STRUCTURAL ANALYSIS OF THE
HUMAN IMMUNODEFICIENCY
VIRUS-BINDING DOMAIN OF CD4

Epitope Mapping with Site-directed Mutants and Anti-idiotypes

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CD4 is a 55-kD glycoprotein found predominantly on the surface of a subset of
T lymphocytes. This monomeric surface protein has an extracellular region of 372
amino acids, a single transmembrane segment of 21 residues, and a short cytoplasmic
region of 40 residues. The extracellular region consists of four tandem domains that
have sequence and predicted structural homology with Ig L chain V regions (1, 2).
On lymphocytes, CD4 defines T cells that recognize antigen presented by MHC
class II-bearing cells. In this interaction, CD4 appears to promote efficient T cell
activation through binding to a monomorphic determinant on the class II proteins
of the presenting cell. This association with class II proteins may increase the avidity
of the interaction between the T cell and APC (3–5). However, the recently observed
association of the T cell-specific tyrosine kinase p561ck with CD4 (6, 7) also sug-
gests a direct role for CD4 in signal transduction in response to antigen presenta-
tion. This potentially complex role of CD4 may explain the reported inhibition (8–16)
or enhancement (17–19) of the T cell response in vitro by different CD4 mAbs.

In man, CD4 is also the target for virus attachment by HIV (20–22), a process
mediated by the viral envelope glycoprotein gp120 (23). Through extensive genetic
analysis, the binding site for gp120 on CD4 was localized within the first Ig-like
domain, V1, to a predicted loop structure of ~12 amino acids (24–28). Based on the
sequence alignment of V1 with human κ chain V domains (Vκ), we and others
(24–28) have proposed that the gp120-binding site encompasses a region within V1
that corresponds to the complimentarity-determining region 2 (CDR2) of Ig L
chains. In this report we provide evidence for this model of the V1 domain through
epitope mapping of a panel of 26 CD4 mAbs.
Previous studies have grouped a panel of CD4 mAbs on the basis of their cross-interference in binding to CD4+ cells and their ability to inhibit HIV-induced syncytium formation of CD4+ cells (29, 30). We have extended the cross-interference analysis to include additional antibodies and have regionally localized the binding sites of the mAbs using truncated soluble CD4 proteins. The epitopes for these antibodies were then defined with a panel of single and multiple amino acid substitution mutants in the V1 region. Finally, the fine specificity of a subset of these antibodies was compared in crossblocking studies with polyclonal and monoclonal anti-idiotype antibodies.

Several antigenically distinct sites were identified within the V1 domain, some of which were common to many antibodies. Most of the epitopes mapped to sites of predicted exposed loops, with the predominant sites lying within sequences corresponding to CDR1, -2, and -3 in Ig L chain V regions. Epitopes for two groups of mAbs consisted of nonlinear sequences spanning residues in CDR1 and -2 or in CDR1 and -3, regions predicted to be close in space in an Ig fold.

Our results agree with and extend other analyses of CD4 mAb epitopes (24, 25, 27, 29, 31) and support a V1-like structure for the V1 domain of CD4. This epitope analysis also has significant implications for an HIV vaccine strategy predicted on the development of anti-idiotype antibodies against CD4 mAbs.

Materials and Methods

Soluble CD4 and Derived Mutants. The soluble CD4 protein, consisting of only the extracellular domains of CD4, truncated derivatives, and site-directed mutants were expressed as exported proteins in CHO cells or in Escherichia coli and isolated as previously described (28, 32, 33).

Expression of CD4 Lacking the V4 Domain. Using synthetic linkers, the V4 region of CD4 was deleted from the plasmid pT4 (1) to yield pT4DV4, in which residues 23-253 are fused to residues 299-437. This DV4 coding sequence was introduced into HeLa cells using the retroviral vector pMV7, as previously described for CD4 (22). Cell clones were screened for expression of the AV4 protein by immunoblot analysis with a polyclonal antibody prepared against a recombinant CD4 protein expressed in E. coli. HeLa cells transfected with the AV4 gene expressed a 40-kDa CD4 protein as compared with a 56-kDa protein in cells transfected with the full-length CD4 gene (not shown).

mAbs. Many of the CD4 mAbs were obtained through the Third International Workshop on Leukocyte Differentiation Antigens (1986). Others were obtained from the following sources: Leu3a (N. Warner, Becton Dickinson & Co., Mountain View, CA); OKT4, OKT4A, OKT4B, OKT4C, OKT4D, OKT4E, and OKT4F (P. Rao, Ortho Diagnostic Systems Inc., Westwood, MA); F101-69, 111-364, and F14 (P. Poncelet, Sanofi Corp., Montpellier, France); RFT4 (G. Janossy, Royal Free Hospital, London, UK); NU-TH/1 (M. M. Yokoyama, Kurosame University, Kuyume, Japan); and MT310 and MT151 (P. Reiber and G. Reithmuller, Munich University).

Mapping of mAb Epitopes with CD4 Mutants. Mutant soluble CD4 proteins were used to inhibit the binding of CD4 mAbs to SupT1 cells. Antibodies at concentrations of between 10 and 50 ng/ml were incubated for 30 min with a 10-fold excess of inhibitory protein, in a total volume of 50 μl. 50 μl of cells at 2 x 10^6/ml was then added. After 30 min, the cells were washed and labeled with FITC-conjugated goat anti-mouse IgG. 30 min later, the cells were washed and analyzed by flow cytometry on an EPICS 753 (Coulter Electronics Inc., Hialeah, FL). All incubations were done at 4°C. Background fluorescence was determined on cells stained with FITC conjugate only. The results are expressed as the mean fluorescence intensity of SupT1 cell staining. For negative controls, conditioned medium from CHO cells or E. coli not expressing CD4 were processed as for cells expressing CD4 proteins. These preparations gave values equivalent to the staining without CD4 proteins. Percent blocking
with each CD4 mutant protein was computed by: \( 100 \times \frac{(MF - MF_m)}{(MF_r - MF)} \),
where \( MF \) is the mean channel fluorescence without CD4 protein (c), with mutant protein (m), and with \( V_1V_2 \) soluble CD4 protein standards (s).

**Preparation of Anti-idiotype to CD4 mAbs.** CD4 mAbs at a concentration of 1 mg/ml were coupled to an equivalent amount of Keyhole Limpet hemocyanin (KLH) with 0.1% glutaraldehyde in PBS-A for 1 h at room temperature, then dialyzed against 1,000 vol of PBS-A. BALB/c mice were immunized with 100 mg of the mAb/KLH conjugate in CFA. 1 mo later, the same amount of antigen was administered in IFA. Mice were then bled \(~10 \, \text{d after the last immunization, and sera tested for anti-idiotypic activity. Further immunizations consisted of the antibody alone without carrier or adjuvant. mAbs were prepared from mice immunized as above by standard procedures (34).}

**Assay of Anti-idiotype Antibodies.** CD4 mAbs were labeled with \(^{125}\text{I as previously described (29). A dilution of labeled mAb yielding \( \sim2,000 \) cpm of specific CD4 binding activity was mixed with dilutions of anti-idiotype antiserum or mAb, incubated for 30 min at 4°C, then added to \( \sim10^6 \) CEM cells. Controls were normal mouse sera or control supernatants. Inhibition ofidiotype binding to cellular CD4 by anti-idiotype antibody was calculated by the formula \( 100 - 100 \times \frac{(T - C/M - C)}{C} \), where \( C \), counts bound in the presence of saturating amounts of blocking anti-idiotype reagent; \( M \), counts bound in the presence of an irrelevant antibody or normal mouse serum; \( T \), counts bound in the presence of anti-idiotype raised against different CD4 mAbs.

**Crossblocking between CD4 mAbs.** These were carried out as previously described (29).

**Results**

**Crossblocking Analysis.** Previous studies have defined groups of CD4 mAbs on the basis of their ability to block the binding of selected CD4 mAbs or HIV-gp120 to CD4+ cells and to inhibit virus-induced syncytium (29, 30, 35). Interference between antibodies (or gp120) suggests that they recognize a common surface on CD4, although in this type of analysis a shared binding site cannot be readily distinguished from inhibition by indirect conformational or steric effects. Conversely, the simultaneous binding of two antibodies (or gp120) indicates that their epitopes are spatially distinct, although apparent simultaneous binding may result from significant differences in affinity. We have extended the previous studies (29, 30) by examining the inhibition of additional antibodies. Each of a series of mAbs was labeled with \(^{125}\text{I and then added to CD4+ CEM cells that had been preincubated with a saturating level of a second, unlabeled CD4 mAb. Each labeled antibody was blocked by an excess of the same antibody and by some, but not all, of the other mAbs (Table I). For example, Leu 3a was strongly inhibited by EDU-2, MT310, l11-364, F101-69, NU-TH/1, and Leu 3a itself, moderately inhibited by OKT4A, and unaffected by all of the other mAbs. Several of the antibodies gave inconsistent crossblocking results. For example, MT151 blocked OKT4C and OKT4D (Table I) but neither antibody blocked MT151 (29). These results, together with previous studies, suggest the following grouping of the antibodies: (a) EDU-2, MT310, l11-364, F101-69, F101-5, Leu 3a, T4/18T3A9, T4/19Thy-5D7, 91d6, 94b1, NU-TH/1, and to a lesser extent, CLBT4/1 and OKT4A; (b) OKT4D and RFT4; and (c) VIT4, MT321, and MT151. Set \( a \) is the best defined due to a more extensive crossblocking analysis of this collection of antibodies.

**Domain Mapping with Truncated, Soluble CD4 Proteins.** We have described the expression and characterization of a soluble form of the CD4 protein, consisting of the entire extracellular domain of this receptor (32), and truncated derivatives consisting of the first two NH2-terminal domains (V1V2J4) or the first domain alone.
Table I
Crosscompetition between \(^{125}\text{I}-\)labeled and Unlabeled CD4 mAbs

| Unlabeled mAbs | EDU-2 | MT310 | 111-364 | F101-69 | Leu3a | OKT4A | OKT4D | OKT4C | OKT4F |
|----------------|-------|-------|---------|---------|-------|-------|-------|-------|-------|
| EDU-2          | +     | +     | +       | +       | +     | ++    | ++    | ++    | -     |
| MT310          | +     | +     | +       | +       | +     | ++    | ++    | ++    | -     |
| 111-364        | +     | +     | +       | +       | +     | ++    | ++    | ++    | -     |
| F101-69        | +     | +     | +       | +       | +     | ++    | ++    | ++    | -     |
| Leu3a          | +     | +     | +       | +       | +     | ++    | ++    | ++    | -     |
| NU-TH/1        | +     | +     | +       | +       | +     | ++    | ++    | ++    | -     |
| OKT4A          | +     | +     | +       | +       | +     | ++    | ++    | ++    | -     |
| OKT4D          | -     | -     | -       | -       | -     | +     | -     | +     | -     |
| 66.1           | -     | -     | -       | -       | -     | +     | -     | +     | -     |
| OKT4E          | -     | -     | -       | -       | -     | -     | -     | +     | -     |
| MT151          | -     | -     | -       | -       | -     | +     | +     | +     | -     |
| OKT4F          | -     | -     | -       | -       | -     | +     | -     | +     | +     |

Binding of \(^{125}\text{I}-\)labeled CD4 mAbs to CD4\(^{+}\) SupT1 cells pretreated with unlabeled CD4 mAbs was determined. Scores represent percentage inhibition of binding of labeled mAb by saturating concentrations of unlabeled mAb. ++, >80% inhibition of labeled mAb binding; +, 20-79% inhibition; and -, <20% inhibition.

(V\(_1\)) (28, 32). We used these soluble CD4 proteins to localize the binding of 26 mAbs to specific domains of CD4 by measuring their ability to block antibody binding to the CD4\(^{+}\) cell line, SupT1. The results define three groups of antibodies (Fig. 1): group 1 (V\(_1\) mAbs), those blocked by all three proteins; group 2 (V\(_1\)V\(_2\) mAbs), those blocked by V\(_1\)V\(_2\)J\(_4\) and soluble CD4 but not by V\(_1\); and group 3 (V\(_3\)V\(_4\) mAbs), those blocked only by soluble CD4. As in previous studies with a more limited panel of antibodies (24), all of the mAbs were blocked by soluble CD4 and all but one, OKT4, was blocked by the V\(_1\)V\(_2\)J\(_4\) protein. This localization of the OKT4 epitope agrees with other similar studies (33, 36) and with the absence of this epitope on a V4 deletion mutant of CD4 expressed on the cell surface (Fig. 2). 13 of the mAbs were inhibited by the V\(_1\) protein, indicating that the determinants of high affinity binding for these antibodies reside within the V\(_1\) domain. Two antibodies, VIT4 and OKT4F, were partially inhibited by V\(_1\), suggesting that a portion of their epitope is within this domain. The epitopes for the mAbs blocked by V\(_1\)V\(_2\)J\(_4\) but not by V\(_1\) may lie only in V\(_2\) or may span V\(_1\) and V\(_2\). Alternatively, some regions of the V\(_1\) protein may not be properly folded, thereby disrupting some epitopes within this domain.

The set a and b antibodies defined by the crossblocking studies in the previous section all map to the V\(_1\) domain. These antibodies also efficiently inhibit infection by HIV and SIV, an observation consistent with the recent localization of the gp120-binding site to this domain of CD4 (24–28).

Mapping of the Epitopes within the V\(_1\) Domain. To define the antibody epitopes residing wholly or partially within the V\(_1\) domain, we used a collection of single and multiple substitution mutations introduced into this region and expressed in the context of V\(_1\)V\(_2\) or full-length soluble CD4 proteins (28). To induce only local disruption of structure, the V\(_1\) region of the murine CD4 protein, which shares \~50% sequence homology with the human V\(_1\) sequence, was used as a template.
FIGURE 1. Assignment of CD4 mAb reactivities to Ig-like domains of CD4. Antibodies were incubated with full-length soluble CD4, V1/V2J4, or V1 proteins as indicated before addition of SUPT1 cells. The results are expressed as percent inhibition relative to the level of staining in the absence of CD4 protein. Inhibition of mAb binding to a CD4+ T cell line, SUPT1, by sCD4, V1/V2, and V1: (A) mAbs blocked by all three proteins; (B) by sCD4 and V1V2; or (C) or by sCD4 only.

for mutagenesis. Thus, at positions where the two sequences differed, mouse residues were substituted in place of the corresponding human amino acids; for example, K8E is the substitution of glutamic acid for lysine at residue 8 of human CD4. The fact that the mutants incorporated human to mouse residue switches while the panel of CD4 mAbs used for the mapping study were generated in mice may account for our ability to differentiate between mAbs that map close to each other on V1.

FIGURE 2. Lack of reactivity of OKT4 with HeLa cells expressing T4ΔV4. HeLa cell transfectants were analyzed by flow cytometry after staining with FITC-conjugated mAbs. Histograms represent the relative mean fluorescence after subtraction of background values. Cell surface expression of full-length CD4 was detected with OKT4 as well as Leu 3a but not with the control CD8 antibody (Leu 2a) while the T4ΔV4 was detected only by Leu 3a.
The effect of these mutations on antibody binding was assessed as described above for the domain analysis. Each mAb was incubated with an excess of the mutant protein before addition to SupT1 cells and binding was measured by flow cytometry. The results of these assays are summarized in Table II. Comparable results for Leu 3a, OKT4A, and 13B8.2 were obtained in an ELISA (Table II, legend). None of the mutations affected all of the antibodies, indicating that each mutation does cause only local perturbation of structure. Among the group 1 (V1) antibodies defined above, all were strongly affected by mutations in the region 42–43; all except NUTH/1 were inhibited by mutations at 24–27. Some of this group were also strongly affected by the phenylalanine substitution at position 55 and partially affected by mutations at 34, 40, and 45. Although each mAb in this group shows a distinct pattern of reactivity to mutant CD4 proteins, the common disruption of their epitopes by mutations at 42–43, and often at 24–27, is consistent with the crossblocking results described in Table I. We have designated this set of antibodies as group 1A (Table II), to distinguish them from the other group 1 mAbs. The other four V1 antibodies were not affected by the substitutions at 42–43 and each showed a different pattern of blocking by the panel of mutants. OKT4A is weakly disrupted by the double mutation at 64–66, a result verified in a competition ELISA (Table II, legend). Recently, we found that mutation of the serine residue at position 60 specifically disrupts OKT4A binding (Ping Tsui, unpublished observations) in agreement with a previous report (24). 13B8.2 is affected only by the mutation at residues 88–89. OKT4D and RFT4 were commonly disrupted by alterations at residues 34 and 55, although to different extents, a result in agreement with the blocking of OKT4D binding by RFT4 (Table I). However, unlike OKT4D, RFT4 was also affected by the mutation at position 27.

All of the group 2 mAbs were affected by one or more mutations within the V1 region (Table II), suggesting that their epitopes reside at least partially within the V1 domain. The results suggest two general groupings of these antibodies. Group 2A (OKT4C, OKT4E, F14, MT321, and VIT4) were primarily affected by the substitutions at both 24–25 and 88–89; whereas group 2B (BL4/IOT4, OKT4B, and MT151) were most affected by the mutation at 94–96. OKT4E and F14 were also moderately disrupted by the 42–43 alteration and BL4/IOT4 was severely perturbed by the mutation at amino acid 8. OKT4F and B264/123 did not match either group. OKT4F was uniquely disrupted by the extensive mutation spanning residues 102–105. Among the mutants examined with B264/123, those at the NH2 terminus and 88–89 had the greatest effect.

In summary, most of the mAbs fall into one of the three groups (1A, 2A, or 2B) based on the pattern of disruption by the substitution mutants. The predominant epitopes appear to reside at or near residues 24–27, 42–43, and 88–96. The epitopes for the majority of these mAbs appear to be nonlinear. Of these mAbs, those reactive with the V1 domain alone recognize epitopes affected by mutations at residues 24–27 and at 42–43, whereas those reactive with V1V2J4, but not V1, recognize epitopes primarily affected by mutations at 24–25 and at 88–89.

Previously, we have used this same collection of mutants to define the binding site for HIV gp120 (28) and the results of that analysis are summarized in Table II. Similar to some of the group 1A mAbs, gp120 binds to the V1 protein and its recognition is selectively disrupted by the mutations at 42–43 and 55. Interestingly,
| mAbs   | K8E | T17E | I24T | I24T/Q25K | T17E/Q25K | H27A | I34R | Q46H | S42G* | F43V | F43V* | T431* | K46R | K50P | A55F | Q64K | N66S | D98N | Q89R | L96W | S104F | V103S | V1 | Class |
|--------|-----|------|------|----------|----------|------|------|------|-------|------|-------|-------|------|------|------|------|------|------|------|------|------|------|------|------|
| EDU-2  | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | ++   | +    | ++   | +    | +    | +    | +    | +    | 1A    |
| 94B1   | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| T419THYS17 | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| 111-364 | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| F10169 | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| T418T3AG | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| CLB7   | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| Leu 3a1 | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| MT310  | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| NU-TH1 | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| RF104  | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| OKT4D  | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| OKT4A  | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| OKT4E  | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| F14    | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| MT321  | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| VIT4   | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| B264/123 | ++ | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| BL4/1014 | ++ + | ++ | ++ | ++ | ++ | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | 1A    |
| MT151  | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| OKT4B  | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| OKT4C  | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| OKT4F  | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| OKT4   | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |
| gpl20  | ++  | ++   | ++   | ++       | ++       | +    | -    | -    | +     | +    | ++    | ++    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1A    |

A panel of CD4-derived proteins containing single or multiple residue substitutions was assayed by flow cytometry for their ability to block mAb binding to CD4* SupT1. Amino acid substitutions are designated by position and the residue changed. For example, F43V indicates the substitution of valine for phenylalanine. Results are expressed as the index of inhibition of mAb binding: +++, >80% inhibition; +, 50-79% inhibition; +, 20-49% inhibition; and -, <20% inhibition.

* Mutants expressed as V1V2 proteins in E. coli. All other mutants were expressed as full-length soluble CD4 proteins in CHO cells.

† Comparable results were obtained for Leu 3a and 13B.8.2 in an ELISA.

§ Titration showed a fourfold decrease in activity for OKT4A with the mutation Q64K/N66S and an eightfold decrease for OKT4B with the mutation Q94E/L96W. These results are also in agreement with values obtained in ELISAs.
however, none of these antibodies are affected by the mutants in a manner identical to gp120. Thus, the gp120 binding site differs from that of the group 1A mAbs.

Anti-idiotype Analysis. Crossblocking studies with anti-idiotypic antibodies provide a highly specific approach for identifying shared epitopes between mAbs. An anti-idiotype raised against the combining site of one mAb is likely to bind to other mAbs that recognize the same epitope. We measured the ability of anti-idiotypes developed against selected group 1A mAbs to block the binding of other CD4 mAbs to CD4+ CEM cells. This approach to mapping shared epitopes is more specific than crossblocking analyses and avoids the conformational and steric effects that may result from two antibodies binding to CD4.

Table III presents the results obtained with polyclonal anti-idiotype antisera raised to some group 1A antibodies. All of the anti-idiotype antisera completely inhibited their own idiotypes. In addition, each of the antisera also inhibited the binding of most of the other group 1A mAbs examined, but did not inhibit either MT151 or OKT4. Table IV presents the results obtained with anti-idiotype mAbs raised against the group 1A mAbs MT310, F101-69, and NU-TH/1. Each antibody blocked its idiotype, but only one, F101-69/2H10, weakly blocked any of the other group 1A mAbs. We conclude from the crossblocking by the polyclonal anti-idiotype antisera that the group 1A mAbs recognize very similar epitopes. Apparently, most of the monoclonal anti-idiotypes we examined were not directed against this common recognition site.

The V Region of REI as a Model for the Vι Domain. The Vι domain of CD4 shares sequence and predicted structural homology with Ig κ chain V regions (1, 2). Among human Vκ regions whose structure have been determined, Vι most closely aligns with that of the Bence-Jones protein REI (26, 28). In the alignment shown in Fig. 3 a, 10 of the 18 invariant residues in Vκ domains are analogously positioned in Vι. Based on this alignment, we have superposed the residue numbers of Vι on an α carbon trace (37) of one V domain in REI (Fig. 3 b).

Vκ regions are composed of nine antiparallel β strands that are folded into two parallel β-pleated sheets (37, 38). Six exposed loop regions lie parallel to, and pro-

| Anti-id | Anti-CD4 mAbs |
|---------|---------------|
|          | EDU-2 | MT310 | F101-69 | Leu 3a | NU-TH/1 | MT151 | OKT4 |
| EDU-2   | ++   | ++   | ++     | ++     | +      | -     | -     |
| MT310   | -    | ++   | +      | +      | -      | -     | -     |
| F101-69 | ++   | ++   | ++     | -      | -      | -     | -     |
| Leu 3a  | +    | +    | +      | ++     | -      | -     | -     |
| NU-TH/1 | +    | +    | +      | -      | +      | -     | -     |

125I-labeled CD4 mAbs were preincubated with saturating concentrations of anti-idiotype antisera and then added to SupT1 cells. Results are expressed as the index of inhibition of labeled mAb binding where: ++ , >80% inhibition; + , 20–79% inhibition; and − , <20% inhibition.
TABLE IV
Blocking of mAb Binding to CD4⁺ Cells by Anti-idiotypic mAbs

| Anti-id antisera | Anti-CD4 mAbs |
|------------------|---------------|
|                  | EDU-2 | MT310 | 94B1 | 111-364 | F101-69 | NU-TH/1 | RFT4 |
| MT310            |       | -     | +    | -       | -       | -       | -    |
| 6H10             |       | -     | -    | -       | -       | -       | -    |
| F101-69          |       | -     | -    | -       | +       | -       | -    |
| 4H5              |       | -     | -    | -       | -       | +       | -    |
| 3F9              |       | -     | -    | -       | +       | -       | -    |
| 2H10             |       | +     | +    | +       | +       | -       | -    |
| NU-TH/1          |       | -     | -    | -       | -       | +       | -    |
| 4H11             |       | -     | -    | -       | -       | +       | -    |
| 2D1              |       | -     | -    | -       | -       | +       | -    |

125I-labeled CD4 mAbs were preincubated with saturating concentrations of anti-idiotypic mAbs and then added to SupT1 cells. Results are expressed as the index of inhibition of labeled mAb binding where: ++, >80% inhibition, +, 20-79% inhibition; and - , <20% inhibition.

truding from, the planes formed by these two β sheets. Three of these loops form the three CDRs (CDR1-3), which together with the corresponding regions in the H chain V segment comprise the antigen-binding site. The CDR regions and the loop-encompassing residues 59–66 in Fig. 3 b project from one end of the β sheet and lie relatively close to each other. In the alignment with REI (Fig. 3 a), the residues in V₁ corresponding to CDR1, -2, and -3 are 17–27, 43–49, and 85–97, respectively.

In the context of this model, most of the antibody epitopes defined by the mutagenesis of V₁ occur in the exposed loops. The group 1A mAbs are primarily affected by mutations at 24–27 and at 42–43, regions that lie within the predicted CDR1 and CDR2 loops (Fig. 3 c). Some of these mAbs are also affected by the substitution of phenylalanine for alanine at position 55. This residue lies in the β sheet platform supporting CDR2 and the introduction of the bulky phenyl group is likely to partially distort this region. The OKT4A epitope lies in the 59–66 loop, in agreement with other reports (24, 27). RFT4 and OKT4D appear to recognize, in part, a turn near residue 34 (Fig. 3 e). The group 2A mAbs are most affected by mutations at 24–25 and 88–89, which are in CDR1 and CDR3; group 2B mAbs are primarily disrupted by 94–96, which is also in CDR3 (Fig. 3 a). The 102–105 mutation, which uniquely affects OKT4F, lies in the J-like region between V₁ and V₂. If this is the epitope for OKT4F, the inability of OKT4F to bind to the V₁ protein suggests that this J-like region is not properly folded in the V₁ protein. Other results indicate that the OKT4F epitope lies between residues 37 and 131 (25).

The structural model for V₁ is also consistent with the apparent discontinuous epitopes for the group 1A and 2A antibodies. For group 1A, residues 24–27 and 42–43 in CDR1 and -2 are close in space (Fig. 3 e). Similarly, for group 2A, residues 24–27 and 88–89 in CDR1 and -3 also lie in close proximity. We thus conclude that the V₁ domain of CD4 is folded in a manner similar to that of the Vₑ region in REI.
EPITOPE MAPPING OF CD4
Discussion

Through genetic analysis, the binding site for the HIV env protein on CD4 has been localized within the V1 domain to residues 41-52 (24-28). Based on the alignment of the V1 domain with Ig Vκ consensus sequence, this site encompasses a region corresponding to CDR2 in L chain V regions. In this report, we show that V1 bears structural similarities with Vκ regions through detailed epitope mapping of 26 CD4 mAbs. The binding sites of these mAbs were initially defined relative to one another by crossblocking analysis and were then localized to specific domains of CD4 in blocking studies with truncated, soluble CD4 proteins. These epitopes within the V1 domain were mapped in detail with a panel of 17 substitution mutants, and the specificities of several mAbs that appeared to recognize very similar epitopes were examined in crossblocking studies with anti-idiotypic antibodies. The location of the epitopes is consistent with a Vκ-like structure. Most of the epitopes lie within regions of predicted exposed loops, and the apparent discontinuous epitopes lie closely in space.

With the exception of OKT4, all of these murine mAbs bound to soluble proteins containing only the V1 and V2 domains of CD4 (Figs. 1 and 2). This immunodominance of the NH2-terminal domains is not likely to be due to sequence differences in human and mouse CD4 because the homology between these proteins is approximately equivalent throughout the external domains. Since these mAbs were generated against cells expressing CD4, we speculate that only limited regions of the V3 and V4 domains are exposed on the cell surface.

All the mAbs we have examined recognize conformational epitopes as evidenced by: (a) their inability to recognize denatured CD4 proteins in immunoblots (unpublished observations); (b) the disruption of binding by limited deletions outside of their epitopes (unpublished observation); and (c) the recognition of discontinuous epitopes by these antibodies. About half of the antibodies that bind to V1-V2-J4 do not bind to V1, yet all are affected by mutations in the V1 region. The conformational nature of the epitopes recognized by the V1 antibodies argues that the V1 protein is properly folded. Thus, the V1-V2 antibodies either recognize epitopes that span both V1 and V2, or that reside in V2 and are affected by mutations in V1. This interpretation requires that the V1 and V2 domains lie in close spatial proximity.

**Figure 3.** Modeling of V1 of CD4 on Ig Vκ domains. (a) Alignment of residues 1-106 of CD4 V1 with Ig Vκ domains and REI. Shaded boxes indicate identical residues between the various sequences. Of the 18 invariant residues (>95% conservation) in Vκ, 10 are analogously positioned in CD4 V1 (heavy shading). Based on this alignment, CD4 was modeled on the known structure (34) of the Bence Jones Homodimer, REI. (b) The Ig fold (35), which consists of four (for the Vκ L chain domains) pairs of antiparallel B-pleated sheets joined by loops at either the NH2-terminal or COOH-terminal end of the molecule. The three hypervariable regions of this protein (CDR1, -2, and -3) would be formed by the NH2-terminal loops connecting strands 1 and 2, 3 and 4, and 5 and 6. The disulfide link between the two cysteine residues defining Vκ is located between strands 1 and 7, and is probably buried within the interior of the molecule. The numbers represent those for the aligned sequence of the Vκ domain of CD4. The two positions where gaps were introduced to accommodate additional residues in Vκ are indicated by broken lines. The shaded strands represent the CDR1 (-----), CDR2 (-----), and CDR3 (-----) loops. (c and d) The predicted mAb binding regions for group 1 and group 2 mAbs, respectively.
A similar conclusion was derived from observations that mutations in both \( V_1 \) and \( V_2 \) affect the binding of certain mAbs and gp120 (24-26).

Within the \( V_1 \) domain, most mAbs bind to regions corresponding to the three CDRs in REI. The antigenicity of these regions is consistent with the fact that they are the regions of greatest divergence between mouse and human sequences and with their presentation as exposed loops on an Ig fold. The extensive evolutionary divergence in these regions is somewhat analogous to the hypervariable nature of the CDRs in Igs.

Our detailed epitope analysis is relevant to a vaccine strategy for HIV based on anti-idiotype antibodies to CD4 mAbs. Subunit vaccines based on immunization with purified or recombinant HIV envelope proteins generally result in a weak neutralizing response (39, 40). In addition, extensive antigenic drift in gp120 results in strain-specific neutralization and rapidly leads to neutralization escape mutants (41). However, because HIV associates with CD4, a monomorphic receptor, the combining site on gp120 is unlikely to tolerate change and is thus a target for broad-spectrum neutralization. The high affinity binding site for gp120 is restricted to the CDR2 loop in the \( V_1 \) domain of CD4 (24-28). We have suggested that this small loop may be accommodated by a compact binding pocket in gp120, which by its size excludes recognition by host antibodies (28), as initially proposed by Rossman et al. (42) for the receptor-binding site on rhinoviruses. An anti-idiotype antibody whose epitope closely resembles the gp120-binding site on CD4 may be a better immunogen than gp120 itself and lead to a stronger host response to this binding site. In previous studies, immunization of mice with the group 1A antibody, Leu 3a, elicited an anti-idiotypic response that weakly neutralized diverse HIV isolates (43-45). This weak response implies that Leu 3a does not precisely mimic gp120 binding to CD4, a conclusion borne out by our epitope mapping studies. Moreover, none of the other CD4 antibodies exactly duplicated the binding pattern of gp120, and anti-idiotypes developed against these mAbs do not bind to gp120 in ELISAs, or immunoprecipitate gp120 or gp160 (unpublished results). Epitope analysis of additional CD4 mAbs should help identify an appropriate target for this anti-idiotype strategy.

CD4 mAbs have been used to define the physiological role of CD4 for T cell responses. Some of the mAbs that recognize epitopes within the first two NH2-terminal domains of CD4 inhibit T lymphocyte activation while others enhance it. In contrast, OKT4, an mAb reactive with the \( V_3V_4 \) region, is apparently inert (14). Recently, mAbs against the murine CD4 (L3T4), possibly mimicking physiological ligands, induced phosphorylation of the CD4-associated tyrosine kinase, p56\( ^{kk} \) (46). In this respect, mapping of the epitopes involved in the binding of CD4 with its putative physiological ligands, MHC class II molecules, may provide insight into the role of these molecules in T lymphocyte activation.

**Summary**

The CD4 molecule, a differentiation marker expressed primarily by T lymphocytes, plays an important role in lymphocyte activation. CD4 is also the receptor for HIV. A number of recent studies have localized the high affinity binding site of the HIV envelope glycoprotein, gp120, to the NH2-terminal (\( V_1 \)) domain of CD4, a region with sequence and predicted structural homology with Ig \( k \) chain V domains (\( V_k \)). In this report, we show that \( V_1 \) bears structural similarities with \( V_k \).
regions through detailed epitope mapping of 26 CD4 mAbs. The binding sites of these mAbs were initially defined relative to one another by crossblocking analysis and were then localized to specific domains of CD4 in blocking studies with truncated, soluble CD4 proteins. The epitopes within the V1 domain were mapped in detail with a panel of 17 substitution mutants, and the specificities of several mAbs that appear to recognize very similar epitopes were examined in crossblocking studies with anti-idiotype antibodies. The location of the epitopes is consistent with a V\alpha-like structure of V1. Most of the epitopes lie within regions of predicted exposed loops. A number of these epitopes span discontinuous residues in the linear sequence that lies in close proximity in an Ig fold. Alignment of CD4 V1 with the Ig V\alpha chains places these epitopes within stretches corresponding to the complementarity-determining regions. This epitope analysis is relevant for a vaccine strategy for HIV based on anti-idiotype antibodies to CD4 mAbs and for studies with CD4 antibodies on the role of CD4 in T lymphocyte activation.

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