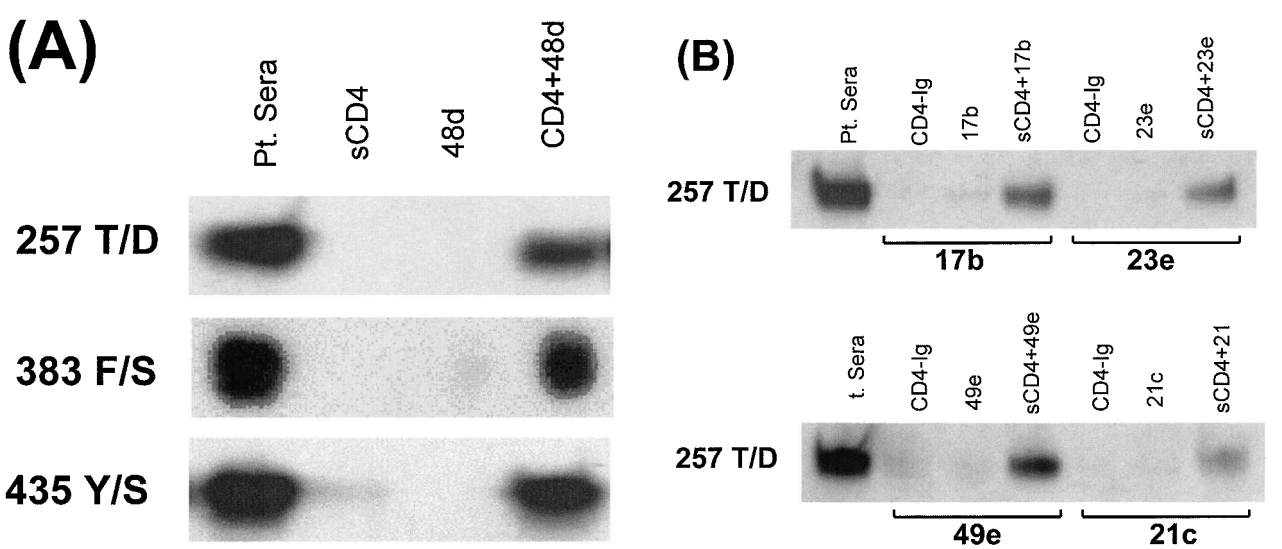


**FIG. 5.** Soluble CD4 and CD4i HMAbs exhibit positive cooperativity in precipitating HIV-1 gp120 glycoprotein mutants. (**A**) HIV-1 YU2 gp120 mutants that exhibit evidence of conformational disruption were radiolabeled and precipitated by a mixture of sera from HIV-1-infected individuals (Pt. Sera), sCD4, the 48d antibody, or a mixture of sCD4 and the 48d antibody. In the experiments in which sCD4 was added, the anti-CD4 polyclonal antibody T45 was included to allow precipitation of the sCD4–gp120 complex. The precipitated protein was analyzed by SDS–PAGE and autoradiography. (**B**) The radiolabeled 257 T/D mutant was precipitated by CD4-Ig, 17b, or a combination of sCD4 and 17b. The 257 T/D mutant was also precipitated by a mixture of sera from HIV-1-infected individuals (Pt. sera). The precipitated protein was analyzed by SDS–PAGE and autoradiography.

fly luciferase pseudotyped with the envelope glycoproteins of laboratory-adapted and primary HIV-1 isolates. Ghost cells expressing CD4 and CXCR4 were used as target cells for viruses with the X4 HXBc2 and MN envelope glycoproteins and the X4/R5 89.6 envelope glycoproteins. Cf2Th cells expressing CD4 and CCR5 were used as target cells for viruses with the R5 ADA, JR-FL and YU2 envelope glycoproteins. Figure 6 shows that the viruses with the laboratory-adapted HXBc2 and MN envelope glycoproteins were neutralized comparably by all of the CD4i HMAbs. Of the viruses with primary HIV-1 envelope glycoproteins, the ADA virus but not JR-FL, 89.6, or YU2 was neutralized at the HMAb concentrations studied. Thus, primary HIV-1 isolates are more resistant to neutralization by the CD4i HMAbs than laboratory-adapted isolates.

## DISCUSSION

The CD4-binding site (CD4BS) antibodies and the CD4i antibodies generated in HIV-1-infected humans are directed against overlapping, conserved, and conformation-dependent gp120 structures implicated in virus binding to CD4 and the chemokine receptors.[28] Numerous examples of CD4BS HMAbs exist, allowing identification of the common elements of the HMAb epitopes and an appreciation of the range of neutralization potencies for primary HIV-1 isolates exhibited by this group of antibodies. Until recently, CD4i antibodies were thought to be rare, as only two such HMAbs, 17b and 48d, existed. The apparent rarity of these antibodies has been attributed to the relative inaccessibility of the CD4i epitope on gp120.[22,39] In this report, we have identified three additional

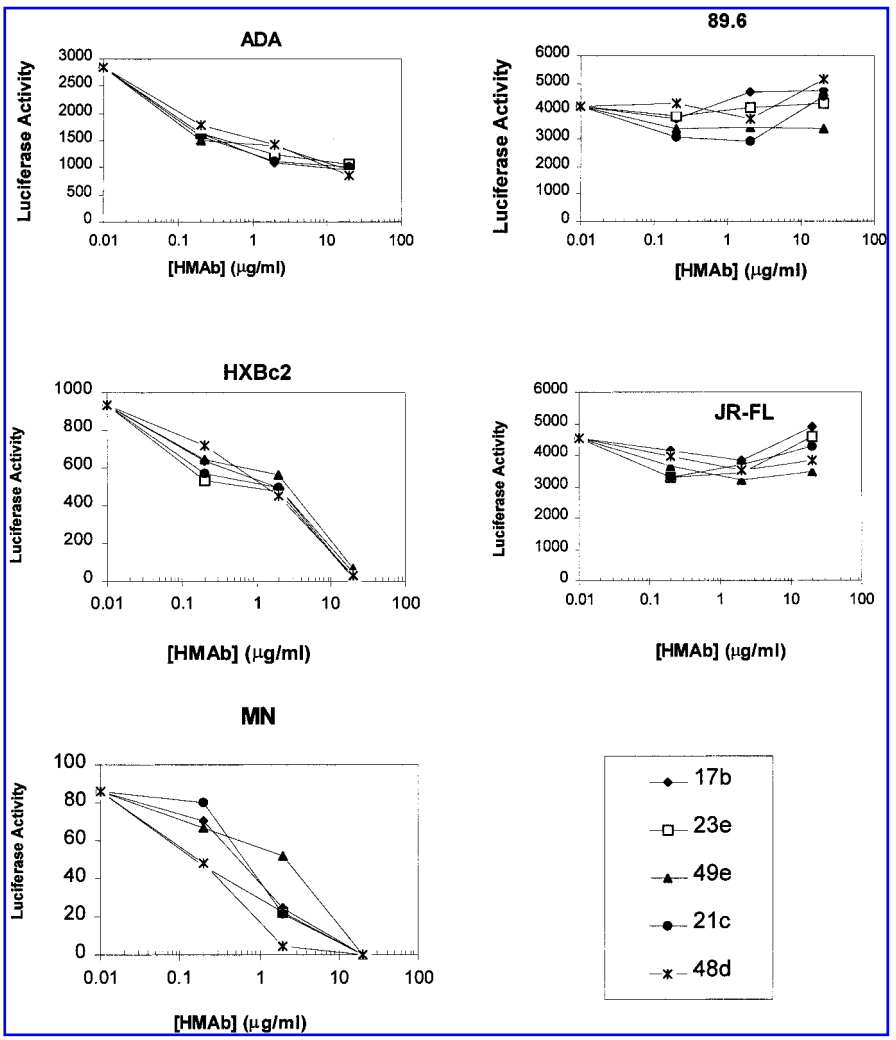


**FIG. 6.** Neutralization of HIV-1 by CD4i HMAbs. Recombinant HIV-1 expressing luciferase and containng *env* deletions were pseudotyped with HIV-1 envelope glycoproteins from the HXBc2, MN, ADA, JR-FL, and 89.6 isolates. The viruses were incubated with the indicated concentration of HMAb for 1 hr at 37°C prior to exposure to the appropriate target cells. Luciferase activity in the target cells was measured as described under Materials and Methods.

CD4i HMAbs derived from an asymptomatic HIV-1-infected, long-term nonprogressor individual (AD19). In addition, we derived another two CD4i HMAbs from later samples of PBMC of this same patient, and studies to further characterize these HMAbs are in progress. We were unable to derive CD4i HMAbs from six other long-term nonprogressor patients, suggesting that patient AD19 exhibited an unusually high frequency of circulating B cells capable of yielding such HMAbs.

The identification of the three new CD4i HMAbs allowed generalizations to be made regarding the structural characteristics of the epitope(s) that these antibodies recognize. The mutagenic data support the notion that all of the CD4i HMAbs recognize similar structures on a conserved portion of the gp120 surface that is thought to face the target cell after CD4 binding occurs. The epitopes for all of the CD4i HMAbs appear to be centered around a sequence in the fourth conserved (C4) gp120 region at the junction of the $\beta$19 and $\beta$20 strands. The critical residues 418–422 (CRIKQ) are highly conserved in primate immunodeficiency virus gp120 glycoproteins and have been implicated in binding the N-terminus of the CCR5 receptor.[48,49] Consistent with this, all of the CD4i HMAbs blocked the binding of gp120–CD4 complexes to CCR5. The importance of a localized gp120 region for the binding of all of the CD4i HMAbs examined suggests that only limited patches of conserved sequence on the gp120 chemokine receptor-binding surface are available to be accessed by antibodies. This limitation is thought to be imposed by the V2 and V3 variable loops, which project from the gp120 core and are thought to flank the conserved chemokine receptor-binding region, partially masking it from the humoral immune response.[22,24,29,39] The CD4i HMAbs apparently have evolved to contact conserved gp120 epitopes in this region and bypass the adjacent variable loops.

Based on thermodynamic studies,[50] CD4 binding has been suggested to limit an unusually high degree of interdomain flexibility present in the free HIV-1 gp120 glycoprotein. Because the conserved CD4i epitope component (residues 418–422) is located at the interface of the gp120 outer domain and bridging sheet,[28,29] ligands like CD4 that modify the spatial relationships among the gp120 domains would be expected to influence the conformation of the CD4i epitopes. The ability of CD4i antibodies and sCD4 to mutually induce binding to gp120 glycoprotein variants that exhibit conformational perturbations (Fig. 5) suggests that these ligands recognize related gp120 conformations.

Although the CD4i HMAbs contact a conserved gp120 element, changes in the major gp120 variable loops can influence the binding of these HMAbs. Removal of the V1/V2 loop has been shown to expose the epitopes for some CD4i HMAbs.[22] At least some of CD4-induced increase in CD4i HMAb binding can be attributed to movement of the V1/V2 loops from a position that masks the CD4i epitopes. The deletion of the V3 variable loop from the wt$\Delta$ protein resulted in a loss of recognition by the CD4i HMAbs, unless sCD4 was present.[23] Most of this effect is probably an indirect consequence of V3 loop removal on the local conformation of the gp120 bridging sheet. However, current structures on the gp120–17b antibody complexes leave open the possibility of V3 loop–antibody contacts.[28,29] The relatively efficient binding of the CD4i antibodies to V3 loop-deleted gp120 in the presence of CD4 suggests that such contacts, if they occur, contribute minimal binding energy to the antibody–gp120 interaction.

The results of our virus inhibition studies indicate a remarkable similarity in the potency and breadth of neutralization exhibited by the different CD4i HMAbs. This may result from the close relatedness of the epitopes for these HMAbs imposed by the gp120 variable structures discussed above. Differences in the conformations of the V2 and V3 variable loops have been shown to account for at least some of the neutralization resistance associated with primary compared with laboratory-adapted strains of HIV-1.[40] Such differences in V2 and V3 loop configurations provide a natural explanation for the limited sensitivity of primary HIV-1 isolates to neutralization by CD4i HMAbs. One primary isolate, ADA, exhibited some sensitivity to the CD4i HMAbs. Possibly relevant is our observation that removal of the V1/V2 variable loops renders the ADA HIV-1 isolate CD4 independent,[51] suggesting that the ADA CCR5-binding region is either formed or can be formed without prior CD4 binding. Likewise, the CD4i epitopes on the ADA gp120 glycoprotein may be available for antibody binding once the V2 loop is bypassed. As the neutralization potency of CD4i antibodies depends on their ability to bind the viral envelope glycoproteins prior to CD4 binding,[20] a virus that has the CD4i epitope preformed in the absence of CD4 would be more sensitive to neutralization by these antibodies. More neutralization-resistant viruses might employ both V2 loop masking and conformational flexibility of CD4i epitope components to minimize the efficacy of CD4i antibodies. Further understanding of these epitopes and the mehcanisms employed by HIV-1 to protect them from the neutralizing antibody response may suggest strategies for intervention in virus transmission or spread within the host.

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