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Immunology

Oligomerization of CD4 is required for stable binding to class II major histocompatibility complex proteins but not for interaction with human immunodeficiency virus gp120

(antigen recognition/AIDS/T-cell receptor/crosslinking/dominant negative mutation)

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ABSTRACT **Previous studies have failed to detect an interaction between monomeric soluble CD4 (sCD4) and class II major histocompatibility complex (MHC) proteins, suggesting that oligomerization of CD4 on the cell surface may be required to form a stable class II MHC binding site. To test this possibility, we transfected the F43I CD4 mutant, which is incapable of binding to class II MHC or human immunodeficiency virus (HIV) gp120, into COS-7 cells together with wild-type CD4 (wtCD4). Expression of F43I results in a dominant negative effect: no class II MHC binding is observed even though wtCD4 expression is preserved. Apparently, F43I associates with wtCD4 oligomers and interferes with the formation of functional class II MHC binding structures. In contrast, F43I does not affect the binding of gp120 to wtCD4, implying that gp120 binds to a CD4 monomer. By production and characterization of chimeric CD4 molecules, we show that domains 3 and/or 4 appear to be involved in oligomerization. Several models of the CD4–class II MHC interaction are offered, including the possibility that one or two CD4 molecules initially interact with class II MHC dimers and further associate to create larger complexes important for facilitating T-cell receptor crosslinking.**

CD4 is a transmembrane glycoprotein expressed on the surface of thymocytes and mature T lymphocytes (1, 2). The extracellular segment of CD4 consists of four immunoglobulin-like domains (D1–D4) joined in a rod-like structure (3–5). Recent studies using site-directed mutagenesis have identified the C′C″ ridge within the membrane-distal CD4 D1 as essential for binding to class II major histocompatibility complex (MHC) proteins and human immunodeficiency virus (HIV) envelope glycoprotein gp120 (6–9). However, in contrast to gp120, class II MHC binding is also affected by amino acid residues that map to the lateral surfaces of D1 and the upper parts of D2 as well as the D1/D2 interdomain groove (10, 11). Whereas soluble CD4 (sCD4) inhibits the interaction between CD4 and gp120 with up to a nanomolar affinity (12, 13), it fails to inhibit class II MHC-specific responses of T-cell clones (12). To explain this paradox, we considered the possibility that CD4 oligomerization is required to form a functional class II MHC binding site. To this end, we have conducted the following cell adhesion and T-cell activation studies and identified a dominant negative mutation consistent with this view.

MATERIALS AND METHODS

COS-7 Cell Transfection and Cell Binding Assay. Human CD4 (6) and CD2 (14) cDNAs cloned into the *Xba* I site of the CDM8 vector were used for transfection. Fifty thousand COS-7 cells were plated into each well of Falcon six-well dishes and transfected with either 1 μg of CD4 DNA plus 4 μg of CDM8 as a carrier or 5 μg of CD2 DNA by the calcium phosphate/chloroquine method as described (15). Two days after transfection, cell binding was assayed. Binding of class II MHC$^+$ human B-lymphoblastoid Raji cells to CD4 transfected COS-7 cells was assayed as described (6). To monitor the effect of sCD4 on CD4-dependent adhesion, 1×10^7 B cells were preincubated in 1 ml of medium containing various concentrations of a recombinant Chinese hamster ovary-derived soluble four-domain CD4 (13) at 37°C for 30 min and then added to transfected COS-7 cells. To examine the effect of anti-CD4 monoclonal antibody (mAb) 19Thy5D7 (16), COS-7 cells were incubated with 16 μg of mAb in 800 μl of medium at room temperature for 15 min before addition of B cells. The mixture of B cells and COS-7 cells was incubated at 37°C for 1 hr, unbound B cells were removed by aspiration and further washing, and class II binding was enumerated by determining the number of COS-7 cell–B-cell rosettes. Binding between CD2-expressing COS-7 cells and CD58-expressing sheep red blood cells (SRBCs) was assayed as described (14). In brief, 0.5% SRBCs ($\approx 1 \times 10^8$ cells per ml) in 1 ml of medium were preincubated in the presence or absence of recombinant soluble two-domain CD2 (sCD2) (17) at 4°C for 1 hr. SRBCs and transfected COS-7 cells were incubated at 37°C for 1 hr and washed, and rosettes were counted as described (14). Percent inhibition was calculated as $100 \times [(R_C - R_I)/R_C]$, where R_C is the number of rosettes in the absence of inhibitor and R_I is the number of rosettes in the presence of inhibitor. For SDS/PAGE analysis, 5 μg of sCD4 and sCD2 were electrophoresed in 12.5% polyacrylamide gels under reducing conditions and stained with Coomassie blue.

Cotransfection Experiments, Immunofluorescence, and gp120 Binding Assay. COS-7 cells were transfected with 5 μg of DNA per well as above and after 48 hr, cells were allowed to react with anti-CD4 mAb 19Thy5D7 (1.25 μg/ml) or OKT4 (10 μg/ml) or the anti-CD2 mAb 3T4-8B5 (1:100 ascites dilution) and then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Caltag, South San Francisco, CA; 1:100 dilution). Ten thousand cells were examined on a FACScan (Becton Dickinson). B-cell binding was assayed as described above. For the assay of gp120 binding, 0.6×10^6 COS-7 cells were plated onto a 10-cm dish, transfected with 4 μg of wild-type CD4 (wtCD4) DNA plus 16 μg of CDM8 or F43I mutant CD4 DNA. Binding of HIV-1 IIIB gp120 (Celltech, Berkshire, U.K.) was determined as described (18) by using anti-gp120 mAb F59.1 (DuPont) and fluorescein-

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Abbreviations: TCR, T-cell receptor; mAb, monoclonal antibody; MHC, major histocompatibility complex; sCD4 or sCD2, soluble CD4 or soluble CD2; HIV, human immunodeficiency virus; gp120, HIV envelope glycoprotein; D, domain; wt, wild type; MFL, mean linear fluorescent intensity; IL-2, interleukin 2; FACS, fluorescence-activated cell sorting.

conjugated second antibody. Cells were analyzed by fluorescence-activated cell sorting (FACS). gp120 binding was calculated as follows: gp120 binding = (FL − FL_{neg}/FL_{19Thy} − FL_{neg}) × 100, where FL = mean linear fluorescence (MFL) of cells stained with anti-gp120 at a given gp120 concentration, FL_{neg} = MFL of cells stained with anti-gp120 in the absence of gp120, and FL_{19Thy} = MFL of cells stained with 19Thy5D7.

T-Cell Transfection and Activation Assay. wtCD4 and mutant CD4 cDNAs were cloned into the COSMSVtkneo vector (19) and introduced into 171.3 T cells (20) by means of retroviral transduction (15, 20). Subsequently, T cells expressing CD4 were sorted by anti-CD4 mAbs plus Dynabeads coated with goat anti-mouse IgG (Dynal, Great Neck, NY). Cells were allowed to react with 10 μg of 19Thy5D7 or OKT4 per ml, stained with fluorescein isothiocyanate-conjugated anti-mouse IgG, and analyzed by FACS. For the activation assay, parental 171.3 cells or transfectants were cocultured with a class II MHC-expressing fibroblast, FT7.1, in the presence of various concentrations of synthetic lysozyme peptide as described (20). The amount of interleukin 2 (IL-2) released in the culture supernatant was measured by using IL-2-dependent CTLL cells (20).

Generation of Chimeric CD4 Molecules. The leader sequence plus domains 1 and 2 of wtCD4 (aa −25 to 184) (21) were amplified by PCR techniques. An *Xba* I site was added at the 5′ end. The third codon of Ser-183 was changed from C to G to create an *Xho* I site. To replace D3/D4 of CD4, domains 1 and 2 of mutant CD4 F43A (18) (aa 1–184) (21) or mutant CD2 D32A (14) (aa 1–185) (22) were fused with the transmembrane and cytoplasmic domains of CD4 (aa 373–433) (21) by PCR techniques, and an *Xho* I site and *Xba* I site were added on the 5′ and 3′ ends, respectively. PCR products were digested with *Xho* I, gel purified, and ligated. The appropriate ligation products were gel purified, digested with *Xba* I, and subcloned into the *Xba* I site of CDM8. The resulting chimeric plasmids were identified by restriction enzyme mapping and DNA sequence analysis. The sequences of specific oligonucleotides and PCR conditions are available upon request. Transfection of COS-7 cells, FACS analysis, class II MHC and gp120 binding assays were performed as described above. For gp120 binding, COS-7 cells were transfected with 5 μg of wtCD4 DNA plus 15 μg of CDM8, 20 μg of CD4 $(D1/D2)_2$ DNA, or 20 μg of CD4/CD2 DNA. For the D3/D4 swaps, mutant sequences were used to avoid additional binding of class II MHC and CD58 to the membrane proximal segments of CD4 $(D1/D2)_2$ and CD4/CD2, respectively (7, 14).

RESULTS AND DISCUSSION

sCD4 Fails to Inhibit B-Cell Binding to CD4-Transfected COS-7 Cells. We have previously established a conjugation assay between class II MHC-expressing human B cells and COS-7 cells transfected with CD4 as a means to assess CD4–class II MHC interaction (6). The effect of the soluble four-domain CD4 extracellular segment (sCD4) on the interaction between CD4 and class II MHC was examined in this system. In contrast to the inhibitory effect of sCD2 on CD2–CD58 binding, sCD4 cannot inhibit B-cell binding to $CD4^+$ COS-7 cells even at a concentration of 100 μM, while the D1-specific anti-CD4 mAb 19Thy5D7 inhibits at 0.1 μM (Fig. 1). These results imply that the affinity of monomeric sCD4 is $<10^{-4}$ M. Consistent with these findings, Weber and Karjalainen (23) reported that pentameric forms of soluble mouse CD4 fused with human C_μ, but not monomeric sCD4 fused with mouse C_κ, could inhibit the interaction between polymer-bound mouse sCD4 and B lymphocytes (23).

F43I Is a Dominant Negative CD4 Mutation for Class II MHC Binding but Not HIV-1 gp120 Binding. The above findings prompted us to examine the idea that oligomerization

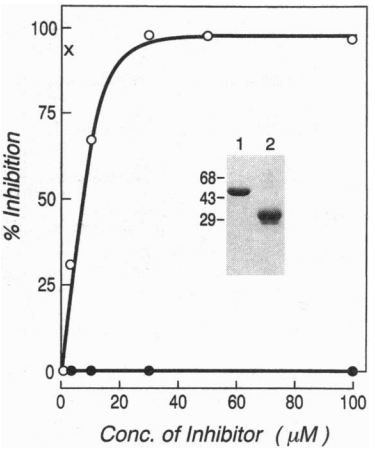

FIG. 1. Soluble monomeric CD4 fails to inhibit the interaction between $CD4^+$ and class II MHC^+ cells. Effect of sCD4 (●) or an anti-CD4 mAb, 19Thy5D7 (X), on the binding of class II MHC-expressing cells to CD4 transfected COS-7 cells and, by comparison, the effect of sCD2 (○) on the binding of CD58-expressing cells to CD2 transfected COS-7 cells. (*Inset*) SDS/PAGE analysis of sCD4 (lane 1) and sCD2 (lane 2) used for assay. Molecular mass markers are bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa).

of CD4 molecules on the cell surface may increase the avidity of interaction between CD4 and class II MHC molecules and stabilize the CD4–class II MHC complex. To examine this idea, we tested for a dominant negative CD4 mutation that would itself be incapable of binding to class II MHC and, at the same time, prevent the binding of wtCD4 to class II MHC. One candidate mutant CD4 molecule, F43I, was transfected into COS-7 cells together with wtCD4. In this mutant, Phe-43, a residue in the C″ strand of D1 essential for the binding of CD4 to both class II MHC (7) and gp120 (18), is changed to Ile. Consistent with our previous reports (7, 10, 18), F43I binds neither to class II MHC (Fig. 2*B*) nor to gp120 (data not shown). F43I reacts with the mAb 19Thy5D7 poorly, if at all, but is recognized by the D3-specific mAb OKT4 (Fig. 2*A*). As shown in Fig. 2*A*, similar amounts of wtCD4 are expressed on the COS-7 cells regardless of coexpression of F43I (left column). Nevertheless, binding of B cells to the COS-7 cells cotransfected with wtCD4 and F43I is virtually eliminated compared to that of the COS-7 transfected with wtCD4 alone (Fig. 2*B*). This inhibition is dependent on the amount of F43I DNA transfected, reaching a maximum level at 3 μg (Fig. 2*C*). In terms of surface protein, the F43I effect is maximal when the copy number is equivalent to or greater than that of wtCD4 (Fig. 2*A* and data not shown). Because F43I cannot bind to class II MHC by itself, it is unlikely that F43I inhibits the binding of wtCD4 to class II MHC through direct competition. Nor does F43I interfere with the binding of wtCD4 to class II MHC by merely occupying space on the COS-7 cell surface: CD2 does not inhibit class II MHC binding (Fig. 2*B*). Rather, the most likely explanation for the effect of F43I is that F43I oligomerizes with wtCD4 and, hence, interferes with the self-association of wtCD4 necessary to create a stable class II MHC binding site. Given the sensitivity of the CD4–class II MHC-dependent adhesion assay (10) and the fact that the oligomer that consists of only wtCD4 should be present at a frequency of $1/2^n$ (where n = number of CD4 molecules within a single oligomer), a trimer, tetramer, or larger oligomer could account for the observed data in the F43I plus wtCD4 cotransfection experiments.

In contrast to class II MHC binding, F43I does not affect the binding of wtCD4 to gp120 (Fig. 2*D*). This result clearly implies a different mechanism for the CD4–class II MHC interaction compared to the CD4–gp120 interaction. Given

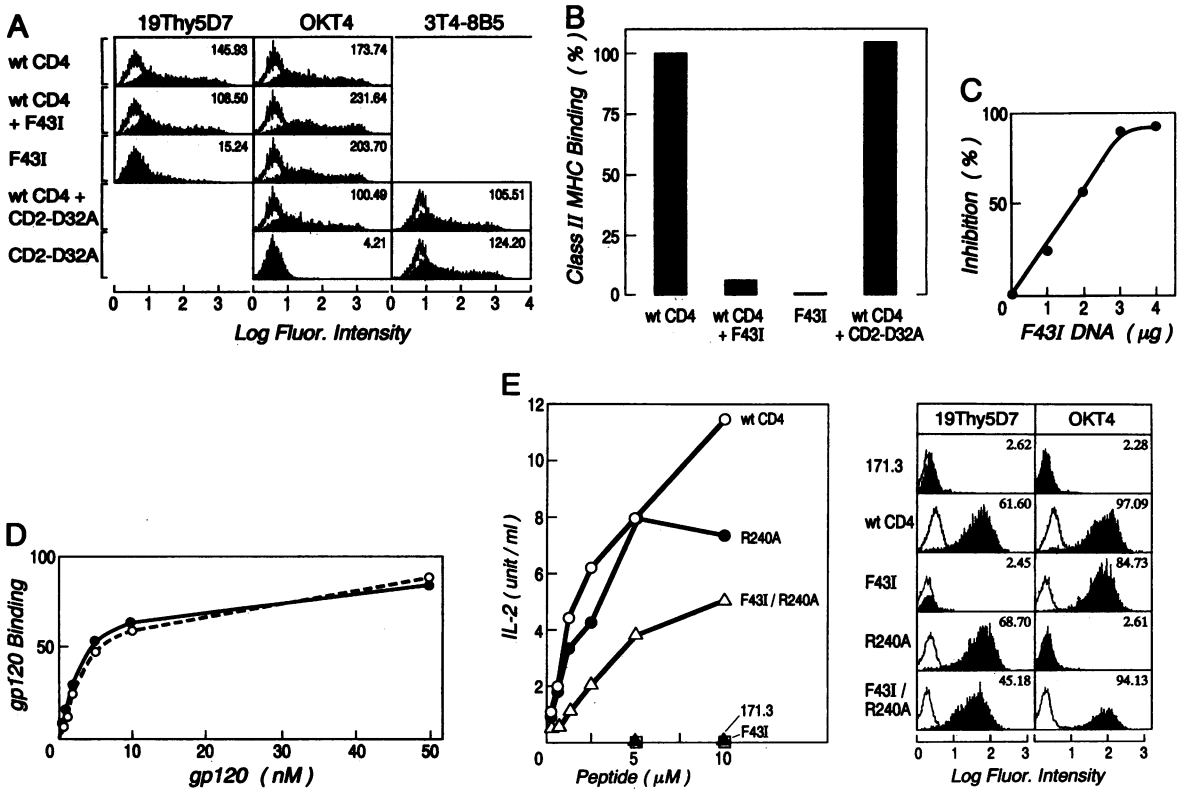

FIG. 2. F43I inhibits class II MHC but not HIV-1-gp120 binding to wtCD4 transfectants. (*A*) FACS analysis of transfected COS-7 cells. For transfections, the following DNA amounts were used: wtCD4, 1 μg of wtCD4 plus 4 μg of CDM8; wtCD4 plus F43I, 1 μg of wt plus 4 μg of F43I; F43I, 4 μg of F43I plus 1 μg of CDM8; wtCD4 plus CD2-D32A, 1 μg of wtCD4 plus 4 μg of CD2-D32A; and CD2-D32A, 4 μg of CD2-D32A plus 1 μg of CDM8. The thin line in each histogram shows cells transfected with CDM8 alone. Numbers in the fluorescence histograms represent MFL values. (*B*) Coexpression of F43I mutant CD4 molecules with wtCD4 molecules prevents class II MHC$^+$ B cells from binding to COS-7 cell transfectants. DNA amounts used for transfection were the same as in *A*. Binding is expressed as a percentage of the average number of rosettes in duplicate wells relative to the numbers in the wells of cells transfected with wtCD4 (240–280 rosettes). Results are representative of four independent experiments. (*C*) Inhibition of B-cell binding as a function of the amount of F43I DNA transfected. One microgram of wtCD4 DNA and 1–4 μg of F43I DNA (abscissa) were mixed, and CDM8 DNA was added to maintain the total amount of transfected DNA at 5 μg. (*D*) Coexpression of F43I does not alter HIV gp120 binding to wtCD4. Binding of gp120 to the COS-7 cells transfected with wtCD4 (●) or wtCD4 plus F43I (○) is shown. (*E*) Coexpression of F43I inhibits CD4-dependent T-cell activation. IL-2 production (*Left*) and FACS analysis (*Right*) of T cells transfected with wt or mutant CD4. The thin line in each histogram shows staining with the secondary antibody alone. Results of T-cell activation assay are representative of five separate experiments using two independent clones of each type.

these data and the earlier finding that monomeric sCD4 binds to gp120 (12, 13), it is likely that binding of CD4 to class II MHC requires oligomerization of CD4 molecules, while binding of CD4 to gp120 does not.

Effect of F43I on T-Cell Activation. Next we examined the dominant negative effect of the F43I mutant on the activation of the 171.3 T cell line that lacks endogenous CD4 (20). Activation of 171.3 T cells by class II MHC plus its specific peptide resulting in IL-2 secretion depends on expression of CD4 (Fig. 2*E Left*). As expected from the results of cell binding assay, T cells expressing F43I are not activated, although they express an equivalent number of mutant CD4 molecules relative to that of the wtCD4 transfectant (Fig. 2*E*). Another CD4 mutant, R240A in which Arg-240 in D3 essential for the recognition by the mAb OKT4 (24) is changed to Ala, reacts with 19Thy5D7 but not OKT4 (Fig. 2*E Right*). This mutation does not affect binding to class II MHC in the adhesion assay (data not shown) and therefore, not unexpectedly, R240A-transfected 171.3 T cells show activation comparable to cells bearing wtCD4. Given the unique anti-CD4 mAb reactivity pattern of R240A and F43I, it was then possible to introduce F43I into the R240A$^+$ cell line, thereby creating an F43I/R240A double transfectant. Importantly, the F43I/R240A transfectant requires a 5- to 10-fold molar increase in peptide concentration to achieve the same level of IL-2 production as R240A or wtCD4 171.3 transfectants (Fig. 2*E Left*). We take this as additional evidence for the important dominant negative effect exerted by F43I. That F43I does not abrogate IL-2 production in T cells as effectively as it inhibits adhesion in the COS cell system may be a consequence of the smaller number and/or size of CD4 oligomers needed to participate in T-cell activation in contrast to cell adhesion. It is also of interest that receptor antagonism has been ascribed to the failure to form effective patches of T-cell receptors (TCRs) and their ligands in the case of peptide antagonists (25).

CD4 Chimeras Lacking D3/D4 Suggest a Role for These Domains in Oligomerization. Previous studies failed to identify residues in D3 or D4 involved in class II MHC binding (10, 11). In view of the unknown role of D3 and D4 in CD4 function, we tested the possibility that these domains might participate in the oligomerization process. To this end, we created two chimeric CD4 molecules that consist of CD4 D1 and D2 (D1/D2) but lack CD4 D3 and D4 (D3/D4). In one chimera, D3/D4 was replaced by D1/D2 of CD4 [CD4(D1/D2)$_2$] (Fig. 3*A Center*). In the other, D3/D4 was replaced by the extracellular domain of CD2 (CD4/CD2) (Fig. 3*A Right*). These replacements were chosen because of the known structural similarities between CD4 D3/D4, CD4 D1/D2, and CD2 D1/D2 (3–5, 26, 27). Both chimeras retain the transmembrane and cytoplasmic domains of CD4. Each chimera is reactive

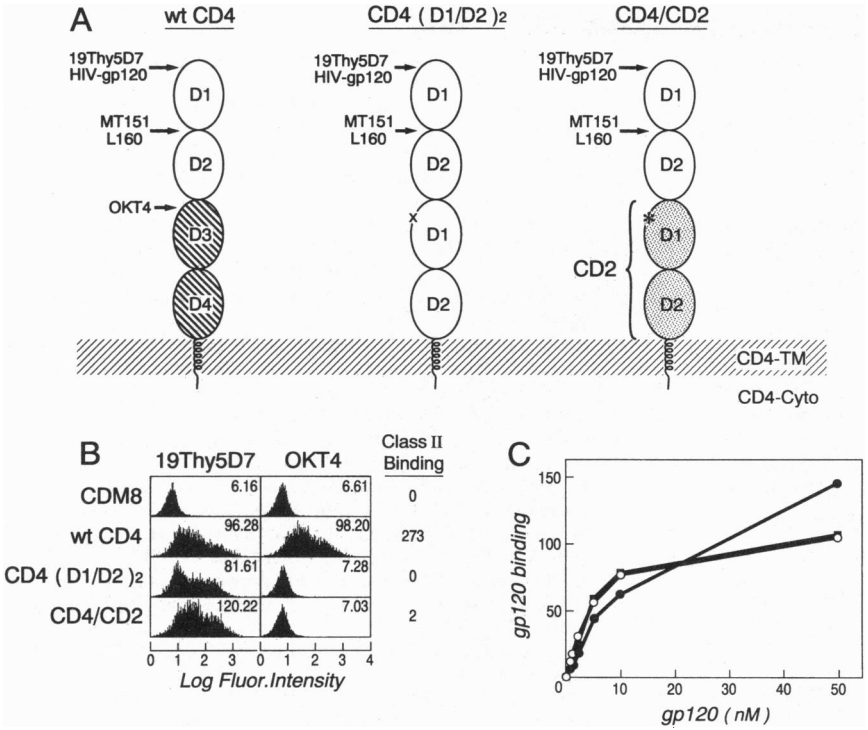

FIG. 3. CD4 D3 and D4 are required for stable class II MHC binding. (*A*) Schematic diagram of wtCD4 (*Left*) and two chimeric CD4 molecules, CD4 $(D1/D2)_2$ (*Center*) and CD4/CD2 (*Right*). Open ovals, D1 and D2 of CD4; hatched ovals, D3 and D4 of CD4; stippled ovals, D1 and D2 of CD2. Areas where mAbs 19Thy5D7, MT151, L160, and OKT4 and HIV-gp120 bind are shown. X and * indicate mutated Phe-43 of CD4 and Asp-32 of CD2 residues in chimera molecules, respectively. (*B*) Chimeric molecules cannot bind to class II. COS-7 cells were transfected with 0.25 μg of wtCD4, 5 μg of CD4 $(D1/D2)_2$, or 5 μg of CD4/CD2 DNA. Results of FACS analysis and class II binding assay (numbers of rosettes) are shown. For FACS analysis, saturating mAb concentrations were used throughout. Numbers in the fluorescence histograms represent MFL values. Results are representative of two independent sets of experiments. (*C*) Chimeric CD4 molecules bind to gp120 in a manner equivalent to wtCD4. Binding of gp120 to COS-7 cells transfected with wtCD4 (○), CD4 $(D1/D2)_2$ (●), and CD4/CD2 (■) was assessed as in Fig. 2.

with the conformation-dependent anti-CD4 mAbs 19Thy5D7 (Fig. 3*B*), MT151, and L160 (data not shown), specific for known epitopes in D1 and/or D2. In addition, gp120 binds to the chimeras in a manner equivalent to wtCD4 (Fig. 3*C*), collectively supporting the view that the structure of D1/D2 within the chimera is native. Nevertheless, unlike wtCD4, neither chimera when expressed on COS-7 cells binds to class II MHC^+ B cells (Fig. 3*B*). Since mutations of multiple D3/D4 residues do not affect class II MHC binding (10, 11), it is unlikely that D3/D4 directly contacts class II MHC. The lack of class II MHC binding in these D3/D4 replacement chimeras is consistent with the idea that association of CD4 molecules is necessary for the binding to class II MHC and suggests that D3/D4 may be involved in oligomerization. Consistent with this notion and in contrast to the effect of F43I, cotransfection of either CD4 $(D1/D2)_2$, CD4/CD2, or F43ICD4/CD2 with wtCD4 in COS-7 cells does not interfere with class II MHC-dependent B cell binding (data not shown).

Assuming that D3 and D4 facilitate CD4 oligomerization, the affinity of self-association must be low because CD4 multimers do not form in solution (28) and because the dimer found in crystals of D3/D4 from rat CD4 is likely the result of lattice contacts (26). Since OKT4 mAb fails to block class II MHC-dependent T-cell proliferation (1) and does not inhibit class II MHC binding in the CD4 transfected COS-7 cell/B-cell assay (data not shown), the proposed oligomerization interface between independent CD4 molecules presumably lies on a surface of D3/D4 opposite to Arg-240. A recent report by Kinch *et al.* (29) indicates that the cell–cell adhesion mediated by CD4–class II MHC interaction requires ATP and is dependent on cytoskeletal function. The CD4 transmembrane and cytoplasmic domains are present in each of the CD4 chimeras characterized here, and the loss of class II MHC binding function of these molecules must therefore be a consequence of alteration of CD4 D3 and/or D4. Aggregation of CD4 molecules into adherens junctions via cytoplasmic interactions may, however, enhance multivalency further.

Implications. Several models could account for the results reported here. As shown in Fig. 4*A*, it is possible that one CD4 molecule binds to one class II MHC molecule and subsequently, oligomerizes via D3/D4, thus forming "flower"-like structures. Three or four copies of CD4 might constitute the stem of a single flower and still leave sufficient space for the TCR to interact with the peptide binding surface. Mutagenesis studies of mouse class II MHC molecules in the β2 domain have identified a region between residues 137 and 143 as important for CD4 binding (30). The role of the α subunit is currently untested, however, leaving open the possibility that α2 could be involved as well. If this were true, then two CD4 molecules could bind to a single MHC heterodimer (Fig. 4*B*), assuming CD4 utilized different regions to interact with the individual subunits of the class II MHC molecule. For example, the CD4 C′C″ ridge might interact with class II MHC β2 and the CD4 D1/D2 interdomain groove might contact class II MHC α2. Recent studies on the crystal structure of human class II MHC suggest that two α/β heterodimers of class II MHC molecules can associate to form a "dimer of dimers" (31). If this is the case on the cell surface, then two or four CD4

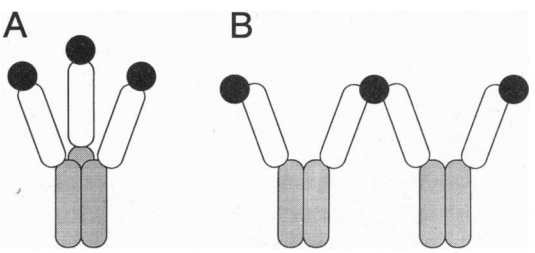

FIG. 4. Hypothetical models of the CD4–MHC class II interaction. Solid circle, MHC class II heterodimer; open oval, D1/D2 of CD4; shaded oval, D3/D4 of CD4. (*A*) One CD4 molecule binds one class II MHC molecule. The CD4–class II MHC complex forms a stem structure by association of independent CD4 molecules via D3/D4. Although not shown, interactions between D2 domains are also possible. (*B*) Two CD4 molecules bind to one class II MHC dimer presumably via β2 and α2 domains. In addition, class II molecules are "bridged" by association of several (two shown) CD4 molecules via D3/D4. *A* and *B* are not necessarily exclusive of one another. Additional oligomerization will result if class II MHC exists as a dimer of dimers as discussed in the text. For ease of illustration, molecules are not drawn to scale.

molecules could bind to one class II dimer of dimers according to the model in Fig. 4 *A* and *B*, respectively. In either case, there would still be sufficient access for the TCR to bind to the MHC surface harboring the antigenic peptide. The possibility remains open as to whether the binding site resides on a single class II MHC α/β heterodimer or the α component of one MHC molecule and the β component of another MHC molecule within the dimer of dimers.

A "network" of CD4–class II MHC multimolecular complexes would serve to concentrate and crosslink the TCR with its associated signal transduction components (i.e., $p56^{lck}$ noncovalently associated with the CD4 cytoplasmic tail) at the site of the T-cell–antigen-presenting cell interface. Crosslinking of TCRs is known to be critical for T-cell activation (32). Because we have been unable to detect CD4 oligomers in the absence of class II MHC interaction by energy transfer methodology (data not shown), it is likely that initiation of oligomerization requires the presence of class II MHC. Such oligomerization might be further modulated by TCR interaction through direct contact with D3/D4 of CD4 or other associated structures.

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