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# Strain Specificity and Binding Affinity Requirements of Neutralizing Monoclonal Antibodies to the C4 Domain of gp120 from Human Immunodeficiency Virus Type 1

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**The binding properties of seven CD4-blocking monoclonal antibodies raised against recombinant gp120 of human immunodeficiency virus type 1 strain MN (HIV-$1_{MN}$) and two CD4-blocking monoclonal antibodies to recombinant envelope glycoproteins gp120 and gp160 of substrain IIIB of $HIV_{LAI}$ were analyzed. With a panel of recombinant gp120s from seven diverse HIV-1 isolates, eight of the nine antibodies were found to be strain specific and one was broadly cross-reactive. Epitope mapping revealed that all nine antibodies bound to epitopes located in the fourth conserved domain (C4) of gp120. Within this region, three distinct epitopes could be identified: two were polymorphic between HIV-1 strains, and one was highly conserved. Studies with synthetic peptides demonstrated that the conserved epitope, recognized by antibody 13H8, was located between residues 431 and 439. Site-directed mutagenesis of gp120 demonstrated that residue 429 and/or 432 was critical for the binding of the seven antibodies to gp120 from HIV-$1_{MN}$. Similarly, residues 423 and 429 were essential for the binding of monoclonal antibody 5C2 raised against gp120 from HIV-$1_{IIIB}$. The amino acids located at positions 423 and 429 were found to vary between strains of HIV-1 as well as between molecular clones derived from the MN and LAI isolates of HIV-1. Polymorphism at these positions prevented the binding of virus-neutralizing monoclonal antibodies and raised the possibility that HIV-1 neutralization serotypes may be defined on the basis of C4 domain sequences. Analysis of the binding characteristics of the CD4-blocking antibodies demonstrated that their virus-neutralizing activity was directly proportional to their gp120-binding affinity. These studies account for the strain specificity of antibodies to the C4 domain of gp120 and demonstrate for the first time that antibodies to this region can be as effective as those directed to the principal neutralizing determinant (V3 domain) in neutralizing HIV-1 infectivity.**

The identification of epitopes recognized by virus-neutralizing antibodies is critical for the rational design of vaccines effective against human immunodeficiency virus type 1 (HIV-1) infection. One way in which antibodies would be expected to neutralize HIV-1 infection is by blocking the binding of the HIV-1 envelope glycoprotein, gp120, to its cellular receptor, CD4. However, it has been surprising that the CD4-blocking activity, readily demonstrated in sera from HIV-1-infected individuals (29, 41, 43) and animals immunized with recombinant envelope glycoproteins (1a–3), has not always correlated with neutralizing activity (1a, 3, 26, 41). Results obtained with monoclonal antibodies (MAbs) have shown that while some of the MAbs that block the binding of gp120 to CD4 possess neutralizing activity, others do not (4, 7, 16, 26, 33, 35, 44, 46). When the neutralizing activity of CD4-blocking MAbs is compared with the activity of those directed to the principal neutralizing determinant (PND) located in the third variable domain (V3 domain) of gp120 (10, 39), the CD4-blocking antibodies appear to be significantly less potent. Thus, CD4-blocking MAbs typically exhibit a 50% inhibitory concentration ($IC_{50}$) in the range of 1 to 10 μg/ml (4, 16, 26, 33, 35, 44, 46), whereas PND-directed MAbs typically exhibit an $IC_{50}$ in the range of 0.01 to 1.0 μg/ml (23, 33, 42).

In the present study we describe the properties of a panel of seven MAbs raised against recombinant gp120 (MN-rgp120) from the MN strain of HIV-1 (HIV-$1_{MN}$) (2) and two MAbs raised against recombinant envelope glycoproteins (IIIB-rgp120 and IIIB-rsgp160) from the IIIB substrain of HIV-$1_{LAI}$ (HIV-$1_{IIIB}$) (3, 7, 21, 33). It was observed that all seven of the MAbs to MN-rgp120 and the 5C2 MAb to IIIB-rgp120 (7, 22, 33) exhibited highly strain-specific binding, whereas the 13H8 MAb raised against IIIB-rgp160 (33) was broadly cross-reactive. Epitope mapping demonstrated that all of the antibodies were reactive with three distinct adjacent or overlapping epitopes located in the fourth conserved domain (C4 domain) of gp120. In vitro mutagenesis of MN-rgp120 demonstrated that lysine residue 429 or 432 or both were critically important for the binding of the CD4-blocking MAbs and accounted for their strain specificity. Similar analysis revealed that the strain specificity of MAb 5C2 depended on a unique phenylalanine residue at position 423 and a glutamic acid residue at position 429. Binding studies with synthetic peptides localized the epitope recognized by the broadly cross-reactive 13H8 MAb to a site C terminal to the others in a region corresponding to residues 431 to 439. Comparison of the amino acid sequences of the

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C4 domain of diverse isolates of HIV-1, as well as molecular clones HIV-$1_{IIIB}$ and HIV-$1_{MN}$, revealed that positions 423 and 429 are polymorphic and that a finite set of amino acid substitutions, critical for the binding of the CD4-blocking MAbs, occur at these positions. These residues appear to constitute a set of common antigenic variants that enable HIV-1 to escape virus-neutralizing MAbs. A comparison of the binding properties of the CD4-blocking MAbs revealed a direct log-linear relationship between their neutralizing activity and their MN-rgp120 binding affinity. One MAb, 1024, that exhibited neutralizing activity ($IC_{50}$ of approximately 0.1 μg/ml) comparable to that of MAbs reactive with the PND was identified. These studies define the requisite conditions of affinity and concentration whereby antibodies to the C4 domain can neutralize HIV-1 infectivity in vitro and may provide an explanation for the poor correlation between CD4-blocking activity and virus-neutralizing activity previously observed with polyclonal (1a, 3, 41) and monoclonal (7, 26, 33) antibodies.

## MATERIALS AND METHODS

**gp120 sequences and nomenclature.** Amino acid residues are designated by the standard single-letter codes. The location of amino acids within the gp120 protein is specified by using the initiator methionine residue as position 1. The designation LAI is used to describe the virus isolate from which the substrains (molecular clones) of HIV-$1_{LAI}$ (HIV-$1_{BH10}$, HIV-$1_{IIIB}$, HIV-$1_{BRU}$, HIV-$1_{HXB2}$, HIV-$1_{HXB3}$, and HIV-$1_{HX10}$) were obtained. The sequence of gp120 $HIV_{IIIB}$ is that determined by Muesing et al. (30). The sequence of gp120 from HIV-$1_{MN}$ is given with reference to the MN-gp120 clone ($MN_{GNE}$) obtained by Berman et al. (1). The sequence of this clone differs by approximately 2% from that of the $MN_{1984}$ clone described by Gurgo et al. (13). The sequences of gp120 from the NY-5, JRcsf, Z6, Z321, and HXB2 strains of HIV-1 are those listed by Myers et al. (32), except where noted otherwise. The sequence of the Thai isolate A244 is that described by McCutchan et al. (24). The variable (V) domains and conserved (C) domains of gp120 are specified according to the nomenclature of Modrow et al. (28).

**MAb production and binding assays.** Hybridomas producing MAbs to MN-rgp120 (2) were prepared and screened for CD4-blocking activity as described previously (7, 33). The binding of MAbs to MN-rgp120 and to rgp120s from the IIIB, NY-5, Z6, Z321, JRcsf, and A244 strains of HIV-1 was assessed by enzyme-linked immunoadsorbent assays (ELISAs) as described previously (33). Antibody binding to synthetic peptides was assessed as described previously (33) by using synthetic peptides purchased from American Bio-Technologies, Inc. (Cambridge, Mass.) or synthesized by the Genentech Peptide Synthesis Laboratory. The sequence of the HIV-$1_{MN}$ V3 domain peptide employed for MAb-binding studies was NKRKRIHIGPGRAFYTTKNIIGTIC.

**Virus-binding and neutralization assays.** The ability of MAbs to neutralize HIV-1 infectivity in vitro was assessed in a colorimetric MT-2 cell cytotoxicity assay similar to that described previously (35). MT-2 cells and H9 cells infected with (HIV-$1_{MN}$) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (contributed by Douglas Richman and Robert Gallo, respectively). Briefly, serial dilutions of antibody or serum were prepared in 50-μl volumes of complete medium, and then 50 μl of a prediluted HIV-1 stock was added to each well. After incubation for 1 h at 4°C, 100 μl of a solution of $4 \times 10^5$ MT-2 cells per ml was added. After incubation of the plates for 5 days at 37°C in 5% $CO_2$, viable cells were determined by using metabolic conversion of the formazan MTT dye. Each well received 20 μl of a 5-mg/ml MTT solution [3(4,5-dimethylthiazole-2-yl)2,5-diphenyl tetrazolium bromide] in phosphate-buffered saline (PBS). After a 4-h incubation at 37°C, the dye precipitate was dissolved by removing 100 μl of the cell supernatant, adding 130 μl of 10% Triton X-100 in acid isopropanol, and then pipetting the mixture until the precipitate was dissolved. The optical density of the wells was determined at 540 nm ($OD_{540}$). The percentage of inhibition was calculated as 1 − (virus control − experimental inhibition of cytopathicity)/(virus control − medium control).

**Cell surface staining of HIV-1-infected cells with MAbs.** H9 cells ($2 \times 10^5$) chronically infected with the IIIB, HXB2, HXB3, and HX10 substrains of HIV-$1_{LAI}$ or with HIV-$1_{MN}$ were incubated for 30 min at room temperature with MAbs (10 μg/ml) in 100 μl of RPMI 1640 cell culture medium containing 1% fetal calf serum. The cells were washed and then incubated with 20 μg of fluorescein-conjugated, affinity-purified, goat antibody to mouse immunoglobulin G $(Fab')_2$ (Cappel, West Chester, Pa.) per ml for 30 min. The cells were washed and fixed with 1% paraformaldehyde, and the bound antibody was quantitated by flow cytometry with a FACSCAN (Becton-Dickinson, Fullerton, Calif.). The fluorescence intensity of the cells was expressed as the log of the mean channel number.

**Fragmentation of the MN-rgp120 gene.** Fragments of the MN-rgp120 gene were generated by using the polymerase chain reaction (PCR) (17). Briefly, forward 30-mer oligonucleotide DNA primers incorporating an *Xho*I site and reverse 36-mer oligonucleotide DNA primers containing a stop codon followed by an *Xba*I site were synthesized and used for the PCRs. Thirty cycles of the PCR were performed with 0.3 μg of a plasmid containing the gene for gp120 from HIV-$1_{MN}$ (pRKMN.D533) and 0.04 nM designated primers. The PCR buffer consisted of 0.1 M Tris buffer (pH 8.4), 50 mM KCl, 0.2 mM deoxynucleoside triphosphates (Pharmacia, Piscataway, N.J.), 0.15 M $MgCl_2$, and 0.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). A typical PCR cycle consisted of a 60-s denaturation step at 94°C, followed by a 45-s annealing step at 55°C and then an extension step at 72°C for 45 s. Following the PCR amplification, the PCR products were purified by phenol-chloroform extraction and then ethanol precipitated. The purified products were then digested with the restriction endonucleases *Xho*I and *Xba*I. The resulting PCR products were gel purified with 1% agarose (SEAKEM; FMC Bioproducts, Rockland, Maine) or 5% polyacrylamide gel electrophoresis (PAGE) and then isolated by electroelution.

**Site-directed mutagenesis of the MN-rgp120 C4 domain.** A recombinant PCR technique (15) was utilized to introduce single amino acid substitutions at selected sites into a 600-bp *Bgl*II fragment of MN-rgp120 that contained the C4 domain. This method entailed the PCR amplification of overlapping regions of the C4 domain of gp120 by using primers that incorporated the desired nucleotide changes. The resultant PCR products were then annealed and PCR amplified to generate the final product. For these reactions, 18-mer outside primers encoding the wild-type sequence (*Bgl*II sites) were amplified with 36-mer inside primers that contained the desired nucleotide changes. The first PCR included 1× Vent polymerase buffer (New England Biolabs, Beverly, Mass.), 0.2 mM deoxynucleoside triphosphates

(Pharmacia), 0.04 nM (each) synthetic oligonucleotide, and 0.3 μg of linearized plasmid pRKMN.D533, which contained the MN-rgp120 gene. Thirty PCR cycles were performed, in the following sequence of steps: 45 s each of denaturation at 94°C, annealing at 55°C, and extension at 72°C. Following PCR amplification, the product pairs were gel purified by using a 1% solution of low-melting-point agarose (SeaPlaque, FMC Bioproducts). The agarose containing PCR product was melted at 65°C, combined with the PCR product of the overlapping pair, and equilibrated to 37°C. Added to this (20 μl) was 10 μl of 10× Vent polymerase buffer, 10 μl of 2 mM deoxynucleoside triphosphates, 0.04 nM (each) outside wild-type 18-mer oligonucleotide, 57 μl of $H_2O$, and 1 U of Vent polymerase. Thirty PCR cycles were performed as described above. The resulting PCR products were purified and digested with the *Bgl*II endonuclease. The digested PCR product was then ligated into the mammalian cell expression vector pRKMN.D533, which had been digested with *Bgl*II to allow for the removal of a 600-bp fragment. Colonies containing the correct insertion were verified by supercoil DNA sequencing by using Sequenase 2.0 (U.S. Biochemical Corp., Cleveland, Ohio).

**Expression of gp120 fragments in mammalian cells.** Fragments of the MN and IIIB gp120 genes were expressed in mammalian cells as fusion proteins incorporating N-terminal sequences of the herpes simplex virus type 1 (HSV-1) glycoprotein D gene (gD-1) as described previously (14, 21). Briefly, isolated DNA fragments generated by PCR were ligated into a plasmid (pRK.gD-1) designed to fuse the gp120 fragments, in frame, to the 5′ sequences of the gD-1 gene and translational stop codons to the 3′ end. The fragment of the gD-1 gene encoded the signal sequence and 25 amino acids of the mature form of HSV-1 protein. To allow for expression in mammalian cells, chimeric gene fragments were cloned into the pRK5 expression plasmid (8) that contained a polylinker with cloning sites and translational stop codons located between a cytomegalovirus promoter and a simian virus 40 polyadenylation site. The resulting plasmids were transfected into the 293s embryonic human kidney cell line (12) by a calcium phosphate technique (11). Growth-conditioned cell culture medium was collected 48 h after transfection, and the soluble proteins were detected by ELISA or by specific radioimmunoprecipitation when metabolically labeled proteins from cell culture supernatants were resolved by sodium dodecyl sulfate (SDS)-PAGE and visualized by autoradiography as described previously (1, 18).

**Radioimmunoprecipitation of MN-rgp120 mutants.** Plasmids directing the expression of the MN-rgp120 C4 domain mutants were transfected into 293s cells as described above. Twenty-four hours following the transfection, the cells were metabolically labeled with [$^{35}$S]methionine or cysteine as described previously (3). The labeled cell culture supernatants were then harvested, and 0.5-ml aliquots were reacted with 1 to 5 μg of MAb or with 2 μl of the polyclonal rabbit antisera to MN-rgp120 and immunoprecipitated with Pansorbin (CalBiochem, La Jolla, Calif.) as described previously (3). The resulting Pansorbin complex was pelleted by centrifugation, washed twice with a solution containing PBS–1% Nonidet P-40–0.05% SDS, and then boiled in a PAGE sample buffer containing 1% 2-mercaptoethanol. The processed samples were then analyzed by SDS-PAGE and visualized by autoradiography (3, 18).

**Assays to measure the binding of MAbs to mutagenized MN-rgp120 polypeptides.** An ELISA was developed to screen for reactivity of MN-rgp120 fragments and mutant proteins with various MAbs. In this assay, 96-well microtiter dishes (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight with mouse MAb, 5B6, specific for the amino terminus of gD-1, at a concentration of 2.0 μg/ml in PBS. The plates were blocked in a PBS solution containing 0.5% bovine serum albumin and then incubated with growth-conditioned cell culture medium from transfected cells expressing the recombinant gp120 variants for 2 h at room temperature. The plates were washed three times in PBS containing 0.05% Tween 20 and then incubated with horseradish peroxidase-conjugated MAbs to rgp120. Following a 1-h incubation, the plates were washed three times and developed with the colorimetric substrate, *o*-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.). The optical densities at 492 nm ($OD_{492}$s) in each well were then read in a microtiter plate-reading spectrophotometer. Data from these experiments were expressed as a ratio of the optical densities obtained with the CD4-blocking MAbs binding to the fragments or MN-rgp120 mutants compared with the full-length wild-type rgp120s. To normalize for different concentrations of MN-rgp120-derived proteins in each cell culture supernatant, envelope glycoprotein concentrations were adjusted on the basis of binding to a V3-specific MAb (1034) or rabbit polyclonal sera to MN-rgp120.

**CD4-binding assays.** The ability of MAbs to inhibit the binding of MN-rgp120 to recombinant soluble CD4 (rsCD4) was determined by two methods. Initial screening of hybridoma supernatants utilized a solid-phase immunoassay, described previously (33), in which the binding of rsCD4 to wells of MN-rgp120-coated microtiter dishes was monitored. For quantitative binding studies, the ability of MAbs to inhibit the binding of $^{125}$I-labeled MN-rgp120 to cell surface CD4 was measured as described previously (22, 33). The effect of single amino acid substitutions on the binding of MN-rgp120 mutants to CD4 was determined in a coimmunoprecipitation assay similar to that described previously (22). Briefly, 293s cells were metabolically labeled with [$^{35}$S]methionine 24 h after transfection with plasmids expressing MN-rgp120 variants. Growth-conditioned cell culture medium (0.5 ml) was then incubated with 5.0 μg of rsCD4 for 90 min at room temperature. Following this incubation, 5.0 μg of an anti-CD4 MAb (465), known to bind to an epitope remote from the gp120-binding site, was added, and the mixture was allowed to incubate another 90 min at room temperature. The gp120-CD4-antibody complexes were precipitated with Pansorbin that had been washed with PBS, preabsorbed with 0.1% bovine serum albumin, and then bound with 50 μg of an affinity-purified rabbit anti-mouse immunoglobulin G (Cappel). The pellet was washed twice with PBS–1% Nonidet P-40–0.05% SDS and then boiled in β-mercaptoethanol containing SDS-PAGE sample buffer. The immunoprecipitation products were resolved by SDS-PAGE and visualized by autoradiography as described previously (3, 22).

**Antibody affinity measurements.** Anti-gp120 antibodies were iodinated with Na $^{125}$I with Iodogen (Pierce, Rockford, Ill.). Briefly, 50 μg of antibody in PBS was placed in 1.5-ml polypropylene microcentrifuge tubes coated with 50 μg of Iodogen. Two millicuries of carrier-free Na $^{125}$I was added. After 15 min, free $^{125}$I was separated from the labeled protein by chromatography on a PD-10 column (Pierce) pre-equilibrated in PBS containing 0.5% gelatin. Antibody concentrations following iodination were determined by ELISA to calculate specific activities. For binding assays, 96-well microtiter plates were coated with 100 μl of a 10-μg/ml solution of MN-rgp120 or IIIB-rgp120 in 0.1 M bicarbonate buffer (pH 9.6) per well and incubated for 2 h at room

TABLE 1. Properties of MAbs to MN-rgp120

| MAb | CD4 inhibition[a] | HIV-$1_{MN}$ neutralization[b] | HIV-$1_{MN}$ V3 peptide | RCM-rgp120[c] | C4 domain peptides[d] | rgp120 cross-reactivity[e] |
|---|---|---|---|---|---|---|
| 1024 | + | 0.08 | − | + | − | 2 |
| 1093 | + | 1.0 | − | + | − | 2 |
| 1096 | + | 1.7 | − | + | − | 2 |
| 1097 | + | 1.2 | − | + | − | 2 |
| 1110 | + | 6.2 | − | + | − | 2 |
| 1112 | + | 29.3 | − | + | − | 2 |
| 1127 | + | 0.33 | − | + | − | 2 |
| 1026 | − | 0.03 | + | + | − | 1,2,3,4,6 |
| 1092 | − | | − | + | − | 1,2,3,4,5 |
| 1126 | − | | − | + | − | 1,2,3,5,7 |
| 1086 | − | | − | + | − | 2 |
| 13H8 | + | | − | + | 1,3 | 1,2,3,4,5,6,7 |

[a] Inhibition of gp120 binding to CD4 was determined by using a solid-phase binding assay described previously (33).
[b] Neutralization assays were carried out as described in Materials and Methods. The data are $IC_{50}$s (micrograms per milliliter).
[c] Chemically reduced and carboxymethylated MN-rgp120.
[d] The synthetic C4 domain peptides used in ELISAs had the following sequences: 1, FINMWQEVGKAMYAPPIS; 2, MWQEVGKAMYAP; 3, GKAMYAPPIKGQIR. MAbs that failed to bind to the peptides indicated gave a background $OD_{492}$ of approximately 0.05. MAbs binding to synthetic peptides exhibited $OD_{492}$s ranging between 0.40 and 0.80.
[e] Studies of rgp120 cross-reactivity employed recombinant proteins (rgp120) derived from the strains indicated by the following numbers: 1, IIIB; 2, MN; 3, NY-5; 4, Rcsf; 5, Z6; 6, Z321; and 7, A244.

temperature or overnight at 4°C. To prevent nonspecific binding, plates were blocked for 1 to 2 h at room temperature with 200 μl of a gelatin solution, consisting of PBS containing 0.5% (wt/vol) gelatin and 0.02% sodium azide, per well. Unlabeled anti-gp120 MAb (0 to 400 nM) was titrated (in duplicate) in situ, and radiolabeled antibody was added to each well at a concentration of 0.5 nM. After 1 to 2 h at room temperature, the plate was washed 10 times with the PBS–0.5% gelatin–0.02% azide buffer to remove free antibody. The antibody-gp120 complexes were solubilized with 0.1 N NaOH–0.1% SDS solution, and the radioactivity was measured in a gamma counter. The data were analyzed by the method of Scatchard (40) with the Ligand analytical software program (31). $K_d$ values reported represent the means of four independent determinations.

## RESULTS

**Characterization of MAbs to MN-rgp120 that block CD4 binding.** MAbs prepared from mice immunized with MN-rgp120 (2, 33) were screened for the ability to bind to MN-rgp120-coated microtiter dishes by ELISA, as described previously (33). Of the 35 clones obtained, 7 (1024, 1093, 1096, 1097, 1110, 1112, and 1127) that were able to inhibit the binding of MN-rgp120 to rsCD4 in ELISA (Table 1; Fig. 1) or cell surface radioimmunoassays (22, 33) were identified. Previous studies have shown that two distinct classes of CD4-blocking MAbs occur: those that bind to conformation-dependent, discontinuous epitopes (16, 26, 33, 35, 46) and those that bind to sequential (linear) epitopes that are not dependent on gp120 secondary structure (4, 7, 22, 33, 44). To distinguish between these two alternatives, the binding of the MAbs to denatured (reduced and carboxymethylated) MN-rgp120 (RCM-rgp120) was measured by ELISA as described previously (33). It was found (Table 1) that all the CD4-blocking MAbs reacted with the chemically denatured protein, indicating that they all recognized sequential epitopes.

The cross-reactivity of these MAbs was assessed by ELISA as described previously (33). In these experiments, the ability of the MAbs to bind to a panel of seven rgp120s, prepared from the *IIIB*, MN, Z6, Z321, NY-5, A244, and JRcsf isolates of HIV-1, was determined by ELISA (33). It was found that all the CD4-blocking MAbs were strain specific and bound only to gp120 from HIV-$1_{MN}$ (Table 1). However, other MAbs from the same fusion (MAbs 1026, 1092, and 1126) exhibited much broader cross-reactivity (Table 1), as did a CD4-blocking MAb to IIIB-rgp120 (13H8) described previously (33).

Further studies were performed to characterize the neutralizing activity of the MAbs to MN-rgp120. In these

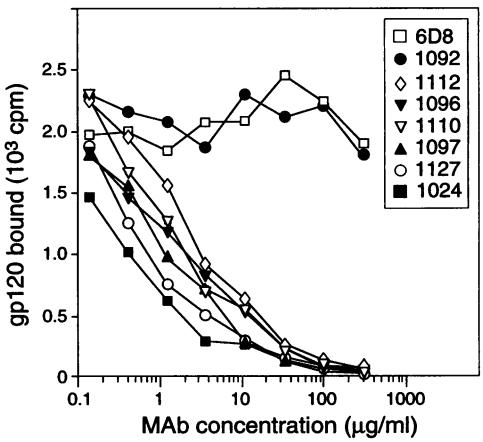


FIG. 1. Inhibition of CD4 binding by MAbs to MN-rgp120. Mice were immunized with MN-rgp120, and the resulting splenocytes were fused with the NP3X63.Ag8.653 cell line as described previously. Thirty-five stable hybridoma clones, reactive with MN-rgp120, were identified by ELISA. Secondary screening (Table 1) revealed seven cell lines (1024, 1093, 1096, 1097, 1110, 1112, and 1127) secreting antibodies able to inhibit the binding of rsCD4. The data shown were derived from binding studies in which the ability of six of the seven MAbs to inhibit the binding of $^{125}$I-labeled MN-rgp120 to cell surface CD4 was measured by the method described previously (22, 33). Data from control experiments measuring the activity of the C1-domain-specific MAb, 6D8 (33), and a non-CD4-blocking MAb (1092) to MN-rgp120 are shown for purposes of comparison.

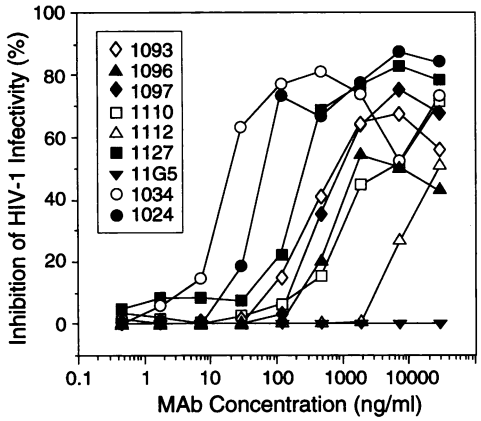


FIG. 2. Neutralizing activity of CD4-blocking MAbs to MN-rgp120. MAbs that blocked the binding of MN-rgp120 to CD4 were screened for the capacity to inhibit the infection of MT2 cells by HIV-$1_{MN}$ in vitro. Cell-free virus was added to wells containing serially diluted antibodies and incubated at 4°C for 1 h. After incubation, MT-2 cells were added to the wells, and the cultures were then grown for 5 days at 37°C. Cell viability was then measured by using MTT as described in Materials and Methods. The $OD_{540}$ of each well was measured with a microtiter plate-reading spectrophotometer. The inhibition of virus infectivity was calculated by dividing the mean OD of wells containing MAbs by the mean OD of wells that received virus alone. MAbs that blocked CD4 binding are the same as those indicated in the legend to Fig. 1. Data from the V3-directed MAb to MN-rgp120 (1034) is provided as a positive control. Data obtained with the V3-directed MAb, 11G5, specific for HIV-$1_{IIIB}$ (33) is shown as a negative control.

studies, MAbs were incubated with cell-free virus (HIV-$1_{MN}$), and the resulting mixture was used to infect MT-2 cells in microtiter plates. Cell viability was determined after 5 days in culture by using the colorimetric dye MTT as described above (see Materials and Methods). It was found (Table 1; Fig. 2) that all the CD4-blocking MAbs to MN-rgp120 were able to inhibit viral infectivity. However, the potency of the MAbs varied considerably with some MAbs (e.g., 1024) able to inhibit infection at very low concentrations ($IC_{50}$ of 0.08 μg/ml) whereas other MAbs (e.g., 1112) required much higher concentrations ($IC_{50}$ of 29 μg/ml). In control experiments with non-CD4-blocking MAbs from the same fusion, one MAb (1026) that inhibited HIV-1 infectivity was identified, while two others (1086 and 1092) were ineffective in this assay. Similarly, one MAb (11G5) to the V3 domain of IIIB-rgp120 known to neutralize the infectivity of HIV-$1_{IIIB}$ (33) was unable to neutralize HIV-$1_{MN}$ (Fig. 2).

**Localization of epitopes recognized by the 13H8 and 10246 MAbs.** The binding of the MAbs to synthetic peptides from the V3 and C4 domains of gp120 was determined by ELISA (33) in an attempt to localize the antibody-binding sites. It was found that none of the CD4-blocking antibodies showed any reactivity with the V3 peptide. In contrast, the non-CD4-blocking MAb 1026 did bind to this peptide, indicating that it was directed to the PND. In other experiments, three synthetic peptides from the C4 domain of gp120 that incorporated sequences recognized by the CD4-blocking, weakly neutralizing MAbs described by McKeating et al. (26) were tested. It was found (Table 1) that none of the CD4-blocking MAbs to MN-rgp120 reacted with these peptides; however, the CD4-blocking MAb 13H8 raised against IIIB-rgp160 did bind two of the three. Because the 13H8 MAb bound to peptide 1 (corresponding to residues 423 to 440 of IIIB-gp120) and peptide 3 (corresponding to residues 431 to 441 of MN-gp120), but not to peptide 2 (corresponding to residues 426 to 437 of IIIB-gp120), the epitope recognized by this MAb could be localized to a 10-amino-acid sequence consisting of residues 431 to 440. Thus, the 13H8 MAb recognized an epitope that was similar, if not identical, to that recognized by the ICR38.1a and ICR38.8f MAbs described by McKeating et al. (26). The observation that the 13H8 MAb, and the MAbs described by Cordell et al. (4) and McKeating et al. (26), reacted with rgp120s from a variety of diverse isolates of HIV-1, whereas the CD4-blocking MAbs to MN-rgp120 were strain specific, suggested that MAbs to MN-rgp120 recognized epitopes distinct from those described previously (4, 26).

**CD4-blocking antibodies recognize epitopes in the C4 domain.** Previously, we described another CD4-blocking MAb (5C2) that, like MAb 13H8, was unable to neutralize HIV-1 infectivity in vitro (7, 33). The observation that 5C2 reacted only with IIIB-rgp120 whereas 13H8 reacted with rgp120s from a number of diverse isolates (33) suggested that the 5C2 epitope was distinct from the 13H8 epitope. Previous studies (22) demonstrated that immunoaffinity columns prepared from 5C2 adsorbed an 11-amino-acid peptide (residues 422 to 432) from a tryptic digest of gp120 (22). However, it was subsequently found that 5C2 was unable to recognize this peptide when it was coated onto wells of microtiter dishes in an ELISA format (32a). To determine whether the CD4-blocking MAbs raised against MN-rgp120 recognized the corresponding 5C2-like epitope of MN-rgp120, a series of overlapping fragments, spanning the V4 and C4 domains of HIV-$1_{MN}$ gp120, were prepared by expression in mammalian cells. A diagram of the fragments expressed is shown in Fig. 3A and B. The fragments were all expressed as fusion proteins that incorporated the signal sequence and amino-terminal 25 amino acids of HSV-1 glycoprotein D as described above (see Materials and Methods). Plasmids directing the expression of the chimeric V4 and C4 domain fragments were transfected into 293 cells, and their expression was monitored by radioimmunoprecipitation studies using a MAb, 5B6, specific for the mature amino terminus of glycoprotein D. It was found (Fig. 3C) that all of the fragments were secreted into growth-conditioned cell culture medium and exhibited mobilities on SDS-PAGE gradient gels appropriate for their predicted sizes. Thus, fMN 368-408 (lane 1) exhibited a mobility of 19 kDa, fMN 368–451 (lane 2) exhibited a mobility of 29 kDa, and fMN 419–443 (lane 3) and fMN 414–451 (lane 4) exhibited mobilities of approximately 6 kDa.

The binding of MAb 1024 to the recombinant fragments was then determined by ELISA. This antibody was selected as a prototype for further studies because it possessed the most potent neutralizing activity (Table 1; Fig. 2). It was found (Fig. 3A) that MAb 1024 reacted with the fragments that contained the entire C4 domain of MN-rgp120 (fMN 368–451 and fMN 404–455) but failed to bind to a fragment derived from the adjacent V4 domain (fMN 368–408) or to another fragment that contained V4 domain sequences and the amino-terminal half of the C4 domain (fMN 368–428). The fact that 1024 bound to the fMN 414–451 and fMN 419–443 fragments demonstrated that the epitope recognized by this MAb was contained entirely between residues 419 and 443 in the C4 domain.

**Residues recognized by MAbs that block binding of MN-rgp120 to CD4.** To identify amino acid residues that might be part of the epitopes recognized by the MAbs to MN-rgp120,

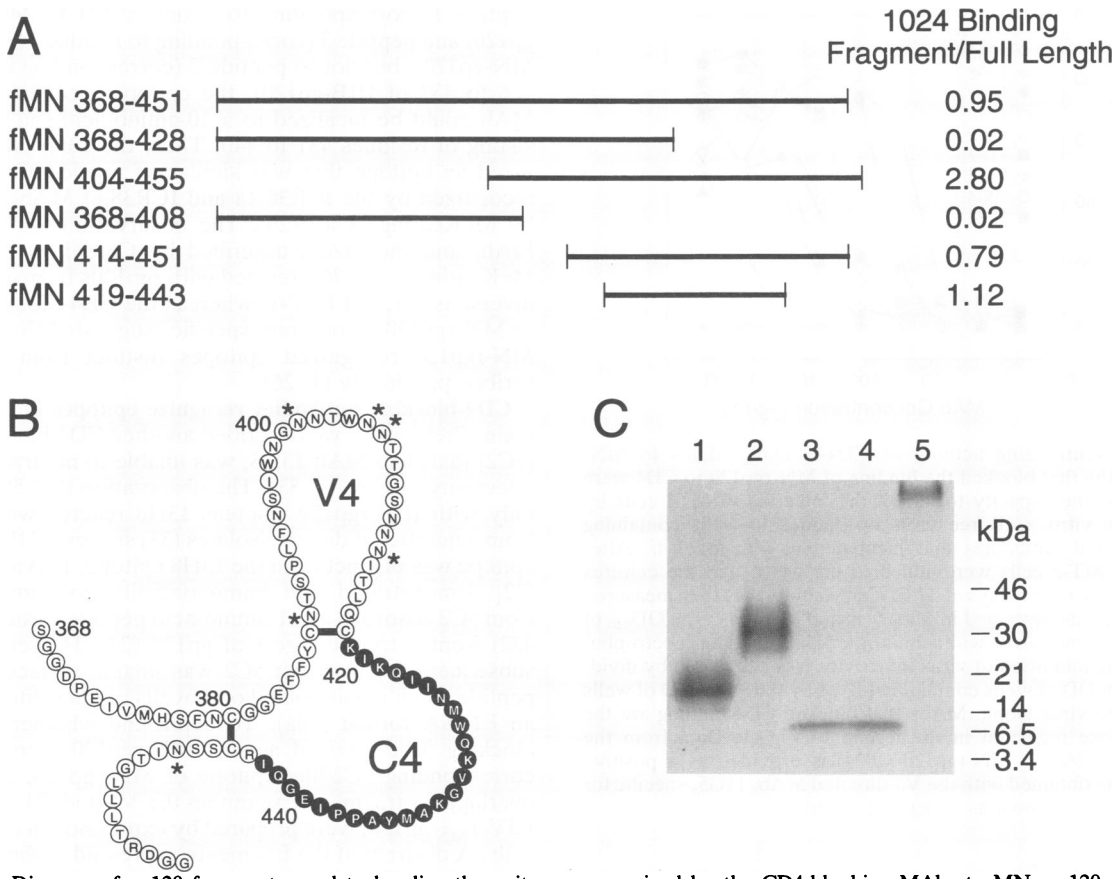


FIG. 3. Diagram of gp120 fragments used to localize the epitopes recognized by the CD4-blocking MAbs to MN-rgp120. A series of fragments (A) corresponding to the V4 and C4 domains (B) of the gene encoding MN-rgp120 were prepared by PCR. The gp120 gene fragments were fused to a fragment of the gene encoding HSV-1 glycoprotein D that encoded the signal sequence and 25 amino acids from the mature amino terminus. The chimeric genes were assembled into a mammalian cell expression vector (pRK5) that provided a cytomegalovirus promoter, translational stop codons, and a simian virus 40 polyadenylation site. The embryonic human kidney adenocarcinoma cell line, 293s, was transfected with the resulting plasmid, and recombinant proteins were recovered from growth-conditioned cell culture medium. Fragments of MN-rgp120, expressed as HSV-1 gD fusion proteins, were produced by transient transfection of 293s cells (see Materials and Methods). To verify expression, cells were metabolically labeled with [$^{35}$S]methionine, and the resulting growth-conditioned cell culture supernatants were immunoprecipitated (C) with an MAb, 5B6, specific for the amino terminus of HSV-1 gD and fixed *Staphylococcus aureus*. The immunoprecipitated proteins were resolved on 4 to 20% acrylamide gradient gels by SDS-PAGE and visualized by autoradiography. Lanes: 1, fMN 368–408; 2, fMN 368–451; 3, fMN 419–443; 4, fMN 414–451; 5, MN-rgp120. The ability of MAb 1024 to bind to the V4 and C4 domain chimeras was determined by ELISA (Materials and Methods). The data in panel A (right column) are $OD_{492}$s.

the sequence of the C4 domain of MN-rgp120 was compared with those of the six rgp120s that failed to bind the CD4-blocking MAbs (Fig. 4A). It was noted that the sequence of MN-rgp120 was unique in that K occurred at position 429 whereas E, G, or R occurred at this position in the other rgp120s. Another difference was noted at position 440, at which E replaced K or S. To evaluate the significance of these substitutions, a series of point mutations were introduced into the MN-rgp120 gene (Fig. 5). Plasmids expressing the full-length mutant proteins were transfected into 293s cells, and expression was verified by radioimmunoprecipitation with a MAb (1034) directed to the V3 domain of MN-rgp120 (Fig. 6B). The effect of these mutations on the binding of the CD4-blocking MAbs was then evaluated by ELISA (Table 2). It was found that replacement of E-440 with an A residue (MN 440A) had no effect on the binding of the 1024 MAb (Fig. 6, lane 6) or any of the other CD4-blocking MAbs (Table 2). The significance of K at position 429 was then evaluated by substitution of either A (MN 429A) or E (MN 429E) at this location. It was found that the A-for-K substitution at position 429 (MN 429A) markedly reduced the binding of the 1024 MAb (Fig. 6A, lane 4) and all of the other CD4-blocking MAbs (Table 2). Similarly, the replacement of E for K (MN 429E) at this position totally abrogated the binding of the 1024 MAb (Fig. 6A, lane 3) and all of the other CD4-blocking MAbs (Table 2). Several other mutants were constructed to evaluate the role of positively charged residues in the C4 domain. It was found that A-for-K substitutions at positions 419 (MN 419A) and 421 (MN 421A) failed to interfere with the binding of any of the CD4-blocking MAbs (Table 3; Fig. 6A, lanes 1 and 2). However, when K at position 432 was replaced with A (MN 432A), the binding of all of the CD4-blocking antibodies was markedly reduced (Table 2; Fig. 6). Because the binding of MAbs 1024 and 1096 was affected by this substitution less than the other MAbs (Table 2; Fig. 6A, lane 5), these data suggest that two different antibody recognition sites can be differentiated within this epitope. Thus, these studies dem-

A

```
418                                  445
CKIKQIINMWQKVGKAMYAPPIEGQIRC     MN_GNE
-R---F-----E----------S-----     LAI_IIIB
-R---------E----------K-----     JRCSF
-R---------G--------------N-     Z6
-R------R--E----I-----S-----     NY5
-R----V----R--Q-------K-V-K-     Z321
-----------GA-Q-------S-T-N-     A244
```

B

```
418                                  445
-R---F-----E----------S-----     LAI_IIIB, LAI_BRU, LAI_HXB3
-----I-----K----------S-----     LAI_HXB2
-R---I-----E----------S-----     LAI_BH10, LAI_HX10
-----------E----------------     MN_1984
```

FIG. 4. C4 domain sequence comparison. The C4 domain amino acid sequences of recombinant and virus-derived gp120s used for MAb-binding studies were aligned, starting at the amino-terminal cysteine. Amino acid positions are designated with respect to the sequence of MN-rgp120. Sequences of the $HIV_{LAI}$ substrains IIIB, BH10, Bru, HX10, HXB2, and HXB3 are shown for purposes of comparison.

onstrated that K-429 and/or K-432 was critical for the binding of all of the CD4-blocking MAbs and that K-419, K-421, and E-440 did not appear to play a role in MAb binding.

**Residues that define the strain specificity of the CD4-blocking MAbs.** The identification of residues 429 and 432 as part of the epitope recognized by the MN-rgp120-specific CD4-blocking MAbs was interesting since an adjacent or overlapping sequence in this region (422 to 432) has previously been implicated in the binding of the 5C2 MAb (22). The properties of the 1024-like MAbs and the 5C2 MAb differed from those of the C4-reactive MAbs described by other investigators (4, 44) in that the former appeared strain specific and the latter were broadly cross-reactive. To account for the strain specificity of these MAbs, the sequence of the 11-amino-acid peptide of IIIB-rgp120 recognized by MAb 5C2 was compared with the corresponding sequence of MN-rgp120. It was found that the IIIB protein differed from the MN protein at positions 429, at which K replaced E, and 423, at which I replaced F (Fig. 4A). Because it was known

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418                                  445
CKIKQIINMWQKVGKAMYAPPIEGQIRC     MN_GNE
-----------E----------------     MN.429E
-----------A----------------     MN.429A
-A-------------------------      MN.419A
---A-----------------------      MN.421A
-------------A--------------     MN.432A
----------------------A-----     MN.440A
-R---F-----E----------S-----     LAI_IIIB
-----F---------------------      MN.423F
-----F-----E----------------     MN.423F,429E
```

FIG. 5. Sequences of C4 domain mutants of MN-rgp120. Nucleotide substitutions, resulting in the amino acid sequences indicated, were introduced into the C4 domain of the MN-rgp120 gene by recombinant PCR. The resulting variants were assembled into the expression plasmid pRK5, which was then transfected into 293s cells. The binding of MAbs to the resulting C4 domain variants was then analyzed (Table 2) by ELISA.

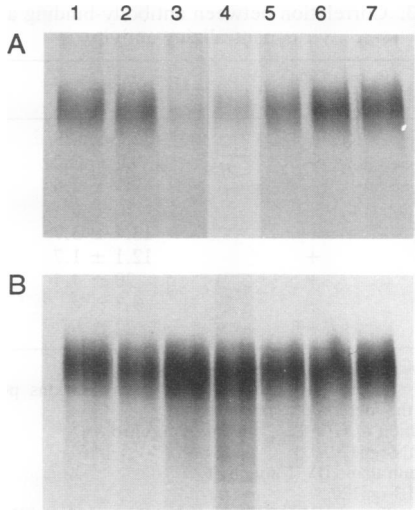


FIG. 6. Immunoprecipitation of C4 domain mutants. 293s cells were transfected with expression plasmids containing the MN-rgp120 variants shown in Fig. 5. Cell culture supernatants were harvested and used for the MAb-binding studies for which the results are shown in Table 2. To verify expression, radioimmunoprecipitation studies using cell culture supernatants from cells metabolically labeled with [$^{35}$S]methionine were performed with the 1024 MAb specific for the C4 domain of MN-rgp120 (A) or the 1034 MAb specific for the V3 domain of MN-rgp120 (B). Immune complexes were precipitated with the use of fixed *S. aureus*, and the adsorbed proteins were resolved by SDS-PAGE. Proteins were visualized by autoradiography. Lanes: 1, MN 419A; 2, MN 421A; 3, MN 429E; 4, MN 429A; 5, MN 432A; 6, MN 440A; 7, MN-rgp120.

from previous studies (33) that the 5C2 MAb was unable to bind to gp120 from two strains (i.e., NY-5 and JRcsf) that also possessed E at position 429, it seemed unlikely that this position could account for the strain specificity of 5C2. Sequence comparison (Fig. 4A) also showed that gp120 from $HIV\text{-}1_{IIIB}$ was unique in that a phenylalanine residue occurred at position 423, whereas the other six strains examined possess an I at this position. To determine whether residue 423 and/or 429 could account for the type specificity of the 5C2 MAb, a mutant of MN-rgp120 which incorporated an F-for-I replacement at position 423 (MN 423F) was

TABLE 2. Binding of CD4-blocking MAbs to C4 domain mutants

| Protein | Relative binding of MAb[a]: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1024 | 1093 | 1096 | 1097 | 1110 | 1112 | 1127 | 5C2 |
| MN-rgp120 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 0.05 |
| MN 419A | 1.11 | 1.10 | 0.94 | 1.21 | 0.78 | 0.95 | 1.10 | ND |
| MN 421A | 1.11 | 1.60 | 0.88 | 1.42 | 1.34 | 0.91 | 1.10 | ND |
| MN 429E | 0.03 | 0.07 | 0.11 | 0.04 | 0.10 | 0.10 | 0.02 | ND |
| MN 429A | 0.10 | 0.07 | 0.14 | 0.04 | 0.09 | 0.11 | 0.05 | ND |
| MN 432A | 0.77 | 0.15 | 0.59 | 0.08 | 0.12 | 0.24 | 0.26 | ND |
| MN 440A | 1.06 | 1.13 | 1.08 | 0.87 | 1.12 | 1.0 | 1.3 | ND |
| IIIB-rgp120 | 0.03 | ND | ND | ND | ND | ND | ND | 1.0 |
| MN 423F | ND | ND | ND | ND | ND | ND | ND | 0.45 |
| MN 423F-MN-429E | ND | ND | ND | ND | ND | ND | ND | 1.09 |

[a] The data represent the relative binding of MAbs to the native and mutant forms of rgp120. Values were calculated by dividing the binding (determined by ELISA) of the CD4-blocking MAbs to the mutants by the binding to native rpg120. Differences in the levels of expression were normalized on the basis of binding to a V3-specific MAb (1034). ND, not done.

TABLE 3. Correlation between antibody-binding affinity and virus-neutralizing activity

| MAb | CD4 blocking[a] | $K_d$ (nM)[b] | $IC_{50}$ (nM)[c] |
|---|---|---|---|
| 1024[d] | + | 2.7 ± 0.9 | 0.53 |
| 1086[d,e] | − | 9.7 ± 2.2 | |
| 1093[d] | + | 9.9 ± 2.6 | 6.7 |
| 1096[d] | + | 10 ± 6 | 11.3 |
| 1097[d] | + | 13.4 ± 3.7 | 8.0 |
| 1110[d] | + | 12.1 ± 1.7 | 41.3 |
| 1112[d] | + | 20 ± 4.4 | 195.3 |
| 1127[d] | + | 9.3 ± 4 | 2.2 |
| 13H8[e,f] | +[g] | 22 ± 6 | |

[a] Blocked binding of MN-rgp120 to CD4.
[b] Means ± standard deviations of four determinations per MAb were calculated by the method of Scatchard (40).
[c] Neutralization of HIV-$1_{MN}$ infectivity in vitro.
[d] MAb to MN-rgp120.
[e] Did not neutralize HIV-1 infectivity.
[f] MAb to IIIB-rgp120.
[g] Blocked binding of IIIB-rgp120 but not MN-rgp120 to CD4.

constructed (Fig. 5). In addition, the MN-rgp120 mutant, MN 429E (described above), was further mutagenized to incorporate an F-for-I substitution at position 423 (MN 423F), thus resulting in a double mutant (MN.423F–MN 429E), whose sequence was identical to that of IIIB-rgp120 within the 11-amino-acid 5C2 epitope (Fig. 4). The expression of these mutants in 293s cells was verified by radioimmunoprecipitation (data not shown) with rabbit polyclonal antisera to MN-rgp120. When the binding of the 13H8 MAb to a set of mutants incorporating substitutions at positions 423 and 429 was examined, it was found that none of the replacements affected the binding of this antibody (data not shown). When the binding of the 5C2 MAb was examined by ELISA, it was found (Table 2) that the F-for-I replacement (MN 423F) conferred partial reactivity. When the double mutant (MN 423F–MN 429E) containing the F-for-I substitution as well as the E-for-K substitution was examined, binding that was indistinguishable from that to IIIB-rgp120 was observed (Table 2). These results demonstrated that F at position 423 and E at position 429 both play a role in binding of the 5C2 MAb and suggest that the strain specificity of 5C2 can be attributed to the residues at these positions.

Examination of the sequences of gp120 from the six most common substrains of HIV-$1_{LAI}$ (IIIB, Bru, HXB3, BH10, HXB2, and HX10) revealed several polymorphisms in the C4 domain (Fig. 4B). Thus, the sequences of the HIV-$1_{LAI}$ clones IIIB (30), Bru (47), and HXB3 (6) were identical at positions 423 and 429 at which F and E residues occurred, respectively. However, the sequence of the HXB2 substrain (36) differed from the others at these positions at which, as in MN-rgp120, K replaced E and at position 423, at which I replaced F. Similarly, the HX10 and BH10 substrains (36, 37) differed only at position 423 at which, as in HIV-$1_{MN}$, I replaced F. On the basis of the mutagenesis experiments described above, it would be predicted that MAb 1024 should be able to bind to gp120 from the HXB2 substrain of HIV-$1_{LAI}$ but not the HXB3 substrain. If I-423 was important for binding, then 1024 should also bind the HX10 substrain. To test this hypothesis, the binding of MAb 1024 to the surface of cells infected with either the IIIB, HXB2, HXB3, or HX10 substrain of HIV-$1_{LAI}$ was determined by flow cytometry (Fig. 7). It was found that MAb 1024 was able to bind only HXB2, providing further confirmation that residues 423 and 429 were important for the binding of this antibody. The fact that MAb 1024 did not bind to HX10-infected cells suggested that I-423 was not the only residue important for the binding of this MAb. Thus, these studies demonstrated that reactivity with the 1024 MAb segregated

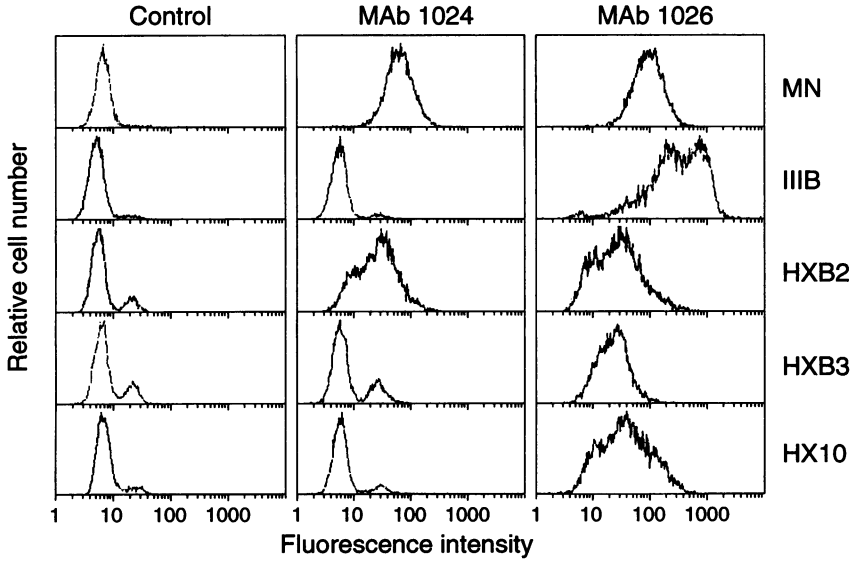


FIG. 7. Reactivity of MAb 1024 with HIV-$1_{LAI}$ substrains. The cell surface binding of the C4-domain-reactive MAb 1024 to H9 cells chronically infected with the $HIV_{LAI}$ substrains IIIB, HXB2, HXB3, and HXB10 or HIV-$1_{MN}$ was analyzed by flow cytometry. Cultures of virus-infected cells were reacted with either MAb 1024, a nonrelevant MAb (control), or a broadly cross-reactive MAb (1026) raised against MN-rgp120. After the unbound MAb was washed away, the cells were then labeled with fluorescein-conjugated goat antibody to mouse immunoglobulin G $(Fab')_2$, washed, and fixed with paraformaldehyde. The resulting cells were analyzed for the degree of fluorescence intensity by using a FACSCAN (Becton Dickinson). Fluorescence was measured as the mean intensity of the cells expressed as the mean channel number plotted on a log scale.

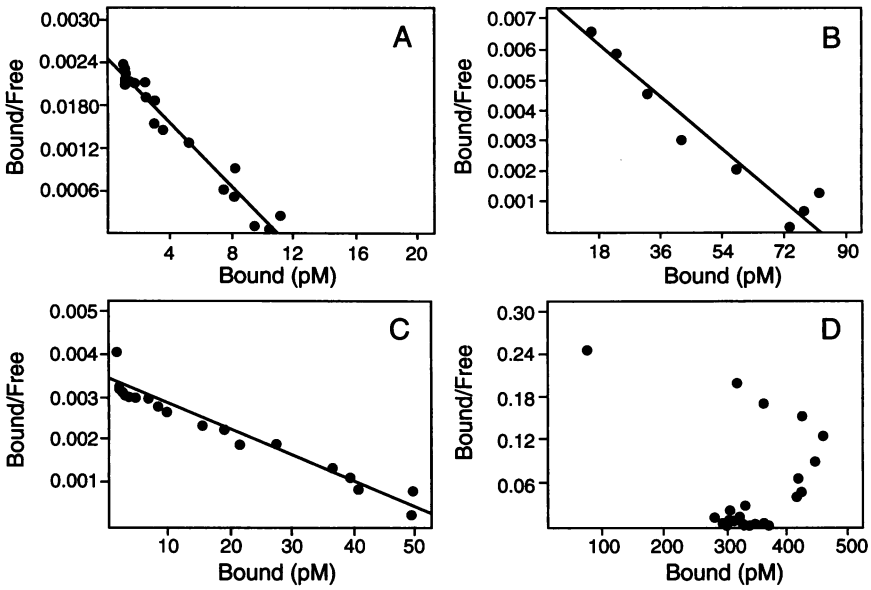


FIG. 8. Determination of the binding affinity of MAbs 1024 (A) and 1112 (B) for MN-rgp120 and 13H8 (C) and 5C2 (D) for IIIB-rgp120. CD4-blocking MAbs raised against MN-rgp120 (1024 and 1112) or IIIB-rgp120 (13H8 and 5C2) were labeled with $^{125}I$, and binding titrations with MN-rgp120 (A and B) or IIIB-rgp120 (C and D) were carried out as described in Materials and Methods.

with the occurrence of I and K residues at positions 423 and 429, respectively, and shows that substrains of HIV-$1_{LAI}$ differ from one another at a functionally significant epitope in the C4 domain.

**Neutralizing activity of CD4-blocking antibodies correlates with their binding affinity.** To account for the differences in virus-neutralizing activity between the CD4-blocking MAbs, their gp120-binding affinities were determined by competitive binding of $^{125}I$-labeled MAbs to rgp120 (Table 3). Typical Scatchard analysis of data from these assays is shown in Fig. 8A to C. Linear, one-site binding kinetics were observed for all the MAbs to MN-rgp120, suggesting that only a single class of sites was recognized and that there was no cooperativity between two combining sites of each immunoglobulin molecule. It was found (Fig. 8A; Table 3) that MAb 1024, which exhibited the most potent virus-neutralizing activity ($IC_{50}$ of 0.53 nM), possessed the lowest $K_d$ (2.7 nM). In contrast (Fig. 8B; Table 3), MAb 1112, the antibody that exhibited the weakest virus-neutralizing activity ($IC_{50}$ of 195 nM) possessed the highest $K_d$ (20 nM). $K_d$s for six additional CD4-blocking MAbs raised against MN-rgp120 were also determined (Table 3). It was found that MAbs that possessed intermediate $K_d$s similarly possessed an intermediate neutralization $IC_{50}$. When the $K_d$ of the MAbs was plotted as a function of the log of the $IC_{50}$, a linear relationship was obtained (Fig. 9). With this analysis, a correlation coefficient ($r$) of 0.97 was obtained. Thus, this graph demonstrates that the virus-neutralizing activity of these MAbs is directly proportional to the gp120-binding affinity and that the threshold for neutralization at this epitope is defined by the slope of the graph in Fig. 9.

A similar binding affinity analysis was performed with the non-neutralizing CD4-blocking MAbs to IIIB-rgp120, 5C2 and 13H8. The binding curve for 13H8 (Fig. 8C) showed that it bound to a single class of sites on IIIB-rgp120 with a $K_d$ of 22 nM. The affinity of 5C2 could not be determined by this assay because at antibody concentrations greater than 5 nM, nonlinear (reduced gp120 binding) was observed (Fig. 8D). This effect suggests steric hindrance under these concentrations or negative cooperativity between combining sites. The binding affinity was also determined for the non-neutralizing, non-CD4-blocking MAb to MN-rgp120, 1086 (data not shown). The fact that this antibody exhibited a binding affinity (9.7 nM) similar to many of the neutralizing MAbs but failed to inhibit infectivity demonstrated that high antibody-binding affinity alone is not sufficient for neutralization.

**Effect of C4 domain mutants on CD4 binding.** Finally, the CD4-binding properties of the series of MN-rgp120 mutants, constructed to localize the C4 domain epitopes, were measured in a qualitative coimmunoprecipitation assay. In these studies the ability of the mutagenized MN-rgp120 variants to coimmunoprecipitate CD4 was evaluated as described previously (22). It was found (Fig. 10) that all of the variants in

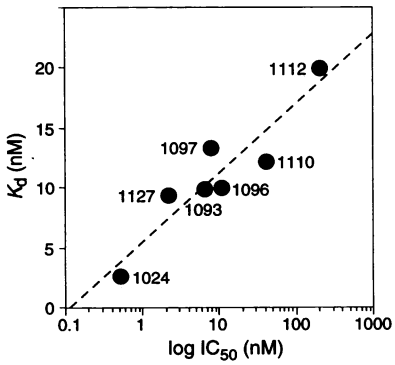


FIG. 9. Correlation between gp120 binding affinity ($K_d$) and neutralizing activity ($IC_{50}$) of MAbs to the C4 domain of MN-rgp120. Binding affinities of MAbs to the C4 domain of gp120 were determined by Scatchard analysis (Fig. 8; Table 3). The resulting values were plotted as a function of the log of their neutralizing activities ($IC_{50}$) (Fig. 2 and Table 3).

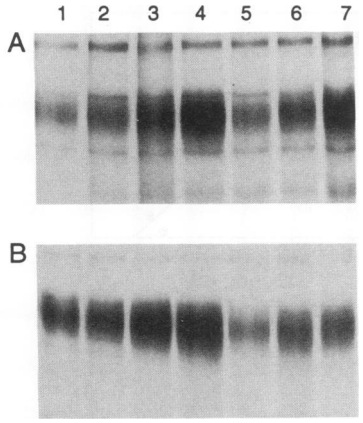


FIG. 10. Coimmunoprecipitation of MN-rgp120 C4 domain mutants with rsCD4. The ability of various C4 domain mutants of MN-rgp120 to bind rsCD4 was assessed in a qualitative coimmunoprecipitation assay similar to that described previously (22). Briefly, 293 cells, transfected with plasmids directing the expression of MN-rgp120 variants shown in Fig. 5, were metabolically labeled with [$^{35}$S]methionine, and the growth-conditioned cell culture supernatants were incubated with rsCD4. The resulting rsCD4:gp120 complexes were then immunoprecipitated by the addition of the CD4-specific MAb, 465 (A), or a positive control MAb (1034) directed to the V3 domain of MN-rgp120 (B). The immunoprecipitated proteins were resolved by SDS-PAGE and visualized by autoradiography as described previously (3). Lanes: 1, MN 419A; 2, MN 421A; 3, MN 429E; 4, MN 429A; 5, MN 432A; 6, MN 440A; 7, MN-rgp120.

which apolar A residue was substituted for the charged K or E residues (e.g., MN 419A, MN 421A, MN 432A, and MN 440A) were still able to coimmunoprecipitate rsCD4. Similarly, the replacement of E for K at position 429 (MN 429E), the replacement of F for I at position 423 (MN 423F), or the mutant which incorporated both mutations (MN 423F–MN 429E) also showed no reduction in its ability to coimmunoprecipitate rsCD4. Thus, radical amino acid substitutions at five positions failed to affect the binding of gp120 to CD4. These results were consistent with previous studies (5, 22, 34) in which it was found that only a few of the many mutations that have been induced in this region affected CD4 binding.

## DISCUSSION

The present studies demonstrate that MAbs to the C4 domain of rgp120 can be potent inhibitors of HIV-1 infectivity if their concentration and/or affinity exceeds threshold values for virus neutralization. The threshold for neutralization of HIV-$1_{MN}$ is defined by the slope of the graph in Fig. 9, which shows that the log of the $IC_{50}$ for virus neutralization by MAbs binding to the C4 domain is linearly related to the antibody-binding affinity. The log-linear relationship suggests that virus neutralization is a cooperative process in which small changes in antibody affinity result in large differences in the $IC_{50}$. These observations suggest that the results from previous studies (1a, 26, 41) noting the lack of correlation between CD4-blocking antibodies with virus neutralization may simply be a consequence of either low-affinity binding or low antibody concentration. Langedijk et al. (19) have reported that the log of the HIV-$1_{IIIB}$ virus-neutralizing activity of V3-specific MAbs in ascites fluid was linearly related to the log of the gp120-binding affinity. This result differed from the present study in that a log-log, rather than semilog, relationship was noted between antibody-binding affinity and virus-neutralizing activity. The log-log relationship might be a consequence of a neutralization mechanism at the V3 domain different from that at the C4 domain or may be the result of uncertainties arising from the method used to calculate the gp120-specific antibody concentration in the ascites fluid.

The observation that the 5C2 and 13H8 MAbs failed to neutralize HIV-$1_{IIIB}$ infectivity, even at high MAb concentrations, suggests that the sensitivity of different viruses to neutralization at homologous epitopes may vary considerably. It has been known (48) for some time that some viruses (e.g., HIV-$1_{IIIB}$) are more resistant to neutralization in vitro than others (e.g., HIV-$1_{SF-2}$). We do not yet know whether the neutralization curve defined for MAbs to the C4 domain epitope of HIV-$1_{MN}$ is similar to that of MAbs to other neutralizing epitopes (e.g., the V3 domain). However, as more neutralizing antibodies become available, it should be possible to answer this question. Theoretically, the potency of virus-neutralizing antibodies should vary depending on the mechanism by which they interfere with virus function. Thus, antibodies that neutralize infectivity by blocking the high affinity interaction between gp120 and CD4 would be predicted to require higher gp120-binding affinities for neutralization than would antibodies that neutralize infectivity by disrupting weaker interactions (e.g., gp120-to-gp41 binding, subunit oligomerization).

The epitopes recognized by the virus-neutralizing, CD4-blocking MAbs to MN-rgp120 were found to be located in the C4 domain of MN-rgp120 and were critically dependent on a lysine residue at position 429 and/or 432. The published sequence (13, 38) of gp120 from the HIV-$1_{MN}$ isolate that has been widely distributed ($MN_{1984}$) differs from that obtained from two independent molecular clones of gp120 from HIV-$1_{MN}$ sequenced in our laboratory ($MN_{GNE}$) in that an E rather than a K residue occurs at position 429 (Fig. 5). This result suggests that some stocks of HIV-$1_{MN}$ (13, 38) may contain a mixture of viruses with either a K or an E at position 429. However, our data suggest that the variant with E at position 429 must represent a very small fraction of the mixture or is selected against under the virus neutralization assay conditions employed, since the 1024 MAb (that shows absolute specificity for K at position 429) is able to neutralize stocks of HIV-$1_{MN}$ (originally obtained from the National Institutes of Health AIDS Research and Reference Reagent Program) maintained in two different laboratories (3a, 33a).

Because of these observations, we wondered whether similar polymorphisms might occur in the envelope glycoproteins of other viruses at position 429. Examination of the sequences reported for six HIV-$1_{LAI}$ molecular clones (substrains Bru, IIIB, HXB2, HXB3, BH10, and HX10) revealed amino acid changes virtually identical to those observed for HIV-$1_{MN}$, with either K or E occurring at position 429 (6, 30, 36, 37, 47). Flow cytometry studies, in which MAb 1024 bound to cells infected with HIV-$1_{LAI}$ substrain HXB2, but not to cells infected with the HIV-$1_{LAI}$ substrains IIIB, HXB3, and HX10, demonstrated the importance of K-429 and confirmed the published sequence of $HIV_{HXB2}$. Examination of the Los Alamos Human Retrovirus and AIDS Data Base (32) for polymorphisms at position 429 showed that the residues known to occur at this position are limited, with only E, K, R, G, or V residues reported. In addition, particular amino acids were sometimes associated with different international subtypes. For example, R at position 429 was most common in the A subtype, and E at this

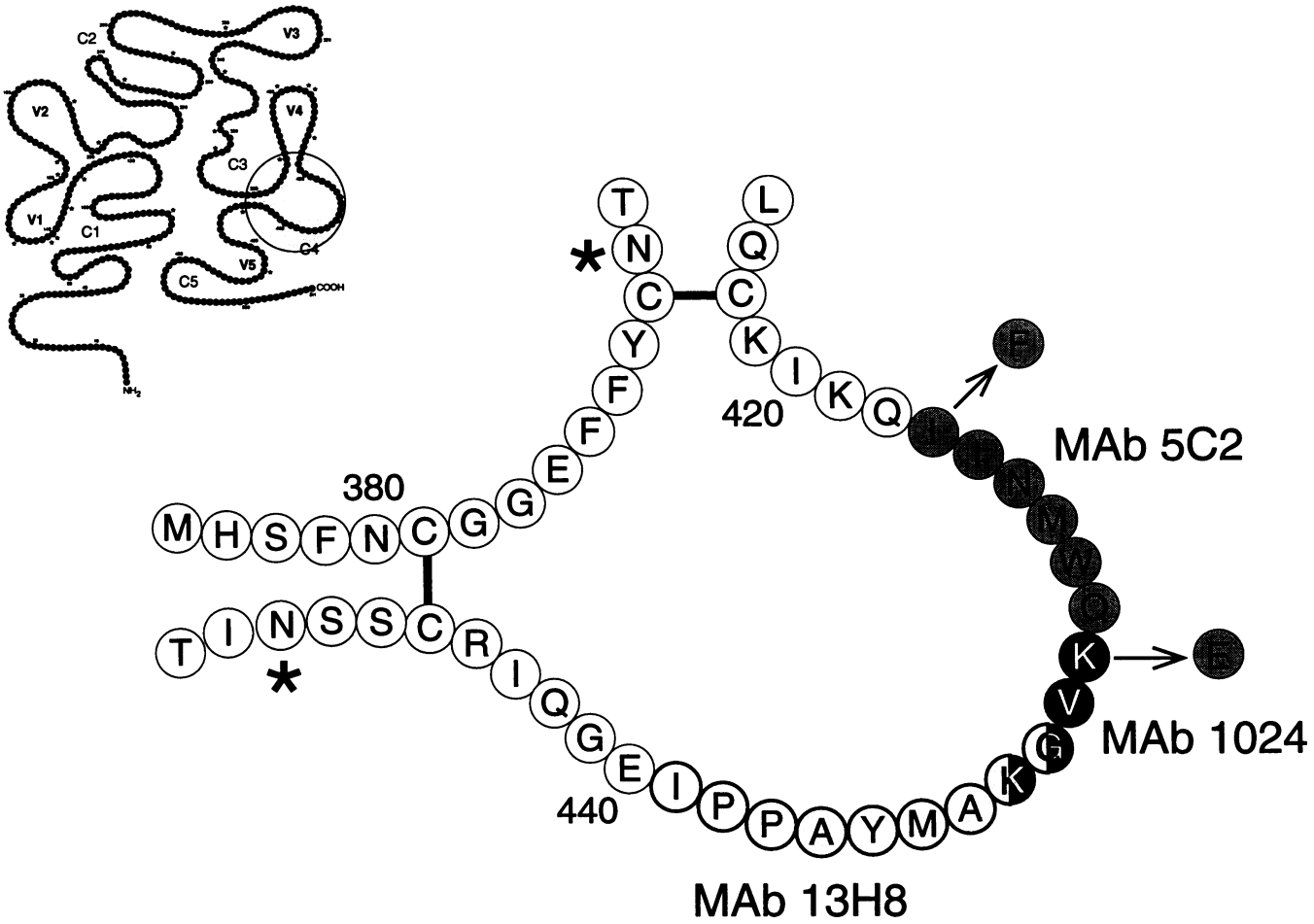


FIG. 11. Location of epitopes recognized by CD4-blocking MAbs in the C4 domain of MN-rgp120. The sequence shown is that of the C4 domain of gp120 from the $MN_{GNE}$ clone of gp120. Shaded residues indicate the sequences recognized by the strain-specific MAbs, 5C2 and 1024, and the type common MAb, 13H8. Polymorphisms that inhibit the binding of the IIIB-rgp120-specific MAb 5C2 (I for F substitution) and the MN-rgp120-specific MAb 1024 (E for K substitution) are indicated. Asterisks indicate the locations of glycosylation sites. The relative location of the C4 domain within the secondary structure of MN-rgp120 is shown in the upper left corner.

position was most common in the B subtype (representative of viruses in North America and Europe).

The polymorphisms that we observed at positions 423 and 429 in substrains of HIV-$1_{MN}$ and HIV-$1_{IIIB}$ suggest that sequences obtained from single clones of a virus isolate must be approached with caution and that seemingly minor differences in sequence can be antigenically significant. Amino acid substitutions of the type documented for positions 423 and 429 may result from yet-to-be-defined selective factors in virus culture conditions. Factors, such as the cellular substrate (45) for virus culture (e.g., peripheral blood mononuclear cells or T-cell lines), the time after infection in which the sample was collected, and the clinical state of the individual from whom the virus sample was recovered (9, 27), may result in inadvertent selection for different envelope glycoprotein sequences and different virus phenotypes. The hypothesis that the C4 domain is mutable and responsive to selective conditions is supported by the results of McKeating et al. (25), who found that selection of HIV-$1_{IIIB}$ in the presence of rsCD4 resulted in a T-for-M substitution at position 435 in the C4 domain and was associated with an rsCD4-resistant, enhanced syncytium-forming phenotype. Analysis of molecular clones from two unrelated strains, HIV-$1_{LAI}$ and $HIV_{MN}$, has shown that either E or K can occur at position 429. We suspect that as more molecular clones of other virus isolates are examined, replacements at these positions will be seen more frequently.

The present studies document three distinct epitopes in the C4 domain of gp120 that are recognized by MAbs able to inhibit the CD4 binding (Fig. 11). Two of these epitopes (5C2 type and 1024 type) are strain specific, while the third (13H8 type) is broadly cross-reactive. Previously (33), we deduced that the 13H8 epitope lies in a highly conserved region located C terminal to the 5C2 epitope. The present studies confirm this conclusion and locate the 13H8 epitope to a nine-amino-acid peptide located between residues 431 and 439. This site is virtually identical to that described by McKeating et al. (26), who concluded that the sequence from W-427 to P-438 is exposed on the surface of gp120 and that the binding site for the broadly cross-reactive, weakly neutralizing MAbs, ICR38.8f and ICR38.1a, lies between residues W-427 and A-436. Although the C4-directed MAbs described by Sun et al. (44) were also broadly cross-reactive, it is not clear whether they bind to the 13H8-like or 5C2-like

epitope. The immunogen used by these investigators appears to be from a heterogeneous HIV-$1_{LAI}$ culture that could have contained a mixture of BH10- and HXB2-like sequences. The former would be expected to yield a more cross-reactive response than the latter.

For purposes of vaccine development, HIV-1 strains have been serotyped primarily on the basis of the most prevalent sequence at the PND (the V3 domain) which, for many populations, is represented by the sequence present in HIV-$1_{MN}$ (20). Does the characterization of a neutralizing site in the C4 domain represent a second criterion by which HIV-1 can be serotyped? In our view, the definition of serotypes should not rely solely on the discovery of a new neutralizing MAb but must relate to a significant biological property of virus or the ability of a vaccine to elicit a biologically significant immune response. While MAbs to the C4 domain have been isolated from mice and rats immunized with gp120, there is no data to show whether antibodies to this site are elicited as part of the normal response to HIV-1 infection in man. There are two reports (4, 44) in which investigators have been unable to detect antibodies to synthetic C4 domain peptides in the sera of HIV-1-infected humans. However, it is not clear from these studies whether the failure to detect antibodies to the C4 domain resulted from the actual absence of such antibodies or whether the sequence of the synthetic peptides used to detect these antibodies (HIV-$1_{HXB2}$) was unrepresentative of the HIV-1 strains that infected the individuals from whom the sera was derived.

The MAb binding studies described in this paper demonstrate that polymorphism at, or near, position 429 of gp120 is antigenically significant and enables viruses to escape binding by neutralizing MAbs. We have observed that sequence variation in this region occurs among different strains of HIV-1 and within clones of the same strain. These results, along with an analysis of available sequence information (32), suggest that the sequence variation in the C4 domain is finite and that amino acid substitutions at position 429 may represent a set of common antigenic variants. On the basis of this information, immunogens containing the most common C4 domain variants could be selected for inclusion in multivalent HIV-1 vaccines. Since only four or five residues are known to occur at position 429, vaccines of this type should be possible. However, before such efforts are justified, data demonstrating that an immune response to the C4 domain is relevant to protective immunity in vivo will need to be obtained.

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