A Region in Domain 1 of CD4 Distinct from the Primary gp120 Binding Site Is Involved in HIV Infection and Virus-mediated Fusion*

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The high affinity binding site for human immunodeficiency virus (HIV) envelope glycoprotein gp120 resides within the amino-terminal domain (D1) of CD4. Mutational and antibody epitope analyses have implicated the region encompassing residues 40–60 in D1 as the primary binding site for gp120. Outside of this region, a single residue substitution at position 87 abrogates syncytium formation without affecting gp120 binding. We describe two groups of CD4 monoclonal antibodies (mAbs) which recognize distinct epitopes associated with these regions in D1. These mAbs distinguish between the gp120 binding event and virus infection and virus-induced cell fusion. One cluster of mAbs, which bind at or near the high affinity gp120 binding site, blocked gp120 binding to CD4 and, as expected, also blocked HIV infection of CD4+ cells and virus-induced syncytium formation. A second cluster of mAbs, which recognize the CDR-3 like loop, did not block gp120 binding as demonstrated by their ability to form ternary complexes with CD4 and gp120. Yet, these mAbs strongly inhibited HIV infection of CD4+ cells and HIV-envelope/CD4-mediated syncytium formation. The structure of D1 has recently been solved at atomic resolution and in its general features resembles IgV, regions as predicted from sequence homology and mAb epitopes. In the D1 structure, the regions recognized by these two groups of antibodies correspond to the C'C" (Ig CDR2) and FG (Ig CDR3) hairpin loops, respectively, which are solvent-exposed β turns protruding in two different directions on a face of D1 distal to the D2 domain. This face is bracketed by the longer BC (Ig CDR1) loop which bisects the plain formed by C'C" and FG. This structure is consistent with C'C" and FG forming two distinct epitope clusters within D1. We conclude that the initial interaction between gp120 and CD4 is not sufficient for HIV infection and syncytium formation and that CD4 plays a critical role in the subsequent virus-cell and cell-cell membrane fusion events. We propose that the initial binding of CD4 to gp120 induces conformational changes in gp120 leading to subsequent interactions of the FG loop with other regions in gp120 or with the fusogenic gp41 portion of the envelope gp160 glycoprotein.

Attachment of human immunodeficiency virus (HIV) to cells is mediated through a high affinity interaction of the viral envelope glycoprotein gp120 with the cell surface receptor CD4 (1–11). CD4 is a 55-kDa transmembrane glycoprotein predominantly expressed by a subset of T-lymphocytes. The extracellular region consists of four tandem domains that have sequence and structural homology with immunoglobulins (12–14). During normal immune responses, CD4 is believed to promote the activation of T-lymphocytes through association with monomorphic regions of major histocompatibility complex Class II molecules (15–17). In the pathogenesis of AIDS, the receptor is usurped by HIV to facilitate viral attachment and entry into the CD4+ subset of T-cells and other cells expressing CD4 (1–11). Infection of CD4+ cells is followed by their progressive depletion leading to generalized immunological abnormality and a decreased ability to mount an effective immune response to parasitic infections.

The high affinity binding site for gp120 resides within the amino-terminal domain (D1, previously denoted V1) of CD4. Accordingly, a truncated protein containing residues 1–106 is sufficient for this interaction (18). Mutational analysis within domain 1 has implicated a discrete loop, which by analogy to Ig variable region structures is equivalent to CDR2, as the primary determinant for high affinity binding (6, 18–23). In addition, epitope mapping analysis shows that antibodies to this region block gp120 and virus binding, HIV infectivity of CD4+ cells, and HIV-mediated syncytium formation (24, 25). Outside of the CDR2-like region, a single substitution of residue 87 within a loop analogous to CDR3 of IgV domains abrogates the ability of CD4 to facilitate HIV-envelope-mediated syncytium formation without altering its affinity for gp120 (26).

In the present study, we have further analyzed the role of these regions in the initial gp120 binding step and the subsequent events leading to envelope- and CD4-mediated viral-cell and cell-cell membrane fusion. We describe two groups of monoclonal antibodies (mAbs) which map to two different sites within the amino terminal domain of CD4 as determined by cross-blocking and mutant binding analysis. The deduced

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§§ The abbreviations used are: HIV, human immunodeficiency virus; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

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epitopes for these mAbs are interpreted in the context of the recently determined atomic structure of D112 which shows significant structural homology to IgV, domains. One group of antibodies (L77 family) binds to the high affinity gp120 binding site within the CDR2-like region and inhibits the binding of gp120 to CD4. A second group (L71 family) recognizes a localized site within the CDR3-like region. This second group did not prevent gp120 binding to CD4 as demonstrated by the formation of ternary complexes between mAb, CD4, and gp120. Nonetheless, these mAbs blocked HIV infectivity and syncytium formation to an extent comparable to that of the CDR2 binding mAbs. We conclude that the initial interaction with the CDR2-like region is a prerequisite step for HIV infection of CD4+ cells, but is not sufficient for the subsequent events leading to envelope-CD4-mediated fusion of viral and cellular membranes. Furthermore, we propose that the initial attachment leads to conformational changes in gp120 which facilitate its interaction with regions outside of the CDR2-like loop.

MATERIALS AND METHODS

Cross-blocking of CD4 mAb Binding—HPB-ALL cells or peripheral blood mononuclear cells (105) were incubated with unlabeled mAb (fivefold excess) for 30 min at 4°C. FITC-labeled goat anti-mouse antibody was added at titre point and incubated for a further 20 min. Cells were washed, fixed with 1% paraformaldehyde, and analyzed on a FACScan™ (Becton-Dickinson, Sunnyvale, CA).

Mapping of mAb Epitopes with CD4 Mutants—mAbs were mapped using CD4 mutant proteins as previously described (18, 27). Briefly, the antibodies were incubated with soluble CD4 proteins for 30 min followed by the addition of SupT1 cells. After a 30-min incubation, the cells were washed, stained with FITC-labeled goat anti-mouse antibody, washed again, fixed, and analyzed by flow cytometry. Percent blocking with each CD4 mutant was computed using the formula 100 × [(MP - sT4)/MP], where MP is the mean fluorescence without CD4 protein (c), with mutant protein (m), and with V1V2 soluble CD4 protein standards (s).

Inhibition of gp120 Binding by mAbs—HPB-ALL cells at 5 × 105/ml in 100 μl/well were incubated for 30 min with dilutions of CD4 mAbs or medium alone in 96-well round-bottomed microtitre plates, then the appropriate concentration of recombinant gp120 was added. Following a further 30-min incubation and washing, 50 μl of 1 μg/ml biotin-labeled gp120 mAb (Dupont, 9284, Ref. 28) was added. Bound gp120 mAb was detected with streptavidin-FITC (Boehringer Mannheim) and cells were analyzed by flow cytometry.

Mapping of HIV Binding to CD4+ Cells by Abs—HIV-1pHVA was labeled using standard protein labeling procedures by incubation with FITC in bicarbonate buffer, pH 9.5, and the unconjugated FITC was removed by passing the mixture over a Sephadex column. For binding assays, 5 × 104 CEM cells were incubated with or without antibody followed by the addition of FITC-labeled virus (final incubation volume was 200 μl) for 30 min at 37°C, washed, fixed with paraformaldehyde, and analyzed by flow cytometry.

Immunoprecipitation of gp120-sT4-mAb Complexes—To immunoprecipitate ternary complexes of gp120-sT4-mAb, conditions were optimized to favor binding of the CD4 mAbs to sT4 complexed with gp120, i.e., the concentration of sT4 was limiting in the reaction mixture. gp120 (3 μg) was incubated with sT4 (155 ng) for 1 h at 4°C prior to the addition of mAb (3.6 μg). Alternatively, sT4 was first incubated with mAb for 1 h at 4°C, followed by the addition of gp120. Incubations were carried out in Ca2+- and Mg2+-free PBS, containing 0.1% Nonidet P-40 and 0.5% nonfat dry milk. After 1 h, prewashed protein G-Sepharose (10 μl; Genex Corp., Gaithersburg, MD) was added and incubated for a further 1 h. Immunocomplexes were washed three times with ice-cold buffer, boiled in sample buffer, and separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a Mini-Protean III gel apparatus (Bio-Rad Labs, Rockville, NY). The proteins were then electrophoretically transferred to nitrocellulose (40 V, 2 h). The nitrocellulose paper was subsequently incubated with blocking buffer (50 mm Tris-HCl, pH 8, 2 mm CaCl2, 80 mm NaCl, containing 0.2% Nonidet P-40 and 5% nonfat dry milk), then incubated with a 1:100 dilution of either rabbit anti-gp120 or anti-sT4 sera, and then finally with blocking buffer containing 125I-protein A (Amersham Corp.). Quantitation of the 125I-protein A binding was determined by autoradiography and scanning using an LKB Ultra Scan XL laser densitometer (Pharmacia LKB Biotechnology, Inc.).

Binding of gp120 to mAb Captured sCD4 on Plates—2 μg of rabbit anti-mouse Ig (DAKO Corp.; 20 μg/ml in bicarbonate buffer, pH 9.2) were adsorbed to 96-well ELISA microtitre plates for 1 h at room temperature. After washing, nonspecific binding was blocked by a 30-min incubation with PBS containing 2% nonfat dry milk (Marvel), and gp120 (100 μl) of 30 μg/ml of purified CD4 mAb in PBS was added to each well and incubated for 1 h. Following further washing, sT4 at 1 μg/ml was added for 1 h. The plates were washed and varying concentrations of gp120 were added to each well for 1 h. The plates were washed and 50 μl of 1 μg/ml biotinylated gp120 mAb (Dupont, 9284) was added. After washing, streptavidin-horseradish peroxidase (Dako, K277) was added at a dilution of 1:20,000. Following a further 45-min incubation the plates were washed, and the reaction was developed in o-phenylenediamine in citrate buffer (pH 5) and read on an ELISA plate reader.

Virus Strains—HIV isolates were obtained from the following sources: HIV-1 isolates HTLV-IIIB and HTLV-IIIRFP from R. Gallo (National Institutes of Health, Bethesda, MD), CD4A and CD4B from P. Clapham and R. A. Weiss (Chester-Betty Laboratories, London, UK); U455 from P. Greenaway (Porton Down, UK); and HIV-2 isolates LAV2-ROD from L. Montagnier (Pasteur Institute, Paris, France) and CBL20 from P. Clapham.

Cell and Viral Cultures—Persistently infected or uninfected H9 cells (from M. Popovic and R. Gallo) and SupT1 (J. Hoeie, University of Pennsylvania, PA), C8166 (R. Gallo), CEM, and HPB-ALL were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum. HIV-containing supernatants were harvested from 5-day cultures of a 1:1 mixture of uninfected H9 cells with HIV-infected H9, and titrated on C8166 cells by syncytium assay to determine the TCID50.

Virus Infection Assay—Syncytium assays were done as previously described (16) by overnight cocultivation of HIV-infected H9 cells with uninfected C8166 cells at a ratio of 1:2, in the presence or absence of inhibitory antibody.

HIV Infectivity Assay—Infection of C8166 cells was determined following addition of approximately 1000 tissue culture infectious doses50 (TCID50) of virus. Soluble p34 HIV core antigen in the culture supernatant was measured on day 5 after infection by twin site ELISA. Supernatants were inactivated by heating for 30 min at 56°C in a 1% Empigen detergent, diluted 1000-1000-fold into Tris-buffered saline-0.1% Empigen, and 100 μl was added to 96-well microtitre plate wells and incubated with adsorbed sheep anti-p24 polyclonal antibody (D7320 [Aalto BioReagents, Ireland]). Captured p24 was detected with alkaline phosphatase-conjugated mAb EHl2El (29; obtained previously described (16) by overnight cocultivation of HIV-infected H9 cells with uninfected C8166 cells at a ratio of 1:2, in the presence or absence of inhibitory antibody.

RESULTS

Cross-blocking Analysis of CD4 mAbs—The CD4 mAbs were segregated into epitope groups on the basis of their ability to cross-block their binding to CD4+ cells. Interference between antibodies suggests recognition of common or spatially related sites on CD4 (27). Conversely, simultaneous binding of two antibodies indicates their epitopes are spatially distinct. Each of this series of mAbs was labeled with FITC and added to HIV-1-infected cells that had been preincubated with saturating amounts of a specific mAb. It was shown that the mAbs cluster into two main groups. One group that included Leu3a, L77, L60, L93, and L1100 cross-block each other completely. A second group that included L68, L71, and L104 were distinct in that they cross-blocked each other but had no effect on the Leu3a group of mAbs. L120 (a D34D mAb) was not inhibited by antibodies of either group. As expected, binding of all FITC-labeled mAbs was blocked by saturating

2 Ryu, S. E., Kuong, P., Truneh, A., Porter, T., Arthos, J., Rosenberg, M., Dai, X., Xuong, N.-H., Axel, R., Sweet, R., and Hendrickson, W. (1990) Nature 348, 419-426.
amounts of the corresponding unlabeled mAbs (Table I, diagonal).

**Epitope Mapping to CD4 Domains with Truncated, Soluble CD4 Proteins**—Binding of mAbs to specific CD4 domains was localized by measuring the ability of truncated soluble CD4 proteins to block antibody binding to the CD4+ cell line, SupT1. The proteins used were sT4 (which consists of the four extracellular domains of CD4, including residues 1–569), V1V2 (first two amino-terminal domains, residues 1–183), and V1 (domain 1, residues 1–106) (18). All but two mAbs in the current study, bind to domain 1 of CD4 (Table II; data for OKT4 not shown but similar to L120). The V1 protein retains all the HIV-related activities of soluble CD4 including its ability to block gp120 binding, HIV infection of CD4+ cells, and HIV-mediated syncytium formation (18). Two mAbs, L120 and OKT4, bind only to sT4 and not to the V1V2 or V1 proteins. These mAbs were selected as experimental controls for the current study because they recognize regions in the 3rd or 4th domains of CD4 and do not affect gp120 binding to CD4.

**Fine Specificity Analysis of CD4 mAbs**—Site-specific single and multiple substitution mutations expressed in the context of the V1V2 and sT4 proteins were used to define the fine specificity of the mAbs binding within the V1 domain of CD4. Most of this collection of mutants were previously used to define the HIV gp120 binding site on CD4 and for structural analysis of the V1 domain. In general, the proteins contain mouse to human residue switches which results only in localized perturbation of structure.

Table II shows that binding of the Leu3a group of mAbs is disrupted by mutation of residues 24–27 and 41–43. As revealed by the high resolution structure of the human CD4 protein,2 these residues are within loops on one face of domain 1 that approximately correspond to CDR1 and CDR2, respectively, of Ig light chain variable regions. The CDR2-like region contains the primary determinant of the high affinity binding site for gp120. Introduction of the Phe substitution at position 55 which compromises the binding of some of the mAbs in this group as well as gp120, probably perturbs the structure of the CDR2-like loop since it represents replacement of the buried Ala side chain with more bulky Phe.

In contrast, the group of mAbs including L68, L71, and L104 were totally unaffected by mutations in the CDR2-like region. However, binding was almost completely abrogated by the double mutation in residues 88–89 which reside within the CDR3-like region. This mutation has no effect on gp120 binding (18).

**Inhibition of HIV-envelope gp120 and Virus Binding**—Having mapped the mAbs to either the CDR2 or CDR3-like loops...
CD4 Determinants Involved in HIV Binding and Infection

TABLE III

Inhibition of HIV-envelope gp120 and HIV binding by CD4 mAbs

| mAb      | Relative affinity, EC$_{50}$ | Inhibition of gp120 binding, IC$_{50}$ | Inhibition of HIV binding, IC$_{50}$ |
|----------|-------------------------------|---------------------------------------|------------------------------------|
|          |                               | ng/ml                                 |                                    |
| Leu3a    | 1                             | 20                                    | 10                                 |
| L77      | 3                             | 50                                    | 15                                 |
| L68      | 16                            | 1,000                                 | ND*                                |
| L71      | 15                            | 1,000                                 | >150                               |
| L104     | 7                             | 420                                   | ND                                 |
| L120     | 10                            | >10,000                               | ND                                 |
| OKT4     | 200                           | >10,000                               | ND                                 |

* Concentration of mAb required for 50% saturation binding to CD4$^+$ HPB-ALL cells.
* Concentration of mAb required for 50% inhibition of gp120 or HIV binding to CD4$^+$ cells.
* ND, not done.
* This mAb caused a maximum of 75% inhibition of gp120 binding with no further increases above 1,000 ng/ml.

In the addition of a CD4 mAb, alternatively, sT4 and mAb were preincubated followed by the addition of gp120. In both cases, the complexes were immunoprecipitated with protein G-coupled Sepharose. The presence of soluble CD4 and gp120 in the complexes was determined by Western blot analysis. Ternary complexes of mAb, sT4, and gp120 were formed with antibodies L68, L71, and L104 but not with Leu3a and L77 (Fig. 1). Thus, the binding sites for L68, L71, and L104 were not blocked by gp120 and, correspondingly, these mAbs could not be displaced by subsequent binding of gp120. Antibodies that bind directly at or near the gp120 binding site (Leu3a and L77) were not able to coimmunoprecipitate gp120. As expected, these latter antibodies immunoprecipitated sT4 more efficiently when they were incubated with sT4 prior to the addition of gp120 (Fig. 1, Ia and Iib). OKT4, which binds outside of the V1V2 domain of CD4, was able to immunoprecipitate gp120 (Ib and IIb) with the corresponding amount of sT4 (Ia and IIa). Similar results were also obtained with mAb L120 (not shown).

In the second approach to demonstrate simultaneous binding of mAb and gp120 to CD4, the ability of mAb-captured sT4 to bind to gp120 was analyzed on ELISA plates. L120 captured sT4 without interfering with the ability of sT4 to bind to gp120 (Fig. 2). As expected, L77-captured sT4 was unable to bind gp120. However, sT4 captured by L68, L71, and L104 was able to bind gp120 although with varying efficiency, L104 being the most effective. This latter observation may reflect some subtle differences in the way these mAbs orient themselves for recognition of this epitope in D1. All of the antibodies bound equivalent amounts of sT4 (Fig. 2, inset).

**mAb Inhibition of Syncytium Formation**—All mAbs were tested for their ability to inhibit syncytium formation induced by HIV. Five strains of HIV-1 and two strains of HIV-2 were used for this analysis. As expected, Leu3a was very effective at blocking syncytium formation induced by the HIV strains, presumably by interfering with the binding of viral envelope gp120 with CD4 (Table IV). L77 which was also a binding site inhibitor was comparatively less effective, perhaps reflecting a difference in affinity to Leu3a. Surprisingly, the V1 mAbs that do not interfere with gp120 binding were very effective at blocking syncytium formation. Although there were strain to strain differences, all the mAbs of this group (i.e. L68, L71, and L104) showed low IC$_{50}$ with the HIV-2 strains and several of the HIV-1 strains. L120, which binds outside domains 1 and 2, was totally ineffective.

**mAb Inhibition of HIV Infection**—All mAbs were tested for their ability to inhibit HIV infection of C8166 cells. The spread of infection was assayed as the production of soluble within domain 1, we evaluated their ability to interfere with gp120 binding to CD4 on the surface of T-cells. Leu3a and L77 were very effective at blocking gp120 binding while L68, L71, and L104 were ineffective or blocked only weakly (Table III). The inability of this latter group of mAbs to block gp120 binding cannot be fully accounted for by lower affinities for gp120 binding to CD4 on the surface of T-cells. Leu3a and L77 were very effective at blocking HIV infection of CD4$^+$ CEM cells whereas L71 was ineffective.

To determine that the differential effects of these two groups of mAbs in the gp120/CD4 binding assays were not fully attributable to the way soluble gp120 binds to cell surface or soluble CD4, it became necessary to test these mAbs for inhibition of whole virus binding to CD4$^+$ cells (Table III, last column). Leu3a and L77 were very effective at blocking HIV binding to the CD4$^+$ CEM cells whereas L71 was ineffective.

Altogether, the above results suggest that the L71 group of mAbs recognize epitopes outside of the CD4/gp120 complex.

**Formation of gp120-sT4-mAb Ternary Complexes**—Two approaches were taken to confirm the simultaneous binding of the L71 group of mAbs and gp120 to CD4. In the first approach, sT4 and gp120 were preincubated followed by the addition of a CD4 mAb. Alternatively, sT4 and mAb were preincubated followed by the addition of gp120. In both cases, the complexes were immunoprecipitated with protein G-coupled Sepharose. The presence of soluble CD4 and gp120 in the complexes was determined by Western blot analysis. Ternary complexes of mAb, sT4, and gp120 were formed with antibodies L68, L71, and L104 but not with Leu3a and L77 (Fig. 1). Thus, the binding sites for L68, L71, and L104 were not blocked by gp120 and, correspondingly, these mAbs could not be displaced by subsequent binding of gp120. Antibodies that bind directly at or near the gp120 binding site (Leu3a and L77) were not able to coimmunoprecipitate gp120. As expected, these latter antibodies immunoprecipitated sT4 more efficiently when they were incubated with sT4 prior to the addition of gp120 (Fig. 1, Ia and Iib). OKT4, which binds outside of the V1V2 domain of CD4, was able to immunoprecipitate gp120 (Ib and IIb) with the corresponding amount of sT4 (Ia and IIa). Similar results were also obtained with mAb L120 (not shown).

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**mAb Inhibition of HIV Infection**—All mAbs were tested for their ability to inhibit HIV infection of C8166 cells. The spread of infection was assayed as the production of soluble

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**FIG. 1. Immunoprecipitation of gp120-CD4 complexes by CD4 mAbs.** In I, gp120 and sT4 were preincubated for 1 h prior to the addition of mAb. In II, sT4 and mAb were preincubated prior to the addition of gp120.
FIG. 2. Binding of gp120 to mAb captured sCD4.

FIG. 3. Inhibition of HIV infection by CD4 mAbs.

p24 core antigen. Leu3a was very effective at blocking infection while L77 was much less effective (Fig. 3). In line with the syncytium inhibition results, L71, L68, and L104 were all effective at blocking infection, with L71 showing IC50 that is comparable to Leu3a. L120 showed no activity.

Atomic Representation of HIV Binding and Fusion Determinants of CD4—Fig. 4 shows a space-filling model of the atomic structure of D1 (residues 1–98). Residues 18–25 (white), 40–43 (pink), 58–64 (yellow), and 86–89 (green) forming the BC (Ig CDR1), C'C" (Ig CDR2), DE (Ig nonCDR), and FG (Ig CDR3) loops, respectively, have been highlighted. The orientation of the C'C" and FG loops in two different directions appears to be favorable for simultaneous recognition by two different ligands such as gp120 and antibody or two distinct antibodies. These loops are on two sides of a plain on D1 bisected by the BC turn residues, thus accounting for the mAb-recognized epitope clusters (27) BC-C'C" and BC-FG (but not C'C"-FG) which are discontinuous in the linear sequence.

DISCUSSION

Several studies have shown that the binding site for gp120 resides within the amino-terminal domain D1 of CD4 (6, 18–23, 35). Within D1, a loop (C'C") that is analogous to CDR-2 of IgV, chains has been implicated as an important determinant for high affinity recognition of gp120. This putative recognition element lies on the same face of D1 as three other solvent-exposed β-turn loops at the end of β strands that form two anti-parallel β sheets. Two of these loops, BC and FG, are analogous to CDR1 and CDR3, respectively, of IgV, while the third loop, DE, represents a turn whose analogous structure on antibodies does not normally participate in antigen recognition. Epitope mapping with over 60 mAbs had shown that of mAbs that recognized nonlinear epitopes within D1, some bound to BC and C'C", while others bound to BC and FG (27). However, none recognized C'C" and FG simultaneously. These observations along with modeling studies of D1 based on IgV, structures had predicted that the C'DR2- and CDR3-like loops are not in close spatial proximity. Now the crystal structure of this region reveals that, although C'C" and FG form solvent-exposed hairpin loops on the same face of D1 distal to the D2 domain, these two loops protrude at different angles at opposite ends of a plain bisected by the BC turn residues which straddle across this face of D1 (1c, 43 in the C'C" loop and C,-87 in the FG loop form a 69° angle with the central axis of D1 and are at a distance of 17 Å from

ND, not done.

TABLE IV

Inhibition of syncytium formation by CD4 mAbs

| mAb        | HIV-1 | HIV-2 |
|------------|-------|-------|
|            | RF    | IIIB  | Ma2 | CRL4 | U455 | CBL20 | LAV-2 |
| Leu3a      | 0.3   | 0.3   | ND  | <0.3 | ND*  | <0.3  | <0.3  |
| L77        | >20   | 0.6   | 5.0 | 10   | 5.0  | 1.25  | 0.6   |
| L68.1      | 10    | 1.25  | 5.0 | 5.0  | 1.25 | 1.25  | <0.3  |
| L71.1.1    | 2.5   | <0.3  | 0.6 | 0.6  | <0.3 | <0.3  | <0.3  |
| L104.5     | 20    | 0.6   | 2.5 | 2.5  | 1.25 | 0.6   | 0.3   |
| L120       | >20   | >20   | ND  | ND   | ND   | >20   | >20   |

* ND, not done.

1 A. Truene, unpublished observation.
CD4 Determinants Involved in HIV Binding and Infection

Fig. 4. Space-filling model of the D1 atomic structure. a and b show two different views of D1 (residues 1–98) with all background atoms colored in blue. Residues 18–25 (BC loop) have been colored white, 40–43 (C'C" loop) pink, 58–64 (DE loop) yellow, and 86–89 (FG loop) green.

each other; see Fig. 4). This disposition appears to be favorable for simultaneous recognition by two separate ligands such as gp120 and antibody or two distinct antibodies.

The current study implies that at least two distinct regions on D1 are involved in HIV infection of CD4+ cells. One region corresponds to the high affinity binding site for gp120. Blocking of this site with specific mAbs causes inhibition of gp120 binding as well as HIV infection and HIV-induced syncytium formation. The second region, which lies outside of the high affinity binding site, is required for events that occur downstream of the initial binding step. Inhibition of this site causes only limited interference with gp120 binding to CD4, as can be shown by cross-blocking analysis with CD4 mAbs and gp120, as well as by the formation of ternary complexes of gp120, CD4, and mAbs. However, mAbs that bind to this site strongly block HIV infection of CD4+ cells and fusion between HIV-infected and uninfected cells. Thus, our data are in accord with previous reports that localize the primary high affinity binding site to the C'C" (Ig CDR2) region within D1 (6, 18–23). We also show that the FG (Ig CDR3) region is not critical for the initial high affinity binding of gp120. However, we present evidence that implicates the FG loop in the subsequent events that follow the initial binding of HIV to CD4+ cells, including virus infection and HIV-induced cell fusion.

Other studies have previously implicated residues in the FG loop in HIV-envelope-mediated fusion events. A single amino acid substitution, Glu-87 of human CD4 to Gly which is the equivalent residue on chimpanzee CD4, abolished its ability to support syncytium formation without altering its affinity for gp120 binding (24). Conversely, substitution of Gly for Glu in chimpanzee CD4 conferred fusion competence. However, in that study, the Glu to Gly mutation did not affect virus infection. In contrast, the mAb inhibition studies presented here demonstrate effects on both cell fusion and virus infection. In other studies, benzylated peptides from this region have been shown to interfere with HIV infectivity and syncytium formation (31). Although the need for extensive benzylation of these peptides has raised questions as to their mechanism of action, the observation that a substitution from Glu to Gly at the position equivalent to residue 87 dramatically reduces the peptide’s ability to inhibit syncytia without altering their ability to inhibit binding (32) gives further credence to the hypothesis that this loop is critical for fusion events.

There is now evidence which suggests that CD4 induces conformational changes upon binding to gp120. gp120 is non-covalently attached to the fusogenic gp41 portion of the envelope protein on the viral surface, prior to the binding of HIV to CD4 cells. Binding of sT4 to virus or virus-infected cells results in the dissociation of gp120 from gp41 (33, 34) with a consequent exposure of epitopes on gp41.4 In the context of HIV infection, these events may be followed by the fusion of the viral and cellular membranes. Similar dissociation occurs with sT4 treatment of gp160-transfected lymphocytes expressing gp120/41 on their surfaces5 which suggests that syncytium formation through cell-cell fusion may also be preceded by CD4-induced conformational changes on gp120. The sensitivity of HIV-induced membrane fusion to interference with the FG loop either through mutation or mAb binding suggests that this loop participates in the fusion event through a direct interaction with the envelope protein. Conformational changes in gp120 may facilitate secondary interactions with the FG loop, resulting in the enhancement of the avidity between gp120 and CD4. This may explain the limited effect of the FG mAbs on gp120 binding. Since the FG loop is not critical for the initial binding of the envelope protein to CD4 and there is as yet no experimental evidence to implicate it in the proposed secondary interactions with gp120, we have considered an alternative possibility. This loop may interact with the fusogenic gp41 protein upon exposure of new epitopes on the latter, in response to CD4 binding to gp120. Such an interaction would ensure the con-

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4 Hart, T. K., Kirsch, R., Ellens, H., Sweet, R. W., Lambert, D. M., Petteway, S. R., Leary, J., and Bugelski, P. J. (1991) Proc. Natl. Acad. Sci. U. S. A., in press.

5 H. Ellens and colleagues, unpublished observation.
tuned high avidity binding of the virus to the cell, while at the same time facilitating the close appositioning of the viral and cellular membranes prior to fusion. Similar molecular events may also facilitate HIV-envelope/CD4-mediated cell-cell fusion. Further experimental evidence is required to distinguish between the two alternative possibilities.

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Note Added in Proof—Other antibodies recognizing different regions of CD4 were recently described to block fusion without inhibiting GP120 binding (Healey, D., Dianda, L., Moore, J. P., McDougal, J. S., Moore, M. J., Eates, P., Buch, D., and Kwong, P. D. (1990) J. Exp. Med. 172, 1239-1242; Celada, F., Cambiaggi, C., Maccari, J., Burastero, S., Gregory, J., Patzer, E., Porter, J., Mcdanell, C., and Matthews, T. (1990) J. Exp. Med. 172, 1143-1150.

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