Involvement of Both Major Histocompatibility Complex Class II α and β Chains in CD4 Function Indicates a Role for Ordered Oligomerization in T Cell Activation

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Summary

CD4 is a membrane glycoprotein on T lymphocytes that binds to the same peptide-major histocompatibility complex (MHC) class II molecule recognized by the antigen-specific receptor (TCR), thereby stabilizing interactions between the TCR and peptide:MHC class II complexes and promoting the localization of the src family tyrosine kinase p56Lck into the receptor complex. Previous studies identified a solvent-exposed loop on the class II β2 domain necessary for binding to CD4 and for eliciting CD4 coreceptor activity. Here, we demonstrate that a second surface-exposed segment of class II is also critical for CD4 function. This site is in the α2 domain, positioned in single class II heterodimers in such a way that it cannot simultaneously interact with the same CD4 molecule as the β2 site. The ability of mutations at either site to diminish CD4 function therefore indicates that specifically organized CD4 and/or MHC class II oligomers play a critical role in coreceptor-dependent T cell activation.
Experimental Approach. Structural homologies (19, 20, 33-36) and similar functions (37) of MHC class I and class II molecules suggest analogies in the mechanism of CD8-MHC class I and CD4-MHC class II interactions. Indeed, the previous identification of the class II β2 domain’s interaction with CD4 (amino acid residues 137-143) was based in part on the assumption that the binding site for CD4 might lie in a position analogous to the CD8-binding site on MHC class I molecules. An x-ray crystallographic structure had been determined for the human class I molecule, HLA-A2 (38), and a binding site for CD8 on the α3 domain of HLA-A2 had been identified (39) before these published CD4 studies. Although the class II β2 domain was the predicted homologue of the class I α3 domain, the lack of a crystallographic structure for class II at that time left open the possibility that the class II α2 domain might actually be the relevant domain for CD4 binding. Sequence alignment of the β2 domain of MHC class II with the class I α3 domain yielded a high degree of similarity, but alignment of the class II α2 domain with the MHC class I-associated β2-microglobulin was not as good. In fact, the MHC class II α2 domain aligns better with the class I α3 domain than with β2-microglobulin. We therefore introduced mutations in both the class II β2 and the α2 domains. Analysis of mutants carrying amino acid substitutions in a surface-exposed loop of the β2 domain rapidly revealed the presence of a critical CD4 contact site, but several clustered mutations in the α2 domain also diminished function with CD4. We have now carefully analyzed this highly conserved second region comprising αα amino acids 125-133 (numbering of residues is according to Kabat et al [40]).

Single or double mutations were made in an Ac-d cDNA throughout this region. Two types of mutations were generated: human for mouse substitutions and alanine replacements. In addition, in order to sterically interfere with CD4-MHC class II interactions, we attempted to introduce potential N-linked glycosylation sites at positions 127 and 129 of the Ac-d molecule. These mutants, however, were not expressed on the cell surface of transiently transfected COS or stably transfected mouse L cells. On the other hand, the corresponding control constructs carrying an asparagine residue at positions 127 or 129 and an alanine or glycine residue at positions 129 or 131, respectively, were surface expressed, and are included in this analysis.

The mutant Ac-d cDNA constructs were cotransfected into L cells together with a cDNA construct encoding wild-type β2 and a plasmid carrying a neomycin-resistance gene. Stable transfectants were selected in G418-containing medium, and cells expressing high levels of the αβ heterodimer were isolated by antibody-mediated magnetic bead sorting.
All mutant $\alpha\beta$ chains included in this report supported efficient surface MHC class II expression. These mutant heterodimers reacted appropriately with a panel of monoclonal anti-$\alpha\beta$ antibodies, indicating that the mutations did not grossly affect the folding and conformation of the class II $\alpha$ chain or the assembled $\alpha/\beta$ heterodimer (data not shown).

The various transfected L cells were used as antigen-presenting cells for the stimulation of two sets of T cell hybridomas, derived from the parental lines DO.11.10 (23) and 171.3 (24). Members of each set express the same TCR, but differ in coreceptor expression, having on their surface either human CD4 (hCD4), mouse CD4 (mCD4), mouse CD8 (mCD8), or no coreceptor at all. The DO.11.10 hybridoma responds to stimulation by APC plus peptide antigen with lymphokine secretion even in the absence of CD4 expression. It is therefore possible to selectively assess the effect of sequence changes in $\alpha\beta$ on the capacity of the $\alpha/\beta$ heterodimer to elicit CD4 coreceptor function by comparing the responses to mutant vs. wild-type class II molecules of DO.11.10 cells with different coreceptor expression. Mutations with selective effects on CD4 coreceptor function but without effects on peptide binding or TCR recognition will not affect the responses of the CD4-negative T cell hybridomas, which therefore can serve as a control to render this assay independent of the precise level of class II expression on the individual transfected L cell lines, or of differences in the absolute level of cytokine production by the individual T hybridoma cell lines. In contrast, the 171.3 hybridoma requires CD4 expression for responses to peptide or toxin. By using transfecants with comparable levels of cell-surface MHC class II expression, the hybridoma set based on 171.3 can be used to confirm data obtained using the DO.11.10-based set of hybridomas. This is the same assay used previously to identify the CD4 interaction site on the class II $\beta2$ domain (22).

**Effects of Mutations.** In the presence of wild-type $\alpha\beta\beta$, DO.11.10 T cells expressing either hCD4 or mCD4 secreted more IL-3 than mCD8-bearing cells (Fig. 1, A and F, and Fig. 2 A), responding more vigorously than the mCD8-expressing line at any given dose of antigen. Substitution of glycine for serine at position 125 in the $\alpha$ chain strongly reduced the response of the mCD4-expressing DO.11.10 cells at all antigen concentrations, but did not affect the response of the hCD4-bearing cells (Fig. 1 C). This mutation is an example of an isotypic substitution at an amino acid highly conserved between alleles of a particular isotype, with serine occurring in all alleles of $\alpha$, and glycine in $\alphaE$ (as well as all human class II MHC isotypes). This finding is consistent with functional data suggesting CD4 interacts less well with $\alphaE\beta\beta$ than with $\alpha\alpha\beta\beta$ (41). Another mutation of this type, substitution of serine for tyrosine at position 133, had no effect on either the hCD4 or the mCD4-expressing hybridomas (Fig. 3). Alanine substitution at positions 129 (Fig. 2 C) and 131 (Fig. 1 D) in the $\alpha$ chain substantially decreased responses from both hCD4 and mCD4-bearing cells, as did substitution of asparagine for threonine at position 129 (Figs. 1 G and 2 D), and two different double mutations also involving residue 129 (Figs. 1 H, 2 E, and 3). In contrast, the capacity of these mutants to elicit responses from the mCD8-expressing DO.11.10 cells remained wild-type-like. As a comparison, we have included the previously described double alanine mutation in the $\alpha\beta$ domain at positions 137 and 142 (Fig. 1 J), which abrogates CD4 coreceptor function in this assay (22). Mutations decreasing CD4 function in these DO.11.10 experiments also reduced cytokine production by both hCD4- and mCD4-expressing 171.3 cells relative to stimulation with wild-type $\alpha\alpha\beta\beta$ molecules (Fig. 1, K–N).

Asparagine substitution of threonine 129 drastically reduced function with both hCD4 and mCD4 (Figs. 1 G and 2 D). Function with hCD4 was also eliminated by combining the alanine substitution at position 125 with one at position 129 (Figs. 1 H and 2 E). Conversely, substituting glycine for serine at position 125 (a change that introduces the conserved human residue at this position) dramatically decreased function with mCD4, but did not affect function with hCD4. To determine the individual contributions of alanine substitutions at positions 125 and 129, we compared the former with the double alanine mutation and the asparagine for threonine substitution at 129 (Fig. 2). This experiment demonstrated the major contribution of the alanine for serine substitution at 125 to the decrease in function with hCD4, and the equivalency of alanine and asparagine substitutions at 129.

The effects of mutations in the $\alpha2$ domain of I-A$^d$ appeared greatest at low to moderate antigen concentrations. Mutations judged to decrease function with CD4 reduced responses by CD4$^+$ T hybridomas to a level equal or only slightly higher than that seen with the mCD8$^+$ control hybridoma cells. As seen in some of the antigen dose-response curves depicted in Fig. 1, the effects of a partial loss of MHC class II–induced CD4 function could be overcome at very high antigen concentrations. To standardize data from multiple experiments in which absolute responses varied, we used a transformation formula that accounts for the range in the dose-response curve covered by low to moderate antigen concentrations. This formula gives an estimate of the residual CD4 coreceptor function induced by the respective mutant $\alpha\alpha\beta\beta$ molecule with wild-type–like function represented by a fractional value of 1 and a value of 0 given to mutant $\alpha\alpha\beta\beta$ molecules that fail to induce an increased response by the CD4$^+$ T hybridoma cells as compared with the CD8$^+$ control cells. The fractional CD4 coreceptor function was defined as: 

$$F = \log f [\text{mutant } \alpha\alpha\beta\beta]/\log f [\alpha\alpha\beta\beta],$$

where $f$ represents the ratio between the antigen concentration necessary to half-maximally stimulate the CD8$^+$ control T hybridoma and the antigen concentration required to cause equal cytokine secretion by T hybridoma cells bearing either mCD4 or hCD4.

Fig. 3 summarizes the results of several independent experiments of the type illustrated in Figs. 1 and 2 using the transformation formula just described. In each individual experiment, this formula attributes a value of $F = 1$ to wild-type $\alpha\alpha\beta\beta$. The shaded area in the panels of Fig. 3 represents the range of $F$ values seen in repeated experiments with mutations we judge to have wild-type function with CD4, because they led to only small effects of inconsistent
Figure 1. Selective mutations in the region of Ac formed by amino acids 125-133 decrease CD4 coreceptor function in T cell assays. Antigen dose-response curves for murine T hybridoma DO.11.10 cells expressing either mouse (closed circles) or human CD4 (open circles), or mouse CD8 (open squares) after stimulation with antigen (ovalbumin peptide 323-339) presented by wild-type or mutant AceAβ transfected L cells (A-J), and for hybridoma 171.3 cells expressing either mCD4 (closed circles) or hCD4 (open circles) after stimulation with staphylococcal enterotoxin A and similar transfectants (K-N). Coreceptor-negative 171.3 cells did not secrete lymphokine at any concentration of antigen tested. Numbering of residues is according to Kabat et al. (40). Mutants are identified by the wild-type residue at the indicated position followed by the new residue present in the mutant protein. Data in panels presented in one row are from the same experiment. Results for the following transfectants are shown: (A) wild-type AceAβ; (B) AceS127T; (C) AceS125G; (D) AceG131A. In E, dose-response curves for the mCD8+ DO.11.10 stimulated with antigen plus wild-type or mutant AceAβ-expressing L cells are depicted to show the comparable capacity of the different AceAβ molecules to provide CD4-independent T cell stimulation (closed circles, wild-type AceAβ; open circles, AceS125G; squares, AceS127T; triangles, AceG131A); (F) wild-type AceAβ; (G) AceT129N; (H) AceS125A.T129A; (I) AceV132A; (J) the previously described mutant AceE137A.V142A is shown for comparison; (K) wild-type AceAβ; (L) AceS125G; (M) AceT129A; (N) AceY133S. In each row of panels, one mutation that did not affect function with either mCD4 or hCD4 is shown (K, L, and N). For experiments with the 171.3 hybridomas, wild-type and mutant transfectants were selected for closely matched levels of surface class II expression.

direction (e.g., 0.78-1.1 for S127T with mCD4; 0.96-1.18 for V132A with mCD4). Bars that do not reach these shaded areas or extend beyond them therefore identify mutations that consistently affect function with CD4 in a single direction (decrease or increase, respectively) and to an extent greater than the experimental variability in this system. The data indicate that amino acids 125-131 of Ace are part of a site necessary for optimal class II-dependent CD4 coreceptor function. Threonine 129 appeared to be particularly important for mediating this interaction, because both alanine substitution and the relatively conservative asparagine replacement drastically reduced function with both hCD4 and mCD4. Glycine for serine substitution at position 125 drastically reduced function with mCD4, but did not affect function with hCD4. On the other hand, alanine for serine substitution at 125 affected function with both. The differential effect of glycine vs. alanine at position 125 on human CD4 vs. mouse CD4 function could reflect the coevolution of the human coreceptor and class II for proper interaction involving this site directly. Alternatively, structural data indicate that residues 125-133 are part of a surface-exposed loop between the C and D strands of the α2 domain (20) (Fig. 4 A). Both ends of this loop are defined by glycines in most class II molecules, and mutating residues 125 and 131 may affect the overall conformation of the loop.

Several of the substitutions augmented function with CD4. For example, substitution of serine for tyrosine 133 strongly enhanced function with both mouse and human CD4. Serine is the amino acid occurring at this position in all alleles of HLA-DR and DQ, whereas tyrosine at this position is conserved among alleles of I-A. The increase in function resulting from the serine for tyrosine substitution may be a reflection


Figure 2. Mutating amino acids 125 and 129 of Acα causes drastic decreases in CD4 coreceptor function. Antigen dose-response curves for T hybridoma DO.11.10 cells after stimulation with ovalbumin peptide 323-339 and wild-type or mutant AcαAβα transfected L cells are shown. For an explanation of symbols refer to Fig. 1.

of the higher affinity of human CD4 for human class II compared to mouse CD4 and mouse class II (21, 42), and the contribution of serine at this position to stronger binding between these proteins. A simple structural explanation is not as readily apparent for the ability of conservative substitutions S127T and V128A to selectively enhance function with human but not mouse CD4.

Structural and Functional Implications. Computer docking studies show that the elongated, rigid structure of the CD4 D1 and D2 domains involved in interaction with class II (43, 44) and the positioning of the β2 and α2 sites on opposite faces of the class II heterodimer (Fig. 4 A) prevent these critical binding regions of single CD4 molecules from simultaneously interacting with both sites on an individual class II molecule (Fleury, S., and R. N. Germain, unpublished observations). Yet the ability of either α or β chain mutations to strongly decrease the capacity of class II molecules to support CD4 function indicates that both sites are required for inducing full CD4 coreceptor activity. Together, these observations provide the first evidence that during T cell activation by physiological ligands, CD4 and class II molecules must undergo a structurally precise dimerization or oligomerization for the effective contribution of CD4 to receptor-dependent signal transduction.

Two different general models for the organization of the CD4-class II complex can be envisioned that satisfy the structural constraints revealed by this study and the available data on CD4 regions affecting interaction with class II (44). In the first model, two CD4 molecules would associate with a single TCR-MHC class II complex, one CD4 molecule binding to the α2 site and the other to the β2 site (Fig. 5 A). The two CD4 molecules thus would form a clamp tethering the class II ligand to the TCR. This would also provide a mechanism for activating the protein tyrosine kinase, p56Lck, upon monovalent TCR-MHC class II interaction, based on CD4 dimerization in this postulated complex. Furthermore, higher order oligomerization of the coreceptor-TCR-MHC class II units might occur, if the free face of each CD4 molecule could engage the alternative site of an adjacent, TCR- engaged class II molecule (Fig. 5 B). This model is consistent with data suggesting that two opposite surfaces of the D1-D2 segment of CD4 contribute to class II recognition (44), and with the possible role of such higher order oligomers (“immunons”) in lymphocyte signal transduction (45).
Figure 3. Residual coreceptor function of mouse and human CD4 in response to mutant Λακββ molecules. A quantitative evaluation of coreceptor function with each mutant Λ-A molecule was derived from dose-response curves as in Figs. 1 and 2 by normalizing responses of CD4 coreceptor positive and negative T cell hybridomas to the responses of the same T cells to wild-type Λακββ. The bars represent mean values of two to six independent experiments (only one experiment was performed for ΛαS125A.S127A with hCD4+ DO.11.10 cells). The shaded area represents the range of F values seen in independent experiments using mutant class II molecules considered wild-type in their function with CD4.
Figure 5. (A) Clamp model of CD4 function. One CD4 molecule binds to each of the functionally significant sites on a single MHC class II heterodimer. These two CD4 molecules and the single class II–peptide complex are postulated to interact with a single T cell receptor complex (not shown). (B) Adapter model of CD4 function. As in A, two CD4 molecules bind to each T cell receptor–MHC class II–peptide complex, but the CD4 also can link individual class II MHC heterodimers to one another by bridging between the α and β chain sites on different class II molecules. (C) MHC class II superdimer oligomerization by CD4 molecules with a single binding site for a conjoint α2/β2 target region and a separate dimerization interface.

An intriguing alternative model comes from recognizing that the α2 and β2 sites lie in close proximity on a single face of the dimer of dimers observed in crystals of the human class II HLA-DR1 molecule (20) (Fig. 5 C). In this configuration, the two discontinuous sites on a single class II dimer would now contribute to a single surface for interaction with CD4, possibly supporting a higher binding affinity than either site alone. CD4 would thus stabilize the interaction of the two heterodimer units of the class II “superdimer” and that of the associated pair of TCR complexes (Fig. 5 C). The mapping of regions contributing to CD4–class II interaction to discrete faces of CD4 might then reflect the presence of the actual binding surface on one face, and a possible dimerization interface on the other, that could link TCR–superdimer complexes into large oligomers.

A large body of literature derived primarily from antibody cross-linking experiments supports the importance of coocclusion or coaggregation of CD4 and TCR for effective T cell activation, but these prior studies provided no information on the specific molecular associations necessary for proper signaling during physiological cell–cell interaction (6, 7, 15). The identification here of a second discrete binding site for CD4 on class II molecules strongly suggests that structurally specific, ordered oligomerization as opposed to generalized colocalization is a key feature in the assembly of useful signal transduction complexes on T cells. This result extends to the T cell receptor–coreceptor complex the general principle of ligand-induced receptor dimerization/oligomerization as a key event in the initiation of transmembrane signaling (46). The critical role of these stable, properly assembled dimers or oligomers in T cell activation also suggests that the partial agonist or antagonist properties of variant TCR–MHC:peptide complexes (47-49) may result from improper formation or altered stability of these critical molecular assemblies (50-53).

Figure 4. (A) The two sites affecting class II–CD4 interaction lie on opposite sides of a single MHC class II heterodimer. The image is a side view of an MHC class II molecule (yellow ribbon drawing), with the residues of both the 137-147 loop in the β2 domain and the 125-131 region in the α2 domain that affect interaction with CD4 shown in red. The domains of the class II molecule are labeled. (B) The two sites affecting class II–CD4 interaction lie next to one another in the dimer of dimers crystal structure of MHC class II. The image is a side view of the dimer of dimers (yellow ribbon drawing), with the residues of both the 137-147 loop in the β2 domain and the 125-131 region in the α2 domain that affect interaction with CD4 shown in red. The domains of each class II molecule are labeled.

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