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Mutations in the D strand of the human CD4 V1 domain affect CD4 interactions with the human immunodeficiency virus envelope glycoprotein gp120 and HLA class II antigens similarly

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ABSTRACT CD4, a cell surface glycoprotein expressed primarily by T lymphocytes and monocytes, interacts with HLA class II antigens to regulate the immune response. In AIDS, CD4 is the receptor for the human immunodeficiency virus, which binds to CD4 through envelope glycoprotein gp120. Delineation of the ligand-binding sites of CD4 is necessary for the development of immunomodulators and antiviral agents. Although the gp120 binding site has been characterized in detail, much less is known about the class II binding site, and it is as yet uncertain whether they partially or fully overlap. To investigate CD4 binding sites, a cellular adhesion assay between COS cells transiently transfected with CD4 and B lymphocytes expressing HLA class II antigens has been developed that is strictly dependent on the CD4–class II interaction, quantitative, and highly reproducible. Mutants of CD4 expressing amino acids with distinct physicochemical properties at positions Arg-54, Ala-55, Asp-56, and Ser-57 in V1, the first extracellular immunoglobulin-like domain, have been generated and studied qualitatively and quantitatively for interaction with HLA class II antigens, for membrane expression, for the integrity of CD4 epitopes recognized by a panel of monoclonal antibodies, and for gp120 binding. The results obtained show that the mutations in this tetrapeptide, which forms the core of a synthetic peptide previously shown to have immunosuppressive properties, affect the two binding functions of CD4 similarly, lending support to the hypothesis that the human immunodeficiency virus mimicks HLA class II binding to CD4.

The T-lymphocyte cell surface glycoprotein CD4 plays a pivotal role in development and differentiation of the immune system and in regulation of immune responses (1) and has been the subject of even more sustained investigation since it was demonstrated to be subverted as the main receptor for the human immunodeficiency retroviruses (2, 3). The multiple facets of CD4 function rely on its ability to interact with multiple physiological ligands (HLA class II antigens, the T-cell antigen receptor–CD3 complex, and the T-cell specific tyrosine kinase $p56^{lck}$), integrating signals for the delivery of inhibitory or stimulatory signals to regulate T-cell adhesion and function (for review, see refs. 4 and 5). Thus CD4–ligand interactions are potential targets for the development of immunomodulators and therapeutic agents in AIDS.

Based on sequence similarities with the fibronectin cell-attachment site, we have proposed (29) that the tetrapeptides RFDS and RADS (amino acid one-letter code for Arg-Phe-Asp-Ser and Arg-Ala-Asp-Ser, respectively) in HLA class II β chains and CD4 might constitute potential interaction sites and that human immunodeficiency viruses (HIVs) might mimic the interaction between the class II molecule and the RADS tetrapeptide. In previous work, we demonstrated that synthetic peptides derived from HLA-DR and CD4, containing the tetrapeptide sequences RFDS and RADS, have immunosuppressive properties toward T-cell proliferation and helper function (6) and have the ability to block conjugate formation between T and B lymphocytes (7).

Extensive site-directed mutagenesis (8–14) and the use of soluble CD4 derivatives (15) have mapped the envelope glycoprotein gp120 binding site to V1, the first immunoglobulin-like domain of CD4, but the site for interaction with HLA class II antigens remains to be defined as precisely. This is due to the fact that variable results have been obtained depending on qualitatively distinct cellular assays, delineating a large region either overlapping the gp120 binding site (16) or distinct from it (10).

We have developed (17) a cellular adhesion assay between B lymphocytes expressing HLA class II antigens and COS-7 cells transfected with a CDM8–CD4 plasmid construct and expressing CD4. This assay has provided sensitive, quantitative, and highly reproducible results of rosette formation, visualized by immunoperoxidase staining with an anti-CD4 antibody, that solved the difficulties of high background encountered with other quantification methods using the same expression system (16), and it has replaced an assay based on a simian virus 40–CD4 construct (18) that proved unreproducible (17). CD4–class II molecule specific adhesion was strongly dependent on the level of cell surface CD4 expression. The pattern of inhibition in this assay with anti-CD4 antibodies, gp120, and peptides derived from gp120 emphasized the great similarity but incomplete identity of the CD4 binding sites for HLA class II antigens and HIV gp120 (16, 17). Conversely, soluble CD4 derivatives that are potent inhibitors of infectivity for laboratory strains of HIV-1 have been shown to be unable to interfere with rosette formation (17), confirming previous results (16, 19) and suggesting a difference in the ability of membrane and soluble CD4 to interact with class II antigens.

We now report on the use of our cellular adhesion assay to measure the properties of a series of point mutations in CD4 at the four amino acids of the RADS tetrapeptide that constitute the core of the immunosuppressive peptide. The results obtained show that the two binding functions of CD4 fully overlap at this level.

MATERIALS AND METHODS

Enzymes, Antibodies, and Recombinant Proteins. Restriction endonucleases and modifying enzymes were obtained from Appligène (Illkirch, France), Amersham, Bethesda Research Laboratories, Boehringer Mannheim, New England

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Abbreviation: HIV, human immunodeficiency virus.
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Biolabs, and United States Biochemical. A panel of monoclonal antibodies specific for various epitopes of the CD4 molecule were kindly provided by P. Rao (Johnson Pharmaceutical Research Institute, Raritan, NJ), OKT4, OKT4A, OKT4B, OKT4C, OKT4D, OKT4E, and OKT4F; by G. Cordier [Institut National de la Santé et de la Recherche Médicale (INSERM), Lyon, France], B14; and by C. Mawas (INSERM, Marseille, France), 13B8.2. T151 was from Boehringer Mannheim, and anti-Leu3a was from Becton Dickinson. Purified soluble recombinant gp120 was a gift from Marie-Paule Kiény and Jean-Pierre Lecocq (Société Transgène, Strasbourg, France) and from Harvery Holmes (Medical Research Council and Celltech, Berkshire, England), and gp160 was from Drs. Michel Kaczorek and Marc Girard (Pasteur Vaccins, Marnes la Coquette, France).

***In Vitro* Mutagenesis and Expression of the CD4 Mutants.** Mutagenesis of the RADS coding sequence was performed on the CD4 cDNA (20) kindly provided by P. J. Maddon. Oligonucleotides were synthesized by the solid-phase phosphoramidite method on a model 381A DNA synthesizer (Applied Biosystems) and purified by either polyacrylamide gel electrophoresis or reverse-phase chromatography on OPC columns (Applied Biosystems). Three methods were used. Four mutants including a deletion (Δ) of Ala-55 were constructed by insertion of an 18- to 21-base-pair synthetic oligonucleotide duplex containing the mutations by using appropriate silent restriction sites introduced in the wild-type CD4 sequence. The other mutants were obtained by saturation mutagenesis of the CD4 600-base-pair *Eco*RI–*Sac* I fragment cloned into M13mp18. Four degenerate 45-mer oligonucleotides were synthesized containing a base mixture in each of the Arg-54, Ala-55, Asp-56, and Ser-57 codons, designed to direct insertion of the other 19 amino acids at these positions. Mutagenesis was performed according to the method of Eckstein (21) by following the specifications of the supplier (Amersham). Transformants were screened by dideoxynucleotide sequencing (22). The CD4 *Eco*RI–*Afl* II fragment containing the mutations was then substituted to the wild-type equivalent in the 1.8-kilobase *Eco*RI–*Bam*HI CD4 cDNA, which had been cloned into the eukaryotic expression vector CDM8 (kindly provided by B. Seed, Harvard Medical School, Boston, MA) at the *Bst* XI sites. Transformants in *Escherichia coli* MC1061/p3 were controlled by dideoxynucleotide sequencing of the double-stranded DNA mutant region. The A55F mutant (substitution of Phe for Ala at position 55), which was not obtained with the previous methods, was constructed by site-directed mutagenesis of the 1.8-kilobase CD4 cDNA in the CDM8 vector using the same method as described above. Single-stranded template DNA was prepared in *E. coli* XS127 (Invitrogen, San Diego) and primed with a 45-mer oligonucleotide containing the appropriate substitutions. COS-7 cells were transfected with the CDM8 constructs, according to the methods described by Seed and Aruffo (23). One day after transfection cells were treated with trypsin, plated either in new 100-mm Petri dishes or in 24- or 12-well culture plates (Nunc), and assayed 1 or 2 days later.

Immunofluorescence and Flow Cytometry. Forty-eight hours after transfection, cells were detached from plates in phosphate-buffered saline (PBS) containing 1 mM EDTA, stained by indirect immunofluorescence using OKT4 (2.5 μg/ml) and a fluorescein-conjugated goat anti-mouse immunoglobulin antibody (Nordic, Tilburg, The Netherlands), and analyzed by flow cytometry with a FACS 440 (Becton Dickinson).

Radioimmunoassay. gp120 or gp160 were labeled with $Na^{125}I$ (Amersham) using the lactoperoxidase method. Adherent COS cells plated in 24-well dishes were incubated in duplicate with 2×10^5 cpm of ^{125}I-labeled gp120 or gp160 (about 25 nM) in PBS/0.1% bovine serum albumin for 1 h at 37°C. Incubation with anti-CD4 antibodies (2 μg/ml) was followed by an incubation with a radioiodinated sheep anti-mouse immunoglobulin antibody (Amersham). After three washes in PBS, cells were treated with trypsin and radioactivity was measured in a γ counter. By assuming that OKT4 binding to mutants was not impaired (see text), the binding percentage of other anti-CD4 antibodies was calculated as follows, after subtraction of cpm bound to mock-transfected cells: % binding = [(anti-CD4 mutant cpm/OKT4 mutant cpm)/(anti-CD4 WT cpm/OKT4 WT cpm)] × 100, where WT is wild type. Binding of gp120/gp160 was calculated similarly. Results are the mean ± SD of three or more experiments.

B-Lymphocyte Adhesion to COS Cells Expressing CD4 Mutants. Twenty-four hours after transfection, COS cells were plated in 12-well dishes (Costar) and assayed 24 and 48 h later for rosette formation with Raji cells, the HLA class II-expressing Burkitt lymphoma line. Enumeration of COS cells expressing CD4, which had bound five B cells or more, was performed under light microscope, after anti-CD4 antibody binding and immunoperoxidase staining, as described (17). Results are expressed as the mean percentage ± SD of three or more experiments.

RESULTS

Expression of the RADS Mutants. Twenty-seven point mutations of the RADS sequence were generated based on the characteristics of the substituting amino acids: small aliphatic residues (Gly or Ala), positively (Lys or Arg) and negatively (Asp or Glu) charged, alcohol function (Ser or Thr), hydrophobic (Val, Leu, or Ile), and aromatic (Phe or Tyr). Three additional residues (Pro, Cys, and Met) were substituted for Asp-56, and a deletion (Δ) of Ala-55 was also tested.

Transient expression of the CD4 molecule in COS-7 cells was assayed 48 or 72 h after the transfection by flow cytometry analysis. All mutant CD4 proteins were expressed at the cell surface, as detected by OKT4 binding. By assuming that the OKT4 epitope, which has been mapped to the fourth extracellular domain (11), was not altered by the mutations, great variability in the mutant expression level was found (Fig. 1). Four groups were defined, according to the distribution of the CD4-expressing cells in the range of 125 to 195 (arbitrary units) of fluorescence intensity, and only 8 out of 27 mutants were as highly expressed as the wild-type CD4. Results were identical 48 or 72 h after the transfection. It should be noted, however, that all mutants expressed CD4 levels higher than the lymphoblastic cell line CEM (17).

Epitopic Phenotype of the CD4 Mutants and gp120 Binding. To evaluate the conformational changes induced by the mutations, binding of nine anti-CD4 antibodies specific for different epitopes of the V1 and V2 domains was tested using a quantitative cellular radioimmunoassay (Fig. 2). By assuming that OKT4 bound to the mutants at 100%, the binding of the nine other anti-CD4 antibodies to mutants was related to OKT4 binding and compared with binding to wild-type CD4. Arg-54 and Ser-57 mutations were the most perturbating, as all mutants impaired 1 to 7 epitopes except the conservative S57T mutant. All substitutions of Asp-56 impaired the T4F epitope and two substitutions that were drastic changes (D56R and D56P) disrupted 7 out of 10 epitopes including Leu3a and T4A. Four out of seven Ala-55 mutants, on the contrary, generated CD4 molecules that bound all antibodies normally. The conservative mutations were the least disturbing except the substitution of Gly for Ala-55, which induced a greater decrease of binding of OKT4E and -F than did A55F, -T, -V, or -R. Conversely, substitutions of Arg and Pro for Asp-56 and of Arg for Ser-57 greatly disrupted most

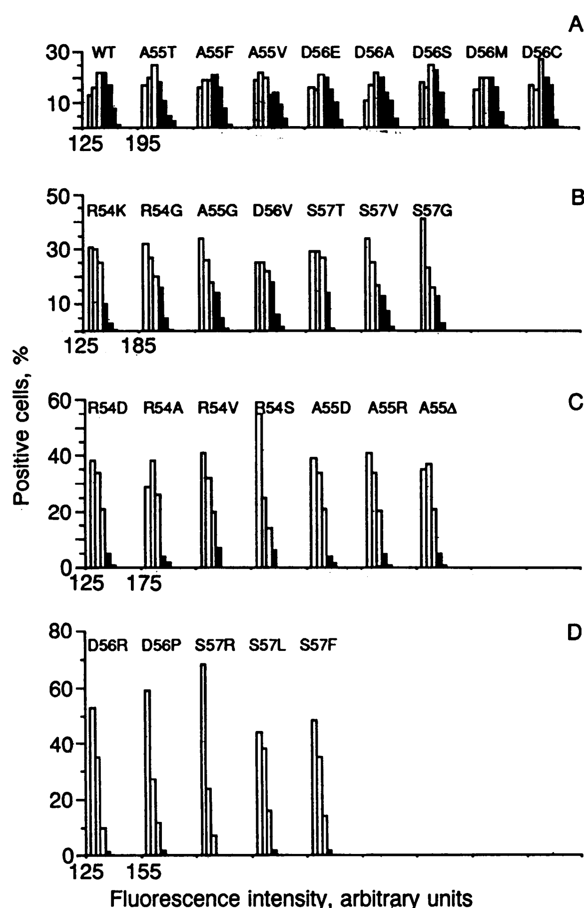

FIG. 1. Expression of CD4 mutants. Distribution of CD4-positive COS cells related to the fluorescence intensity after indirect immunofluorescence staining with OKT4 and flow cytometry analysis. The percentage of cells with different CD4 expression levels was calculated from the population of CD4-positive COS cells with a fluorescence intensity greater than 125 (arbitrary units). Solid bars correspond to cells having a fluorescence intensity greater than 155, defining four groups of expression level with 35–50% of positive cells in group I (*A*), 13–26% in group II (*B*), 5–7% in group III (*C*), and 0–2% in group IV (*D*). Results are the mean of two to four experiments. Mutant names indicate CD4 residue (one-letter symbol), position number, and substituted residue; Δ, deletion; WT, wild-type CD4.

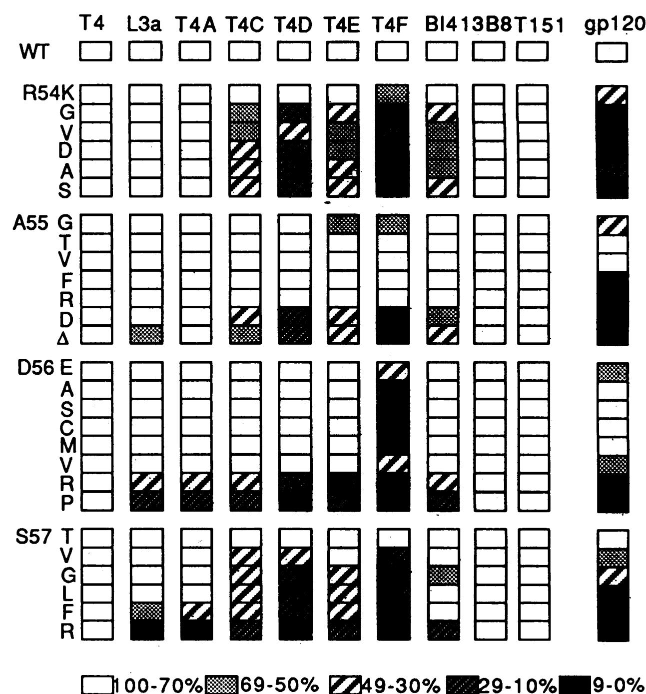

FIG. 2. Epitope mapping of CD4 mutants. The binding percentage of anti-CD4 antibodies and gp120/gp160 to the mutants was calculated (for mutant names, see Fig. 1). T4, T4A, T4C, T4D, T4E, and T4F represent antibodies of the OKT series; L3a is an antibody for Leu3a.

epitopes. Some epitopes were never modified such as 13B8.2 and T151.

HIV gp120 or gp160 binding assays gave similar results and both recombinant proteins were used alternatively or in parallel. Their binding (Fig. 2) was abolished or severely decreased to all Arg-54 mutants, to all Ala-55 mutants but two (A55T and -V), and to four of six Ser-57 mutants. Binding was normal to S57T and moderately decreased to S57V. Normal or slightly decreased binding was also observed to Asp-56 mutants except to those that severely disrupted several epitopes, in which case binding was completely abrogated.

Effect of CD4 Mutations on B-Lymphocyte Adhesion. By using a cellular adhesion assay dependent on CD4–class II interaction (17), adhesion of Raji cells to COS cells expressing mutant CD4 molecules was tested (Fig. 3). Twenty-four of 27 mutant CD4 molecules affected the cellular adhesion assay to some extent (Table 1). Among the 6 mutants that did not alter any epitope (A55G, -T, -F, -V, and -R, and S57T), 3 mutants (A55T, A55V, and S57T) and wild-type CD4 formed rosettes with Raji cells to the same level. Conversely, none of the mutants that impaired two or more epitopes was able to generate rosettes, whereas the single alteration of the T4F epitope produced a moderate decrease of the rosette number. Some mutants such as A55F produced a sharp decrease in the number of rosettes as well as in the number of lymphocytes per rosette (Fig. 3*B*), which could be measured accurately because of the absence of background in the assay. Rosette formation and gp120 binding were impaired by the same mutations indicating that the RADS sequence is similarly involved in both binding functions. It should be noted that all mutants that displayed a low expression level (groups III and IV) were unable to form rosettes with Raji cells, whether they exhibited epitope impairments or not (A55R).

DISCUSSION

Based on our previous hypothesis and experimental results (6, 7), we suggested that the RADS tetrapeptide of CD4 could play an important role in the CD4–class II interaction. This possibility was tested further by producing a series of point mutations at four positions by *in vitro* mutagenesis. We have systematically substituted various amino acids with different properties at the same position, since this is likely to provide more useful information than single-amino acid changes, insertions, or deletions that alone could be silent or too drastic and yield incomplete or conflicting results, as illustrated in several previous reports (8–15). Moreover, we have performed not only a precise quantitative analysis of CD4 expression level and epitopes but also a comparative analysis of the effect of specific mutations on HIV gp120 and HLA class II binding by using a transient CD4 expression assay that makes it possible to visualize the CD4–class II interaction in the absence of the T-cell receptor–CD3 complex through cellular adhesion in a quantitative and highly reproducible manner (17).

Great heterogeneity in the expression of mutant CD4 molecules was observed, 12 of 27 being poorly expressed at the cell surface (groups III and IV), as described for transmembrane and soluble forms of CD4 mutants (9, 14, 16).

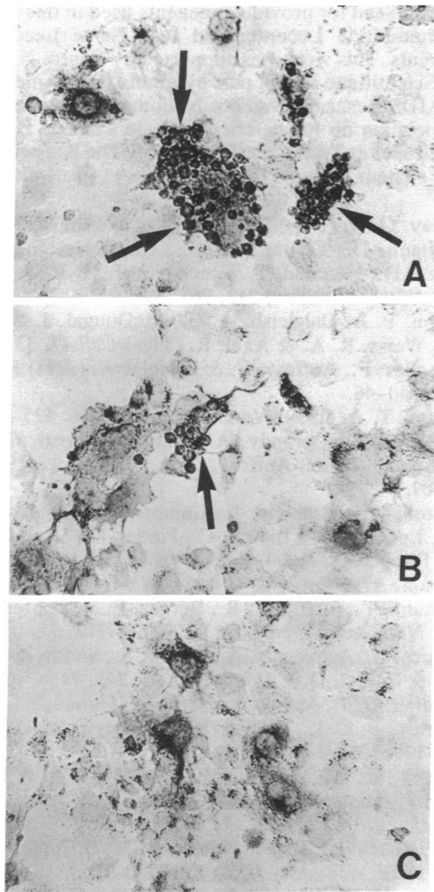

FIG. 3. Rosettes formed between COS cells expressing CD4 mutants and Raji B lymphocytes. CD4 expression was detected by immunoperoxidase staining after binding of the 13B8.2 anti-CD4 antibody. Mutants with high CD4 expression level generate either as many rosettes (arrows) as does wild-type CD4 (*A*) or lower rosette numbers (A55F mutant) (*B*), whereas no rosette is observed for several specific mutations (R54V) (*C*). (×125.)

Table 1. Effects of CD4 mutations

Mutant	CD4 level	% rosettes	% gp120/gp160 binding	Altered V1–V2 epitope(s), no.
WT	I	40 ± 4	100	0
R54K	II	1.6 ± 0.8	30 ± 1	1 (F)
G	II	0	3 ± 5	4 (D, E, F, Bl4)
V	III	0	5 ± 5	2 (D, F)
D	III	0	9 ± 8	3 (C, D, F)
A	III	0	13 ± 2	4 (C, D, E, F)
S	III	0	5 ± 9	5 (C, D, E, F, Bl4)
A55G	II	6.5 ± 1	31 ± 11	0
T	I	43 ± 3	84 ± 7	0
V	I	38 ± 1	123 ± 16	0
F	I	0.7 ± 0.5	9 ± 4	0
R	III	0	0	0
D	III	0	2 ± 3	4 (C, D, E, F)
Δ	III	0	0	4 (D, E, F, Bl4)
D56E	I	26 ± 5	69 ± 1	1 (F)
A	I	25 ± 8	93 ± 2	1 (F)
S	I	24 ± 2	109 ± 11	1 (F)
C	I	22 ± 6	71 ± 3	1 (F)
M	I	11 ± 5	79 ± 13	1 (F)
V	II	18 ± 1	68 ± 6	1 (F)
R	IV	0	3 ± 2	7 (Leu3a, A, C, D, E, F, Bl4)
P	IV	0	4 ± 5	7 (Leu3a, A, C, D, E, F, Bl4)
S57T	II	38 ± 1	96 ± 6	0
V	II	0	59 ± 4	3 (C, D, F)
G	II	0	32 ± 9	4 (C, D, E, F)
L	IV	0	2 ± 3	4 (C, D, E, F)
F	IV	0	4 ± 1	5 (A, C, D, E, F)
R	IV	0	1 ± 2	7 (Leu3a, A, C, D, E, F, Bl4)

CD4 expression levels are defined by groups I to IV (Fig. 1). Rosette formation between COS cells expressing CD4 and Raji B lymphocytes and gp120/gp160 binding is expressed as a percentage (mean ± SD). The number of altered epitopes is defined by antibody binding ≤50% of wild type (Fig. 2). Letters (in parentheses) A, C, D, E, and F represent antibodies OKT4 A, C, D, E, and F, respectively (for mutant names, see Fig. 1).

Although this did not interfere with the antibody and gp120 binding assays, it was impossible to interpret the effect of these mutations on rosette formation since all mutants of groups III and IV were unable to form rosettes and only mutants from groups I and II can be interpreted confidently with respect to CD4–class II interaction. Similar conclusions were drawn in studies of HLA class I mutants with low expression levels that were unable to trigger cell adhesion to cells expressing CD8 (24). Thus quantification of mutant cell surface expression levels is an absolute requirement in these expression assays.

All mutations of Arg-54 but one (R54K) impair several epitopes and completely abrogate gp120 binding and rosette formation, indicating the importance of this residue for the conformation of the V1 domain probably related to its involvement in a salt bridge with Asp-78, as suggested (25). The conservative substitution of Lys for Arg-54 moderately affected the T4F epitope and generated incomplete but severe inhibition of gp120 binding and rosette formation; in addition, the conservative D78E mutant exhibited normal gp120 and B-lymphocyte binding (data not shown), suggesting the possibility that the salt bridge can still form in these mutants, whereas substitution of Ala for Asp-78 induced profound structural changes that inhibited gp120 binding (13). Alternatively, but not exclusively, these results can be interpreted to indicate that part of the side chain of Arg-54 might participate in contacts with gp120 and class II antigens. An effect of the limited impairment of the single T4F epitope on both functions is unlikely since Asp-56 mutations have a more drastic effect on this epitope, whereas Asp-56 mutations affect contacts with gp120 and class II antigens only moderately. Therefore, Asp-56 appears to be an important residue for the T4F epitope and to participate in low-affinity contacts with HLA class II molecules, whereas its involvement in high-affinity binding to HIV gp120 is marginal. Similar conclusions can be drawn from the analysis of Ser-57 mutations, in which the conformational changes affect both interactions more profoundly.

Ala-55 mutants with normal gp120 binding generate normal rosette numbers (A55T and -V) and mutants with abrogated or decreased gp120 binding have a parallel effect on rosette formation (A55G and -F). It should be noted that substitution of Phe at position 55, the murine CD4 residue, abolished both binding functions, whereas the substitution of Val, the chimpanzee CD4 residue (26), had no effect, correlating with the inability and ability, respectively, of CD4 in these two species to bind HIV and suggesting a parallel effect on interaction with HLA class II antigens. As those Ala-55 mutants that are expressed at normal levels are indistinguishable from wild type with regard to antibody binding, a logical tendency would be to consider these effects as the results of the mutations and an indication that Ala-55 participates in direct contacts with both CD4 ligands.

The results presented in this report are in general agreement with those obtained in other studies dealing with a

limited number of substitutions at the same positions. However, there are two cases in which the results are different: (*i*) when the A55F mutant is expressed as a soluble CD4 molecule comprising the four extracellular domains, it no longer binds OKT4D (11), and (*ii*) the R54A mutant does not bind Leu3a, OKT4A, and T151 when expressed as a V1–V2–immunoglobulin chimera (13), whereas these epitopes are not modified in the membrane forms. These discrepancies can be interpreted as reflecting conformational changes that distinguish the membrane and soluble forms of CD4. We already suggested that this might be associated with a differential ability for interaction with HLA class II antigens without a concomitant loss of gp120 binding (17).

With the recent determination of the crystal structure of the first two domains of soluble CD4, which appeared after completion of the present study (27, 28), it is now possible to interpret the effect of the mutations within a three-dimensional model. The RADS tetrapeptide corresponds exactly to the D strand on one of the β-sheets typical of the immunoglobulin fold in the V1 domain, and Arg-54 is indeed engaged in a salt bridge with Asp-78. Ala-55 is buried, indicating on one hand that the absence of conformational change induced by substitutions at this position is not a sufficient criterion to predict a contact residue and on the other hand that the buried state of this residue is required for gp120 and HLA class II binding. Substitutions leading to exposed side-chains would possibly suppress both binding functions, even in the absence of antibody-binding impairment. A more detailed analysis of the contribution of Asp-56 and Ser-57 side chains will be feasible when the atomic coordinates of the models become available.

In this study we have provided evidence that the two binding functions of CD4 overlap at the level of the D strand in the V1 domain. Inhibition of the rosette formation assay by gp120, synthetic peptides derived from gp120, and HIV blocking anti-CD4 antibodies suggests that the class II and gp120 binding sites largely overlap (17). Whether the class II binding site extends to the other extracellular domains is as yet unclear. Simultaneous substitutions of several residues of the V2 domain by the residues present in murine CD4 at positions 99, 104, and 107 and positions 132, 133, and 137 have been described to inhibit rosette formation totally or partially (16). However, a detailed characterization with regard to epitopes and level of expression of these mutants is lacking, and involvement of these residues in interaction with class II antigens cannot be definitively assumed. In addition, mutations of residues 181–186 and 220–226 in the V3 domain have been reported to inhibit interleukin 2 production and aggregate formation of a murine T-cell hybridoma transfected with the mutants in response to recognition of $H\text{-}2D^d$ expressed by murine fibroblasts together with HLA class II antigens (10). The strikingly different pattern of inhibition of this assay by anti-CD4 antibodies (19), when compared with the results obtained in our rosette formation assay (17) is an indication that these mutations are likely to affect interaction of CD4 with the T-cell receptor–CD3 complex. The tools described in the present study, together with the crystal structure of CD4, will be of great help for a more detailed understanding of CD4 interactions with its multiple ligands and the development of drugs for immune intervention in AIDS, autoimmune disease, and organ transplantation.

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