Molecular Interaction of the CD4 and MHC Class II Molecules: Mapping the Contact Sites On CD4

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Abstract

T cells expressing CD4 recognize antigens presented by class II following the contact of CD4 with non-polymorphic regions of class II. CD4 enhances T cell activation by acting as an adhesion molecule (co-ligand function), or by bringing the CD4 associated $p56^{lck}$ to the vicinity of the TCR (co-receptor function).

To dissect the molecular interactions which lead to CD4 function(s), wild-type (WT) and mutant CD4 molecules were expressed in the CD4-dependent 3DT52.5.8 T cell hybridomas. Results showed that multiple sites on CD4 encompassing the CDR1, the CDR3 regions of D1 and the FG loop of D2 are involved in class II interaction. The opposite face containing the CDR2 region also plays a role, either as another class II binding site, or the TCR docking site, or in another function of CD4. Co-receptor function requires a much larger site on CD4, compared to co-ligand function. A stretch of 15 amino acids which links D2 and D3 of CD4 appears to be very important for maintaining CD4 conformation, or to provide CD4 the flexibility required for its interaction with other cell surface molecules, including class II, the TCR, etc.

Crystallographic and functional studies have suggested that CD4 may dimerize, although biochemical evidence is lacking. To investigate the CD4 dimerization issue both human and mouse CD4 WT were co-expressed in 3DT52.5.8 cells. Surprisingly this led to a severe disruption of CD4 functions, although it has been shown that both human and mouse CD4 molecules are capable of interacting with human class II efficiently. As expected, co-expression of h-CD4 WT with class II-interaction-deficient CD4 mutants within the CDR1, CDR3 and the FG loop did not rescue CD4 functions. However, co-expression of CD4 WT with mutants from the CDR2 region resulted in an enhanced response. This result suggests that CDR2 mutants do not dimerize with WT molecule, therefore cannot behave as a dominant negative mutant, which is not the case for class II-interaction-deficient mutants from the CDR1, CDR3 and FG loop. Based on these results we suggest a model whereby dimerization involves, at least in part the CDR2 region. Final confirmation of this model awaits further structural data.

RESUME

Les cellules T $CD4^+$ reconnaissent les antigènes présentés dans le contexte de molécules classe II. Cette interaction est médiée par le contact de CD4 avec les régions non-polymophiques du class II. CD4 accroît l'activation des cellules T en agissant soit comme une molécule d'adhésion (fonction de co-ligand), soit en rapprochant la $p56^{lck}$ associée à la CD4, au récepteur de cellules T (fonction de co-récepteur).

Afin de disséquer les interactions moléculaires impliquées dans la(les) fonction(s) de CD4, des molécules de type sauvage ou mutantes furent exprimées dans des hybridomes T 3DT52.5.8 qui sont CD4-dépendants. Les résultats démontrent que plusieurs sites présents dans les régions CDR1 et CDR3 ainsi que dans la boucle FG de CD4 sont impliqués dans l'interaction avec la classe II. La région CDR2 du côté opposé de la CD4 joue aussi un rôle soit comme un autre site d'interaction avec classe II ou dans une autre fonction de CD4. La fonction de co-récepteur requiert un plus grand site sur CD4. Une séquence de 15 acides aminés reliant les régions D2 et D3 de CD4 apparaît comme très importante pour le maintient de la conformation de CD4 afin de donner à CD4 la flexibilité qu'elle requiert pour interagir avec d'autre molécules de surface.

Des études fonctionnelles et crystallographiques ont montré que la molécule CD4 était capable de former des dimères, par contre aucune évidence biochimique ne le démontre. Afin d'étudier la formation de dimères CD4, les molécules de type sauvage humaines et murines ont été co-exprimées dans des cellules 3DT 52.5.8. Ceci a mené à une désorganisation des fonctions de la molécules CD4. Par contre, it a été montré que les molécules CD4 humaines et murines sont capables d'interagir avec les molécules du Classe II du CMH de façon efficace. La co-expression de molécules CD4 humaines de type sauvage et mutées dans les régions CDR1, CDR3 ainsi que dans la boucle FG n'ont pas restauré les fonctions résultant normalement de l'interaction CD4-Classe II. D'autre part, la co-expression de CD4 sauvage avec des mutants CDR2 résulte en une

augmentation de la réponse. Ce résultat suggère que les CD4 mutés dans la région CDR2 ne dimérisent pas avec la molécule de type sauvage, et par conséquent ne peuvent agir comme mutant négatif dominant. Ceci n'est pas le cas pour les CD4 mutés dans les régions CDR1, CDR3 ainsi que dans la boucle FG qui n'interagissent pas. Nos résultats suggèrent une formation de dimères impliquant en partie la région CDR2. Afin de confirmer ce modèle d'autres études doivent être envisagées.

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Lists of Abbreviations

Ab: Antibody

Ag: Antigen

APC: Antigen presenting cells

CDR1: Complementary Determining Region 1

CDR2: Complementary Determining Region 2

CDR3: Complementary Determining Region 3

D1: Domain 1

D2: Domain 2

D3: Domain 3

D4: Domain 4

Del: deletion

HIV: Human immunodeficiency virus

Ig: Immunoglobulin

IL-2: Interleukin 2

mAb: Monoclonal antibody

MHC: Major Histocompatibility Complex

SEB: Staphylococcal enterotoxin B

TCR: T cell receptor

WT: wild type

Preface

The Guidelines concerning thesis preparation of the Faculty of Graduate Studies and Research of McGill University read as follows:

> Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.
>
> If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory**. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.
>
> The thesis must still conform to all other requirements of the "Guidelines for thesis preparation". **The thesis must include**: a table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.
>
> Additional material must be provided where appropriate (e. G. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.
>
> In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to

make perfectly clear the responsibilities of all the authors of the co-authored papers.

My thesis is presented according to the option mentioned above and consists of 5 chapters. Chapter 1 is the general introduction and overall reviews of background literature. Chapter 2 is a paper that has been accepted by Journal of Immunology. Chapter 3 is a paper which was published in Journal of Immunology. Chapter 4 is a manuscript in preparation. Chapter 5 is the general discussion and conclusion of the thesis. Each chapters contains a reference for the reader's convenience. For each manuscript an abstract, introduction, materials and methods, results, discussion and references are included.

The contributions of each co-authors in all the manuscripts presented in the thesis are as followings:

1. B. Huang, S. Fleury, A. Yachou, W. A. Hendrickson and R-P. Sekaly. Analysis of the contact sites on the CD4 molecule with class II MHC molecule: co-ligand vs co-receptor. accepted by Journal of Immunology, 1996. (Chapter 2)

The CD4 mutants used in this study were derived by a collaborative efforts among the first three authors: the CD4 mutant constructs were obtained from different sources or generated by A. Yachou. I transfected some of the D1 and all the hinge region mutants. I completed part of the co-ligand assay (for some of the D1 mutants, all the D2 and the hinge region mutants); all the co-receptor assays; the serology and the two anti-TCR stimulation assays for all the D1, D2 and hinge region mutants. S. Fleury made transfection for most of the D1 mutants, A. Yachou made transfection of the D2 mutants, S. Fleury did part of the co-ligand assay (for most of the D1 mutants). I wrote up the manuscript which was revised by Dr. Sekaly and S. Fleury. Dr. W. A. Hendrickson is a collaborator on the crystal structure of CD4 by giving advises on CD4 mutagenesis.

2. S. Fleury, B. Huang, A. Zerbib, G. Croteau, E. O. Long and R-P. Sekaly. Mutations in Human CD4 impair the functional interaction with different human and mouse class II Isotypes and alleles. Journal of Immunology 156: 1848-1855 (1996). (Chapter 3)

The study is completed with the collaborative efforts mainly by the first two authors and Dr. Sekaly. S. Fleury completed the KJ12 stimulation; staining of the APCs; and all the D^d stimulation. I finished the staining of the human $CD4^+$ and mouse $CD4^+$ T cells; and all the SEB stimulation. A. Zerbib and G. Croteau helped to transfect some of APCs. E. O. Long is a historic collaborator. This manuscript is written up with the efforts of the first two authors and Dr. Sekaly.

3. B. Huang and R-P. Sekaly. Dimerization of CD4 is required for both the co-ligand and the co-receptor functions and the implication of the CDR2 region in D1 of CD4 in the dimerization event. Manuscript in preparation (Chapter 4).

I completed all the experiments presented in this manuscript: including transfection, serology, anti-TCR stimulation, the co-ligand and co-receptor assays. The manuscript was written by me and revised by Dr. Sekaly.

Chapter 1

General Introduction

1. Immune System

Our environment contains a large variety of infectious microbial agents- viruses, bacteria, fungi and parasites. They can cause pathological damages and even kill their host if they multiply in an uncontrolled way. However, most infections in normal individuals are of limited duration and leave little permanent damage due to the individual's immune system, which combats infectious agents.

The immune system has two functional divisions: innate immune system and adaptive immune system. Innate immunity acts as the first line of defense against infectious agents and most potential pathogens are checked before they establish an overt infection. Innate immunity is conferred by all those elements with which an individual is born and which are always present and available at very short notice to protect the individual from challenges by "foreign" invaders. However, some of the defense mechanisms do not discriminate among most foreign substances, and they are not enhanced by such exposures. The elements of the innate immune system include body surfaces, as well as internal components. The internal components include cellular and soluble factors. The cells are mainly phagocytes such as granulocytes, macrophages, microglial cells, and natural killer cells. The soluble factors are lysozyme, complement, and acute phase proteins of the inflammation.

If the first defenses are breached, the adaptive immune system is activated and produces a specific reaction to each infectious agent which normally eradicates that agent. It is induced by antigen and gives rise to long term protection of diseases. The components of adaptive immunity are mainly lymphocytes and antibodies. Lymphocytes are activated by antigen to give rise to clones of antigen-specific cells that mediate adaptive immunity; this is called clonal selection. Clonal selection is the single most important principle in adaptive immunity. It occurs both for B lymphocytes, which proliferate and mature into antibody-producing cell; and for T lymphocytes, which are involved in the recognition and

destruction of virally infected cells. The other characteristics of adaptive immunity besides specificity include: adaptiveness, discrimination between self and non-self, memory.

1.1 Cells of Immune System

1.11 Effector Cells: T and B Lymphocytes

It is now firmly established that there are two types of cells involved in adaptive immunity: effector cells and accessory cells. Effector cells include two types of lymphocytes that come from a common lymphoid precursor cell but differentiate along different developmental pathways. One matures in the thymus so it is called T lymphocyte while the other matures in the bone marrow in mammals or in the bursa of Fabricius in birds and is thus called B lymphocyte. T and B cells share one of the important properties of the immune system, the specificity toward an antigen; however, they differ in many functional aspects. B lymphocytes mediate the humoral immune response by responding to an antigen (usually soluble and extracellular) through the production of antibodies while T lymphocytes mediate cellular immunity by recognizing and attacking cells infected with intracellular pathogens through the release of lymphokines. Besides, T lymphocytes also help B lymphocytes to make antibodies.

One of the first ways to differentiate human T cells from B cells was by determining the ability of T cells to bind to sheep erythrocytes through the CD2 molecule. However the definitive T cell marker is the T cell antigen receptor (TCR). There are two types of the TCRs: the $\alpha\beta$ TCR and the $\gamma\delta$ TCR. Both receptors are associated with a complex of polypeptides called the CD3 complex. Approximately 95% of T cells express the $\alpha\beta$ TCR and 5% express the $\gamma\delta$ TCR. (The $\alpha\beta$ TCR, the $\gamma\delta$ TCR and the CD3 complex will be discussed in details in 3.11 and 3.12). The $\alpha\beta$ TCR bearing T cells can be further divided into two distinct populations: the $CD4^+$ sub-population which are mainly T helper cells, and the $CD8^+$ subset which are mainly cytotoxic T cells.

T cells act on cells containing foreign proteins which are usually produced by pathogens that infect the cells. They do so by means of the TCR which recognize antigen not in its intact form (as recognized by B cells), but as a peptide fragment derived from the foreign protein and bound to specialized cell surface molecules called major histocompatibility complex molecules (MHC). $CD4^+$ T cells recognize antigen in association with MHC class II molecules; while $CD8^+$ T cells recognize antigens in association with MHC class I molecules. Class I MHC molecules are expressed on all nucleated cells in the body, and they bind to peptides derived from pathogens that replicate in their cytosol, typically viruses and some bacteria; class II MHC are expressed primarily on cells of the immune system and they bind to peptides derived from proteins degraded in intracellular vesicles. The structure and function of class I and II MHC complex will be discussed in detail in 2.1 and 2.2.

T cells destroy intracellular pathogens by killing infected cells and by activating macrophages. They also play a central part in the destruction of extracellular pathogens by activating B cells. The later one is the role of $CD4^+$ T helper cells.

B lymphocytes are defined by the presence of endogenously produced immunoglobulins (Ig-antibody). These molecules are inserted into the cell surface membrane where they act as specific antigen receptors. The majority of human peripheral blood B cells express both IgM and IgD molecules. Very few cells express surface IgG, IgA or IgE in the circulation although they are present in large numbers in specific locations in the body.

B lymphocytes are the only cells capable of producing antibodies (Ab). Resting B cells are activated to proliferate and secrete Abs upon encountering specific antigens (Ags). The specificity and recognition are provided by the membrane forms of Ab expressed on the B cell surface. Interaction of Ags (soluble and extracellular) with these membrane-bound Ig initiates B cell activation, which culminates in the development of effector cells that actively secrete Ab molecules. B cells may undergo heavy chain isotype

switching, allowing the same Ag-binding specificity to be expressed at different times as part of Ig molecules of different isotypes. As consequence, the same Ab specificity for Ag can be utilized to activate different effector function.

Ig structure:

All Igs have a common core structure of two identical light chains (about 24 kD) and two identical heavy chains (about 55-70 kD). One light chain is attached to each heavy chain, and two heavy chains are attached to each other. Both the light and heavy chains contain a series of repeating and homologous units. Each unit is about 110 amino acid residues in length, which folds independently in a common globular motif, called **immunoglobulin domain**. All Ig domains contain two layers of β-pleated sheet with three or four strands of antiparallel polypeptide chain. Many other proteins of importance in the immune system contain regions that use the same folding motif and show structural relatedness to Ig amino acid sequences. All molecules that contain this motif are members of the **Ig gene superfamily**, and all of the gene segments encoding the Ig-like domains are believed to have evolved from the same common ancestral gene. Molecules such as TCR, class I MHC, class II MHC, CD2, CD3γ, δ, ε, CD4, CD8, FcRII, etc, are members of the Ig gene superfamily.

There are two classes of light chain isotypes, κ and λ. Each light chain is folded into separate variable (V) and constant (C) domains, corresponding to the amino and carboxyl terminal halves of the polypeptide, respectively. All the members of the same Ig isotypes share amino acid sequence identity of the carboxyl terminal C region. This V and C domains are about 110 amino acids long. Most of the amino acid sequence variation among different light chains is confined to three separate locations in the V region- the hypervariable regions or complementary determining regions (CDRs). They are named as CDR1, CDR2 and CDR3 regions. Among them, CDR3 is the most variable among the three CDRs since its nucleotides at amino acid sequence results from the rearrangement of the V and J gene segments.

There are five classes of H chains: μ, γ, α, δ and ε. All heavy chain polypeptides contain a tandem series of approximately 110 amino acid sequences. These sequences are homologous to each other and all have Ig domain folding. As in the light chain, the amino terminal variable (V_H) domain displays the greatest sequence variation among heavy chains. CDR1 and CDR2 are encoded by the V_H genes while the CDR3 is derived from the rearrangement of the V, D and J segments. The reminder of the heavy chain, which forms the constant region, differs among different Ig isotypes. In heavy chains, there is a nonglobular region located between the first and second constant region domains which has a random and flexible conformation, permitting the molecular motion between C_H1 and C_H2. This region is called the **hinge**.

The basic pattern of chain association in all Ab molecules is that each light chain is attached to a heavy chain, and each heavy chain pairs with another heavy chain. The associations involve both covalent and noncovalent interactions. Covalent interactions are in the form of disulfide bonds between cysteine residues. Non-covalent interactions arise primarily from hydrophobic interactions. The association of the C_L and the C_H1 brings the V_L and V_H domains into spatial apposition such that the juxtaposed V domains can each contribute to the binding of antigen.

1.12 Accessory cells

Lymphocytes are the cells that specifically recognize and respond to foreign Ags. However, both the cognitive and activation phases of immune responses depend on non-lymphoid cells, called **accessory cells**, which are not specific for different Ags.

The accessory cells are mainly cells from the mononuclear phagocyte system. They originate from the bone marrow, and as they enter the blood they become monocytes. Once they settle in tissues, they are called macrophages. Their principal functions are to phagocytose foreign particles and to produce cytokines. Besides, they also play an

important role in the cognitive, activation and effector phases of specific immune responses. Macrophages can display foreign Ags in association with self major histocompatibility complex molecules that can be recognized by Ag-specific T cells. This function is called Ag presentation and cells capable of doing so are called Ag-presenting cells.

2. Class I and Class II Major Histocompatibility Complex Molecules

The major histocompatibility complex (MHC) is a region of highly polymorphic genes whose products are expressed on the surfaces of a variety of cells. The MHC locus was discovered as the result of intraspecies tissue-grafting experiments. The genetic elements controlling immune responses were then found to lie in the same region as the major histocompatibility loci, through studies in imbred mice. It is now clear that the MHC-encoded class I and class II membrane glycoproteins are involved in antigen presentation for the T cell recognition.

2.1 Class I MHC Molecule

The MHC class I molecule is a polymorphic type I membrane glycoprotein that is expressed on all the nucleated cell surface, although the expression varies widely and is typically highest on hematopietic cells. Class I MHC molecules play critical roles in tissue grafting and in immune responses to viruses and to neoplastically transformed cells by presenting antigens to $CD8^+$ cytotoxic T cells. There are several class I genes in human (HLA-A, -B and -C) and in mice (H-2K, -D and -L) that encode for the MHC class I proteins. These class I proteins are found to be associated with the non-MHC encoded β2-microglobulin (β2-m) on the cell surface.

2.11 Structure of MHC Class I Molecule

MHC class I molecule is a heterodimer of a 45 kD heavy chain, noncovalently associated with a 12 kD soluble subunit, the β2-microglobulin. The heavy chain consists of three extracellular domains, α1, α2 and α3, a transmembrane region and a cytoplasmic tail at the carboxyl terminus. The polymorphic domains α1 and α2 associate intimately to form a peptide-binding groove, while the non-polymorphic α3 domain binds to CD8. β2-microglobulin is a single, compact immunoglobulin-like domain that lacks a membrane anchor. It could either be associated with the class I heavy chain, or be a monomer in the plasma and tissue fluid. The α3 domain of heavy chain and β2-microglobulin show amino-acid sequence similarities to immunoglobulin constant regions and have a similar folded structure.

High resolution X-ray crystallographic structures have been derived for three human class I molecules (HLA-A2, HLA--A68, and HLA-B27) and for the mouse H-2K^b molecule. In all these structures it has been demonstrated all that the α1 and α2 domains form a single peptide binding groove. The peptide appears to be an integral part of class I, since empty class I molecules are thought to be rather unstable. The peptide, which is generally eight to eleven amino acids long, is tightly bound in the groove in an extended configuration (Madden et al., 1991; Fremont et al., 1992). The majority of peptides are generated from proteins located in the nucleus or in the cytoplasm, after they have been degraded in the cytosolic compartment.

2.12 Interaction of Class I MHC Molecules with CD8

MHC class I molecules bind peptide antigens and present them to T lymphocytes expressing CD8. Antibody blocking experiments (Swain, S. L. 1983) and gene transfection studies (Dembic et al., 1987; Gabert et al., 1987) indicate that CD8 binds to a determinant

on class I molecules on the target cells, thereby facilitating the interaction between effector T lymphocytes and the target cells. Later, studies using a substitution mutant at residue 227 of class I H-2D^d molecule showed that this mutation abrogated CD8-dependent but not CD8-independent cytotoxic T cell recognition (Potter et al., 1989), suggesting the α3 domain of class I being the binding site for CD8. This hypothesis was substantiated by an extensive mutagenesis study involving 48 point mutation mutants of class I HLA-A2.1 (Salter et al., 1990). This study showed that three clusters of α3 residues of class I MHC contribute to the binding, and a negatively charged loop (residues 223-229) play a dominant role in the binding to CD8 α chain. It also demonstrated that class I molecules are not just an inert structural support for the antigen recognition, the interaction of class I/CD8 is also important for the antigen recognition by T cells. This provided support for the coreceptor model of CD8 in which a simultaneous interaction of CD8 and TCR with a Ag/class I complex is critical for T cell activation. Furthermore, in vivo studies in transgenic mice carrying mutations in the α3 domain of class I also demonstrated that these mutations affect the positive selection of class I-restricted CD8$^+$ T cells (Aldrich et al., 1991; Ingold et al., 1991).

The β2-microglobulin (β2-m) has a similar structure to the α3 domain of class I (Bjorkman et al., 1987) and may also contribute to the contact with CD8; however, no data addresses this question as β2-m has been invariant on all the class I molecules.

2.2 Class II MHC Molecules

MHC class II molecules are type I heterodimeric integral membrane proteins. Each dimer consists of one α chain (33kD) and one β chain (29kD) in noncovalent association. Class II is expressed primarily on B cells, macrophages, monocytes, dendritic cells and endothelial cells; except under the influence of the cytokine γ-interferon, which induces class II expression on diverse cell types.

2.21 Crystal structure of class II HLA-DR1

The crystal structure of the class II HLA-DR1 molecule has been solved (Brown et al., 1993). It shows a great structural similarity to MHC class I molecules. The two α chain domains, α1 and α2, of DR1 superimpose closely on the α1 domain and β2-microglobulin of class I, respectively. The two β chain domains, β1 and β2 of DR1 superimpose on the α2 and less closely on the α3 domains of class I, respectively. The peptide-binding groove of class II is formed by the α1 and β1 domains

The peptide-MHC interaction between class I and class II molecules are different. The class I peptide-binding pocket is blocked at either end thus might impose severe restrictions on the peptide it can bind (8-10 residues). Peptides longer than that have to bulge out in the middle. However, this is not the case for class II. The class II peptide binding groove is formed by the close association of the α1 and β1 domains and it allows peptide to protrude from it, consequently, longer (average 15-18 residue) peptides can bind and there is no need for bulges.

The major surprise in the crystal structure of HLA DR1 comes from that in all the three crystal forms, DR1 heterodimer occurs as **a dimer of the αβ heterodimers** (**superdimer**). The superdimer contains two parallel class II **αβ heterodimers** that it would allow simultaneous interaction with two CD4 and TCR complexes. Dimerization (or even oligomerization) of cell surface receptors induced by ligand binding often activates the receptor and send signal into the cell. This is a general mechanism of signal transduction (Ullrich and Schlessinger, 1990; Heldin, 1995). Given that, it has been proposed that dimerization of class II is relevant to T cell activation, the TCR will aid the formation of the superdimer (Brown et al., 1993; Germain, 1993). However, only class II molecules loaded with the same peptide can be induced to dimerize via interaction with the TCR. Such superdimer would not exist in large concentrations in the absence of a suitable TCR. However, it is not hard to deduce that the dimerization of class II molecules on APC will induce dimerization of TCR on T cell surface (Germain, 1994).

2.22 Interaction of Class II MHC Molecules with CD4

MHC class II molecule binds to its physiological ligand -the CD4 molecule on the T cells and this interaction enhances the T cell response. Studies mapping the CD4 interaction sites on class II MHC molecules have been carried out. Antibody blocking experiments as well as cell adhesion assays have indicated that CD4 binds to the non-polymorphic determinants of class II molecules (Doyle et al., 1987; Gay et al., 1987; Clayton et al., 1989). However, direct evidence for the class II / CD4 interaction came from the competition experiment in which class II derived peptides compete with soluble recombinant class II for the binding of CD4, and mutagenesis studies on class II (Cammatota et al., 1992; Konig et al., 1992). These studies identified a region analogous to the class I α3 domain (the CD8 binding site)- the exposed region containing highly conserved residues 137-143 in the β2 domain of human class II is critical for binding with both mouse and human CD4.

Besides the conserved β2 region 137-143, additional site(s) in the β2 domain that may modulate CD4/class II MHC interaction has been suggested (Fleury et al., 1995). This study involved stimulation of 3DT52.5.8 cells with alloantigen co-expressed with different human class II DR alleles and isotypes which share a common α chain. As the results show there is a hierarchy of these class II molecules to enhance the T cell response, it lead to the suggestion that differences outside the β2 segment may be responsible for it.

The α2 domain is structurally homologous to the β2 domain, it is relatively non-polymorphic and consists of typical Ig-like domain that are frequently involved in receptor-ligand interactions between hematopoietic cells. Approaches have then been carried out to investigate if α2 domain plays a role in the CD4/class II interaction. This study have led to the identification of a second CD4 binding site on class II, which encompasses residues 125-131 (Konig et al., 1995). This site is positioned in a single class II αβ heterodimers in such a way that it cannot simultaneously interact with the same CD4 molecule which binds

to the β2 site. The ability of mutations at either the α2 or the β2 site to diminish CD4 function indicates that specifically organized CD4 and/or MHC class II oligomers play a critical role in receptor-dependent T cell activation (Konig et al., 1995).

2.23 Superantigen Presentation by Class II MHC Molecules

Superantigens are a group of T cell mitogens produced by a variety of bacteria and viruses (Herman et al., 1991; Irwin et al., 1993). They bind to class II MHC molecules and stimulate a large number of T cells in a Vβ-specific manner. Members of the superantigen family include toxins from Staphylococcal Aureus and other bacteria (Micusan et al., 1993), as well as viral superantigens from mouse mammary tumor virus (MMTV) (Marrack et al., 1993). The mechanism by which superantigens stimulate T cells differs from that of normal antigens. Conventional T cell antigens are short proteolytic peptides from foreign proteins, bound in the peptide-binding groove of class I or class II MHC molecules. In contrast, bacterial superantigen activity is abolished by proteolysis. It is the intact superantigen protein that interacts with class II MHC molecules outside the peptide-binding groove in order to stimulate T cells (Jorgensen et al., 1992; Dellabona et al., 1990). The interaction of conventional peptide antigens and superantigens with the TCR also differs. TCR molecules are structurally related to Ig molecules, with hypervariable regions forming a combining site for a specific peptide-MHC complex. Superantigen bypass this specificity-determining region of the TCR, and interacts with a surface of the TCR predicted to lie outside the Ag-binding site on the variable β-chain domain (Vβ), the so-called CDR4 region (Choi et al., 1990; Cazenave et al,, 1990; Pullen et al,, 1990; Pullen et al., 1991; White et al., 1993). This leads to the stimulation of much larger frequency of T cells (100-1000 fold) than observed with normal peptide Ags.

Recently, the three-dimensional structure of a human class II molecule HLA-DR1 complexed with the bacterial superantigen staphylococcal enterotoxin B (SEB) has been determined (Jardetzky et al., 1994). This study confirms that SEB binds as an intact protein outside the conventional peptide Ag binding site of the class II MHC molecule. No

large conformational changes occur upon complex formation in either the DR1 or the SEB molecules. The structure of the complex helps to explain how different class II molecules and sAgs associate and suggests a model for ternary complex formation with the TCR, in which unconventional TCR-MHC contacts are possible.

From the co-crystal of DR1-SEB, we know that SEB only contacts residues of the α1 domain of DR1, interacting with amino acid from the first and third turns of the β-pleated sheet and from the N-terminal region of the α helix. These residues form a deep, concave surface to one side of the peptide-binding site of DR1. Mutational studies from Dr. Sekaly's lab have confirmed that SEB binding site are located in the α1 domain of DR1. Residues implicated are 13, 36, 39, and 163 (Thibodeau et al., 1994). From the co-crystal of DR1-SEB, what is clear is that there is still room for CD4 to bind the class II molecule, because CD4 binds to the non-polymorphic region of class II, namely, the β2 and the α2 domains of class II.

3. T Lymphocytes

As mentioned before, T lymphocytes arise from the bone marrow and then migrate to and mature in the thymus. T lymphocytes are further divided into two functionally distinct populations, the helper T cells and the cytotoxic T cells. The helper T cells are usually $CD4^+$ and recognize Ag in association with class II MHC molecules; whereas cytotoxic T cells are usually $CD8^+$ and recognize Ag in association with class I MHC molecules.

3.11 T Cell Receptor: TCR αβ and TCRγδ

The T cell receptor complex (TCR) is a multi-component signaling machine that is composed of the products of six genes, all of which are required for efficient plasma membrane expression. The main components of TCR are the hypervariable αβ or γδ

heterodimer that confers the T cell specificity, and its attendant invariant chains CD3 γ, δ, and ε and the ζζ or the ζη (Figure 1). The αβ TCR and γδ TCR differ in cell surface expression, maturation and functional roles. The TCR αβ is expressed on the majority of T cells, while the TCR γδ is expressed on a small subset of T cells which belongs mostly to the $CD4^-CD8^-$ lineage. Only the αβ TCR will be discussed in this thesis.

The α and β chains are polypeptides which are members of the Ig gene superfamily and are linked to each other by disulfide bonds. Together, the α and β chains form the ligand-binding subunit responsible for the recognition of an antigenic peptide bound to an MHC molecule. The α chain is a 40-50 kD acidic glycoprotein, and the β chain is a 40-45 kD uncharged basic glycoprotein. There are striking structural similarities between the α and β chains of the TCR and Ig. Both the α and β chains have a membrane-distal amino-terminal variable domain with homology to Ig V region, a constant domain with homology to Ig C region, and a short hinge-like domain with cysteine residues which form the interchain disulfide bond. Each chain spans the lipid bilayer with a hydrophobic transmembrane domain of positively charged residues. These positively charged residues play an important role in the interaction of the TCR with the negatively charged residues of the CD3 complex. Finally, each chain ends with a short, charged cytoplasmic domain of five amino acids, it is quite unlikely that they could be involved in the transduction of extracellular binding events directly to the intracellular signaling machinery.

The V-like region of the β chain comprises V-, D- and J-like elements, while that of the α chain comprises V- and J-like elements only. Like Ig, the TCR α and β chains are rearranged in a similar manner. The variability of the CDR1 and CDR2 regions is limited to that of the germline V gene segments. The TCR diversity is focused in the CDR3

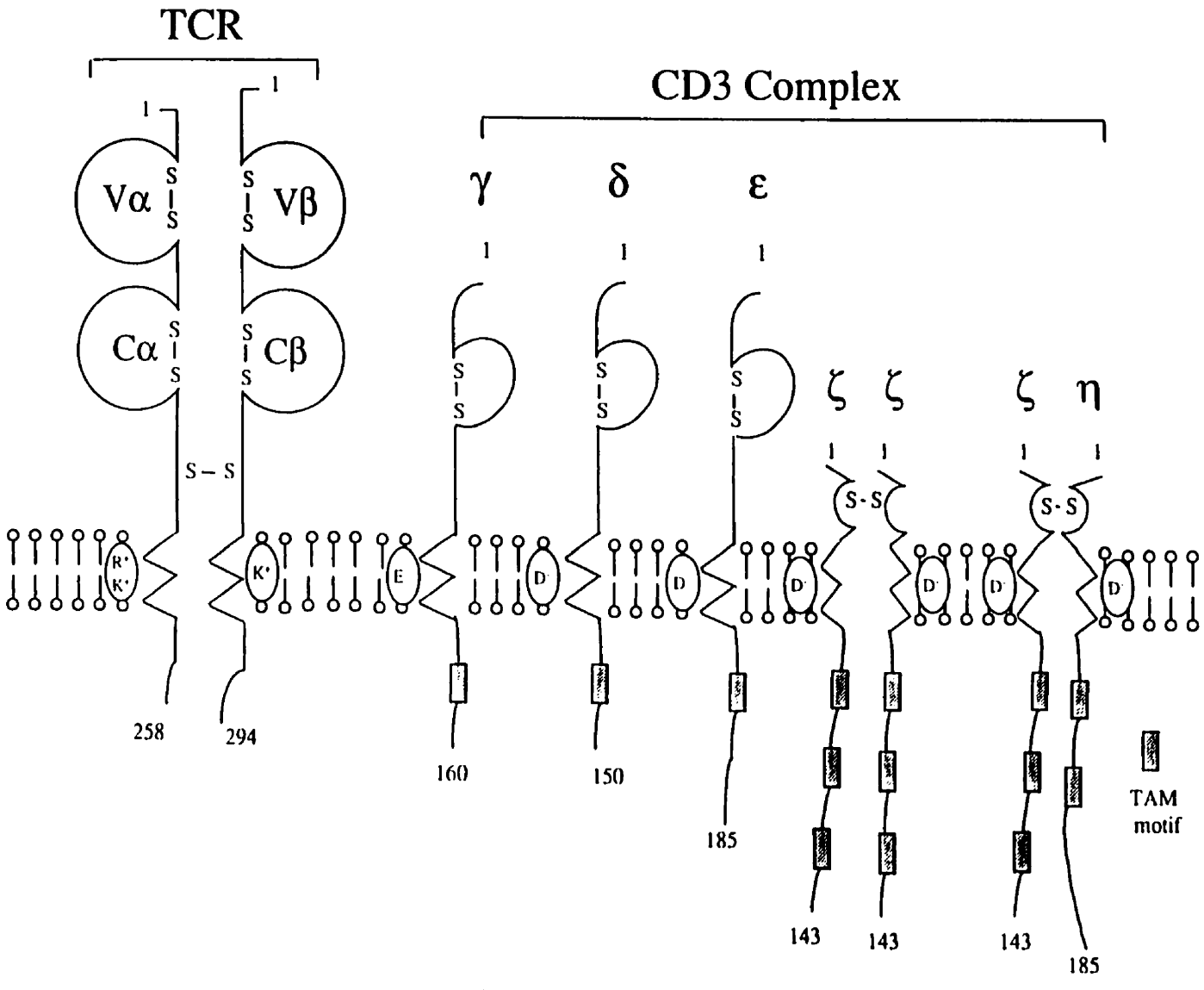

Figure 1. The TCR/CD3 complex and the ARAM Motif.

region, which is generated by the junction diversity and the presence of N nucleotides. The CDR3 will form the center of antigen binding site, while the CDR1 and CDR2 will form the periphery (Jogensen et al., 1992). In addition to the three CDR regions, another hypervariable region has been identified in the V-like domain of TCR which lies between the sequence 65-75. This region is the so-called CDR4 region. It is important for the superantigen binding, however, not important for the conventional peptide antigens (Choi et al., 1990; Cazenave et al., 1990; Pullen et al., 1990; Pullen et al., 1991; White et al., 1993).

3.12 The CD3 Complex

The TCRαβ heterodimer provides T cells the ability to recognize Ag-MHC complexes, but both their cell surface expression and function in activating T cells are dependent on a group of associated proteins that form the CD3 complex. In fact, the CD3 complex and ζ chain are responsible for coupling the TCR subunit to the intracellular signal machinery. The CD3 complex consists of at least five distinct type I integral membrane proteins that non-covalently associate with one another and with the TCRαβ (or TCR γδ) heterodimer. These five distinct members have been defined in both human and mouse. They include a 25-28 kD glycosylated γ chain, a 20 kD glycosylated δ chain, a 20 kD glycosylated ε chain, a 16 kD non-glycosylated ζ chain and a 21 kD non-glycosylated η chain. The CD3 γ, δ and ε chains exist as monomers in the TCR/CD3 complex, while the ζ chain is expressed as a homodimers in 90% of TCR, and as ζη heterodimers in the remaining 10% of TCR.

The CD3 γ, δ and ε chain genes are highly homologous to each other. Their proteins are members of the Ig gene superfamily, and all have an NH2-terminal extracellular Ig-like domain, a transmembrane segment and a cytoplasmic tail. The lack of variability or polymorphism identified in the extracellular domain of the CD3 γ, δ and ε proteins or their genes, suggests that these portions do not contribute to the specificity of

Ag recognition of the T cells. The transmembrane segments of the three chains all have negatively charged aspartic acid residue. This is important for the physical association or functional interaction with the TCR α and β chains, since the latter chains both contain a positively charged lysine in the transmembrane region. The cytoplasmic tail of the three chains ranging from 44 to 81 amino acids in length, are of sufficient size to transduce signals to the cell interior (reviewed in Weiss, 1993; Malissen and Schmitt-Verhulst, 1993; Chan et al., 1994).

The structure of the ζ and η chains are similar to each other, but unrelated to the γ, δ and ε chains. In fact, η chain is the alternatively spliced product of ζ chain. They are members of another distinct family which also includes the γ chain of the high affinity IgE receptor (FcεRγ) (Weiss, 1991). Both ζ and η chains have identical amino acids in their extracellular and transmembrane domains, but differ in their cytoplasmic tails. The extracellular domains are short (9 amino acids), the transmembrane regions contain a negatively charged aspartic acid residues (similar to that of γ, δ and ε chains), and the cytoplasmic tails are long (113 and 155 amino acids for ζ and η chains, respectively). Both chains have multiple possible sites for tyrosine phosphorylation in their cytoplasmic tail (reviewed in Weiss, 1993; Malissen and Schmitt-Verhulst 1993; Chan et al., 1994).

The signal transduction functions of the invariant non-antigen-binding chains of the TCR complex were revealed by studies with mutant cell lines (Wegener et al., 1992) or with chimeric receptors. Chimeric molecules containing the cytoplasmic domains of the ζ, η, or the related FcεRγ chains were fused to the extracellular and transmembrane domains of another receptor (including CD8, CD4, CD16, and the IL-2 receptor α chain) (Irving and Weiss, 1991; Letourneur and Klausner, 1991; Romeo and Seed, 1991). These chimeric receptors could be expressed independently of the TCR, and stimulation of these chimeric receptors with natural ligands or monoclonal antibodies induce the early and late signal transduction events characteristics of those observed with intact oligomeric TCR

receptors. These findings suggest that ζ or the related chains can function to couple the TCR to the intracellular transduction machinery.

Subsequent experiments suggested a similar function for the other CD3 chains. Reconstitution of TCR expression in a distinctive murine hybridoma which contains the ζ chain devoid of the cytoplasmic domain resulted in IL-2 production in response to antigen and superantigen (Hermans and Malissen, 1993). This suggests that a TCR component other than the ζ chain could provide signal transduction function. Further, a chimeric molecule incorporating the CD3 ε chain cytoplasmic domain was shown to be capable of activating a T cell hybridoma (Letourneur and Klausner, 1992). Collectively, these studies indicate that both the CD3 ε and ζ chains, though structurally distinct, appear to have redundant functions in TCR signaling. Thus, the CD3 complex is composed of at least two autonomous transduction modules, the CD3 ε and ζ chains.

The apparent redundancy of function of these invariant chains is explained by the presence of a common cytoplasmic domain sequence motif that couples these proteins to intracellular protein tyrosine kinases (PTKs). The minimal functional segment of this motif, first noted by Reth (1989), consists of paired tyrosines and leucines in the consensus sequence (D/E)XXYXXL$(X)_{6-8}$YXXL and are referred to as the Antigen Recognition Activation Motif (ARAM) (Weiss, 1993) (Figure 1). This motif has also been referred to as the tyrosine-based activation motif (TAM) (Samelson and Klausner, 1992) or the antigen receptor homology 1 (ARH1) motif (Cambier, 1992). There is one copy of the so-called ARAM motif in the cytoplasmic tail of γ, δ, and ε chains, three copies in that of ζ chain, and two copies in that of alternatively spliced η chain. This isolated ARAM can transduce TCR-mediated signal. Chimeric receptors with a cytoplasmic tail containing solely the isolated ARAM, encompassing as few as 17 amino acids, were capable of transducing signal sufficient of activation of the PTK pathway and subsequent TCR-mediated effector functions (Romeo et al., 1992; Irving et al., 1993). Thus, the minimal ARAM is able to function in coupling the TCR to cytoplasmic signaling machinery. Interestingly, this motif was also found to be present in the cytoplasmic domains of other

signaling subunits associated with hematopietic cell antigen receptors, such as the Igα and Igβ associated with membrane Ig on B cells; the β and γ chains of the FcεR on mast cells and basophils (Chan et al., 1994). In addition, this motif is found in the latent membrane protein LMP2A of Epstein-Barr virus (Rowe et al., 1990) and the envelop glycoprotein of the bovine leukemia virus (Sagata et al., 1985).

3.2 T Lymphocyte Development in the Thymus

T lymphocytes develop from bone marrow stem cells, and their progenitors migrate to the thymus where they mature. For this reason they are called thymus-dependent (T lymphocytes or T cells). In the thymus, immature T cells proliferate and differentiate, passing through a series of discrete phenotypic stages. These stages can be identified by distinctive expression patterns of various cell surface molecules, such as the TCR, the co-receptors CD4 and CD8, and other molecules that reflect the state of functional maturation. It is during their development that thymocytes undergo the gene rearrangements that produce the TCR, and the positive and negative selection that shape the mature T cell receptor repertoire. These processes depend upon interactions of the developing thymocytes with cells of the thymic microenvironment. The principal stages of thymic development are summarized in Figure 2.

The most important cell surface molecules in identifying thymocytes sub-populations are CD4, CD8 and TCR (other molecules are also important but are not to the interest of the thesis thus will not be discussed here). The earliest cell populations in the thymus do not express any of these molecules. Since these cells do not express either CD4 or CD8, they are called "**double negative**" (**DN**). (In the thymus, γδ T cells do not express CD4 or CD8, but they are a minor population. Therefore only the αβ T cells are discussed). The TCR genes for the DN cells are in germline configuration. This stage is followed by the next one at which the cells are still $CD4^-8^-$ but the rearrangement of TCRβ locus can be detected. Experiments using mice with mutations in the recombinant genes

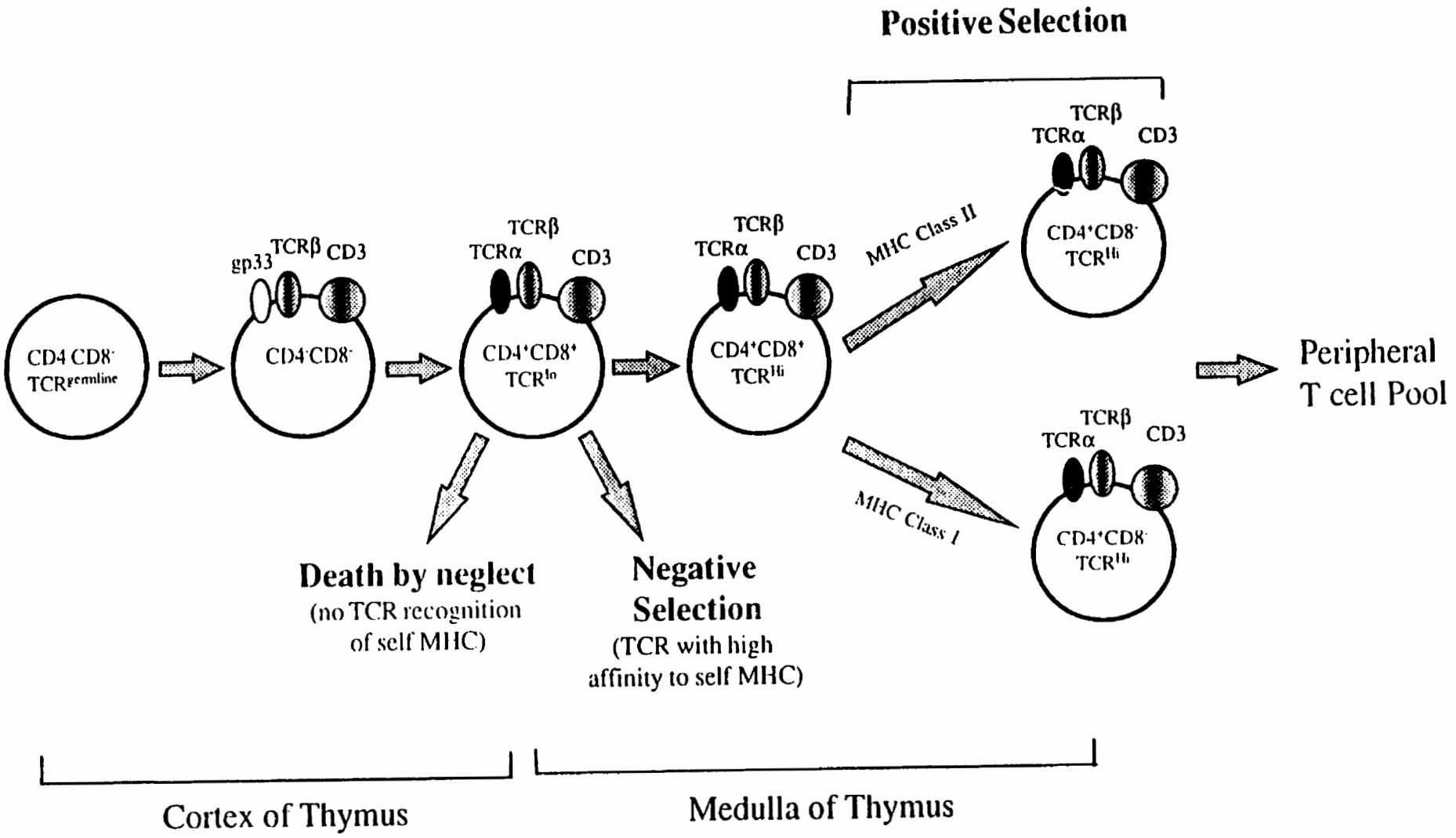

Figure 2. Thymic Selection.

(*Rag-1* or *Rag-2*) or mice with a mutated TCR β gene indicate that the TCR β gene rearrangement is critical for this early phase of T cell development. TCR β chain can be expressed on the surface of immature but not mature T cells, in disulfide linkage to a developmentally regulated protein named gp33 (Groettrup et el., 1993; Groettrup and von Boehmer, 1993a). The TCR β-gp33 heterodimer (the pre-TCR complex) is associated with the CD3 complex, and cross-linking of this pre-TCR complex results in Ca^{++} mobilization (Groettrup et el., 1992; Groettrup and von Boehmer, 1993a; 1993b). It is likely that development of the $CD4^-8^-$ thymocytes by the TCR β chain involves signaling through the CD3 complex and requires the *lck* or *lck*-related tyrosine kinase (von Boehmer, 1994). Under the physiological condition, signals may be induced by a putative intrathymic ligand that may bind to either the TCR β chain, the pg33 protein, or other noncovalently associated proteins of the pre-TCR complex. Once the signal is delivered, TCR α rearrangement may be accelerated, while the expression of the gp33 protein is terminated (Groettrup et al., 1993). Next, thymocytes begin to express CD4 and CD8 and low levels of surface TCR. Transition form the $CD4^-8^-$ to $CD4^+8^+$ (**double positive-DP**) is accompanied by a highly proliferative phase. The DP cells ultimately differentiate to mature $CD4^+$ or $CD8^+$ **single positive** (**SP**) thymocytes with high densities of TCR, during this process positive and negative selection occur. Both selections are mediated by the TCR through the interaction with the polymorphic MHC molecules expressed on the thymic epithelial cells. When the TCR on thymocytes recognizes self-MHC molecules with low affinity, thymocytes are positively selected and rescued from programmed cell death and induced to mature (**positive selection**); while the TCR recognizes self MHC with high affinity, cells are negatively selected, thus prevent the maturation of hazardous autoreactive T cells (**negative selection**). During positive selection, the class I-restricted thymocytes become $CD4^-8^+$, whereas the class II-restricted thymocytes become $CD4^+8^-$. Only a minority of the cells survive these processes and migrate to the periphery, where they provide an effective immune system able to recognize most potential pathogens yet not respond to itself.

The decision by an immature thymocyte to become either a CD4 helper T cell or CD8 killer T cell, like the decision to survive or die, is influenced by the specificity of the TCR expressed by a thymocyte. The mechanism by which thymocytes choose the CD4 or CD8 lineage, and how this is influenced by the TCR specificity is central to our understanding of thymic development. It is also critical to our understanding of the functional difference between CD4 and CD8. Now two models- Stochastic/selective model and instructive model, have been proposed for the CD4 and CD8 lineage commitment. In a **stochastic/selective model** SP T cells are generated irrespective of the T cell receptor specificity, and rescue from cell death requires co-engagement of the TCR and the matched coreceptor. That is, rescue of cells with a class I-restricted TCR from cell death requires co-engagement with CD8, and rescue of cells with a class II-restricted TCR from cell death requires co-engagement with CD4. In the **instructive model** it is postulated that co-engagement of TCR, CD8 and MHC class I on DP thymocytes leads to generation of a signal distinct from that generated by co-engagement of TCR, CD4 and MHC class II, resulting in down-regulation of the non-engaged coreceptor and selection (von Boehmer et al., 1993).

In the past few years, several groups have tested the stochastic model by constitutively expressing coreceptor and examining the effects on thymic selection. Initial experiments using the class I-restricted anti-HY TCR transgene and a constitutive CD8 transgene did not support the stochastic model, but were in favor of the instructive model (Borgulya et al., 1991; Robey et al., 1991). Recently, analyses of thymocytes commitment in class I-deficient and class II-deficient mice have identified "transitional" cells. It is consistent with the stochastic model but not the instructive model. In β2-microglobulin$^{-/-}$ mice a subset of $CD8^{hi}CD4^{lo}$ cells have been found (Chan et al., 1993; Davis et al., 1993; van Meerwijk et al., 1993), while in I-Aβ$^{-/-}$ mice some $CD4^{hi}CD8^{lo}$ cells appeared (Chan et al., 1993;. Chan et al., 1994). These provided evidences for the stochastic/selective model; however, the presence of an instructive mechanism can not be ruled out by these experiments (reviewed by von Boehmer et al, 1993; Robey & Fowlkes, 1994; Jameson et al., 1995).

3.3 T Lymphocyte Recognition of Antigens

T lymphocytes recognize antigen in the form of peptide fragments bound to the polymorphic cleft at the outer end of the MHC molecules. Mature peripheral T cells with αβ TCR express either CD4 ($CD4^+$ T cells) or CD8 ($CD8^+$ T cells). $CD4^+$ T cells recognize peptide fragments bound to MHC class II molecules, while $CD8^+$ T cells recognize peptide fragments bound to MHC class I molecules. This strong association led to the suggestion that CD4 might bind to the same MHC class II molecule which presents antigen to the TCR and serve as part of the antigen receptor, while CD8 bind to the same class I as the TCR. This is now widely believed to be the case.

3.4 Accessory and Signaling Molecules CD4 and CD8

The cell surface molecules CD4 and CD8 are known primarily for their capacity to distinguish between helper T cells and cytotoxic T cells. However, there is more to their story than just being morphological markers. On the contrary, these molecules have additional roles. They can act as an adhesion molecule by interacting with their respective MHC ligands, as well as a coreceptor by providing further signal to those generated through the TCR complex alone and are involved in the T cell activation. While CD4 and CD8 clearly have a number of similarities, they also have distinct differences.

3.41. The CD8 Molecule and its Structural Features

The CD8 molecule is present on virtually all class I-restricted T cells and contribute to activation of these cells by binding to class I proteins on the antigen presenting cells. It is expressed mainly as a disulfide-linked αβ heterodimer, with αα homodimer present as a minor species (Parnes, 1989). The CD8 α and β chains are products of two separate genes. Despite their close chromosomal location, the CD8 α and β genes are not tightly

co-regulated. CD8 α can be found transcribed in the absence of CD8 β and be expressed at the cell surface as the CD8 αα. In contrast, CD8 β polypeptide can not reach the cell surface in the absence of CD8 α chain. Furthermore, differences in the expression and function of the CD8αα and the CD8αβ dimers have been detected (Wheeler et al., 1992; Terry et al., 1990). CD8αβ heterodimers are expressed mainly on the cell surface of some thymocytes and peripheral class I-restricted T cells, while CD8αα homodimers are found on the surface of NK cells, γδ T cells and intestinal intraepithelial cells (which are suggested to be generated extrathymically).

Transfection of the CD8α chain into hybridomas with a class I MHC-restricted TCR is sufficient to reconstitute antigen recognition and to mediate binding to class I MHC, indicating that the α chain alone is sufficient for at least some CD8 functions (Dembic et al., 1987; Gabert et al., 1987). However, co-transfection of both the CD8α and β chains have been shown to broaden the specificity of recognition (Karaki et al., 1992) and to increase an allogenic response (Wheeler et al., 1992), in comparison to hybridomas bearing only the CD8α chain. These results suggest that CD8αβ may have a higher avidity for the class I molecule than the CD8αα. In addition, the almost total exclusion of surface CD8αα in favor of CD8αβ on thymocytes and class I-restricted T cells suggests that for these cells expression of the CD8β chain is important. Due to the fact that expression of the CD8β chain is dependent on the co-expression of the α chain, it has not been possible to examine the β chain's function directly. Recently, the CD8β chain knockout mice have been generated, which gave some information for the role of the CD8β chain (Nakayama et al., 1994; Itano et al., 1994; Fung-Leung et al., 1994). In these knockout mice, CD8 cells development have been affected, as only 20-30% of normal number of $CD8^+$ T cells develop, suggesting CD8α alone is inefficient for thymic development. However, those CD8 T cells which do develop in these mice have normal cytotoxic function, indicating that the CD8β chain is not essential for the cytotoxic function.

The CD8 α and β chains are members of the Ig gene superfamily. The cDNA sequences of the CD8 α (Sukhatme et al., 1985; Littman et al., 1985) and β subunits (Norment et al., 1988; Johnson et al., 1987) revealed that both of them contain an NH2-terminal domain homologous to the Ig variable domains, and a short "hinge" region connecting the Ig-like domain to a putative membrane-spanning region (Figure 3). Although the overall composition of the α and β chains is the same, their sequences are only distantly related. Alignment of the NH2-terminal Ig-like domain of the α and β chains shows that only 17% of the residues are matching, and the hinge region of the β chain is 20 amino acids shorter than that of the α chain. Intracellularly, the α and β chains are also different. The cytoplasmic region of the α chain includes a binding site (the cysteine motif CXCP) for the *src*-related tyrosine kinase $p56^{lck}$, through which the cosignalling effects of CD8 engagement are presumably mediated. The α chain can be expressed as a full length form or an alternatively spliced α' form which lacks the C-terminal cytoplasmic domain containing the $p56^{lck}$ binding site. The cytoplasmic region of the CD8β chain does not contain the cysteine motif (CXCP) which was identified in CD4 and in the CD8α chain as responsible for the association with $p56^{lck}$.

The crystal structure of the NH2-terminal 113 amino acids of the human CD8αα molecule has been resolved (Leahy et al., 1992) (Figure 4). It revealed that the N-terminal domain is homologous to Ig variable domains as expected, with 9 beta strands divided into 2 beta sheets, one sheet of 4 strands and the other of 5 (Leahy et al., 1992). This N-terminal domain of the CD8αα is associated into dimers like the variable domains of the Igs. The CC' and FG loops in the CD8αα dimer, whose counterpart in Igs help to form part of the dimer interface, are similar in both the length and function compared to that of Igs, although these loops are truncated in CD4. In CD8, as in CD4, the C'C" loop (which is homologous to the CDR2 region in Igs) is extended by several residues as compared to that of Igs. Therefore it has been speculated that this loop may be involved in a function common to CD4 and CD8, but not to Igs.

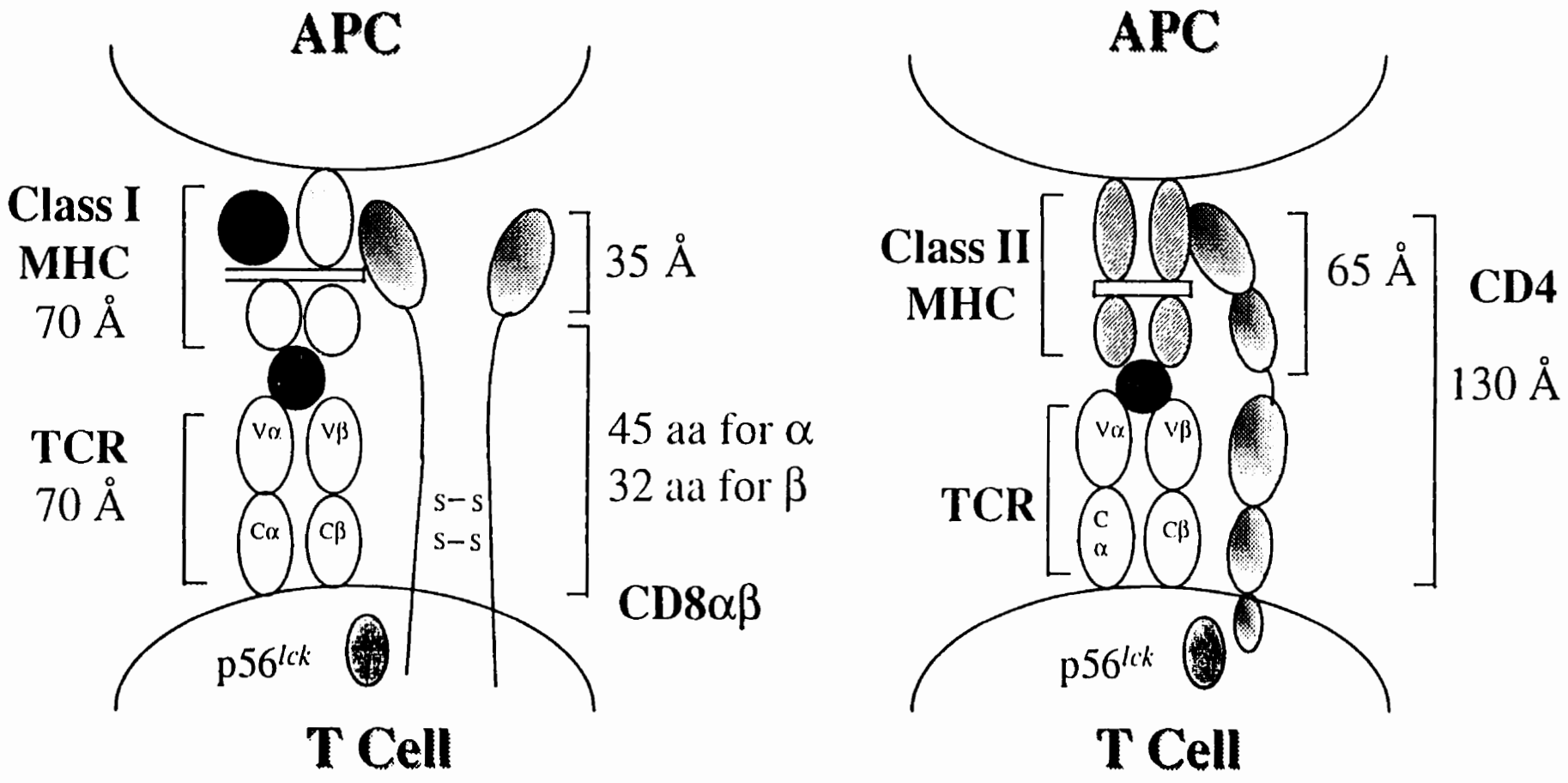

Figure 3. The Schematic Diagram of CD4 and CD8.

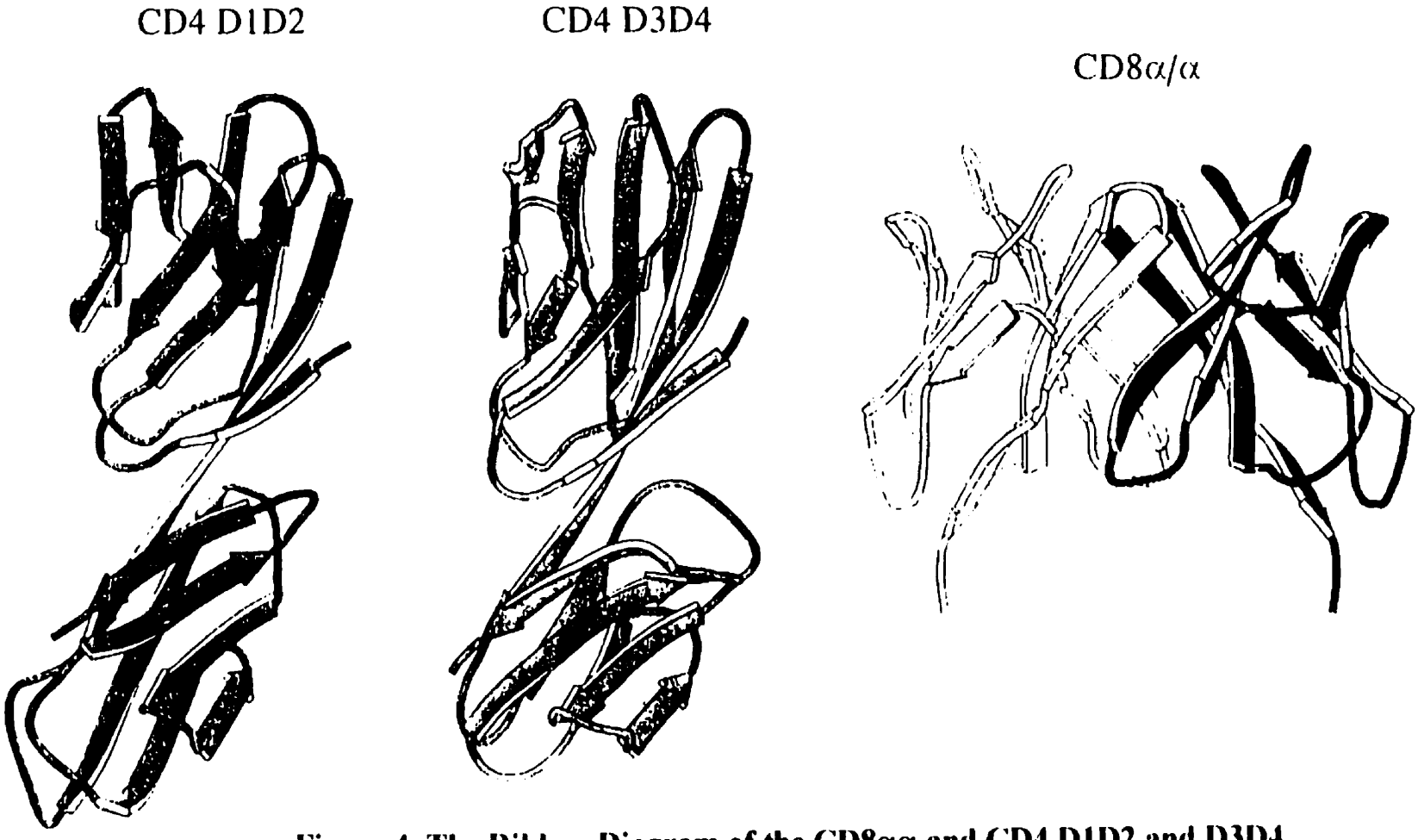

Figure 4. The Ribbon Diagram of the CD8αα and CD4 D1D2 and D3D4 (from Leahy 1995).

The hinge region connecting the NH2-terminal Ig-like domain to the transmembrane segment consists of 50 residues in the α chain and 30 residues in the β chain. The α chain hinge region has been shown to carry extensive O-linked glycosylation (Leahy et al., 1992; Classon et al., 1992; Snow et al., 1985; Pascale et al., 1992), and the β chain hinge region almost certainly share this property. Hydrodynamic and NMR studies have indicated that the hinge region lacks a regular structure (Classon et al., 1992; Boursier et al., 1993), and failure to observe electron density for the hinge region residues in the crystal is consistent with these results. In mucins the presence of O-linked glycosylation correlates with an extended structure for the polypeptide (Shogren et al., 1989). Therefore, the presence of O-linked glycosylation in the CD8 hinge region may lead to an extended structure. Such an extended structure is in fact needed for CD8 to span the length of a TCR and an MHC molecule, so that its NH2-terminal domain can interact with the α3 domain of class I. The O-linked glycosylation found in the hinge region is heavily sialated resulting in several negative charges in this region. Therefore, the purposes of the glycosylation and sialation in the hinge region could be to extend the extracellular portion of CD8 away from the negatively charged cell membrane, to modulate intermolecular interactions with other T cell membrane proteins, and to protect an extended polypeptide from proteolytic cleavage. In fact, the level of sialation on the β chain hinge region has been shown to vary with the activation state of T cells, and this modulation of glycosylation may reflect a regulated change in CD8 properties (Casabo et al., 1994). Although alterations in the hinge region glycosylation may affect the interactions of CD8 with other molecules, the lack of a rigid globular structure in this membrane proximal region makes signaling across membranes via a conformational change in CD8 seem unlikely (Leahy, 1995).

3.42 Interaction of CD8 with Its Ligand Class I MHC Molecules: Adhesion versus Co-receptor Function

The CD8 molecule is present on virtually all class I-restricted T cells. The involvement of CD8 in the class I-restricted CTL recognition was first suggested by studies showing inhibition of conjugate formation and cytolytic function by anti-CD8

antibodies (Swain, 1983) Subsequently, gene transfer experiments also confirmed it by showing that CD8 expression enhanced class I-specific T cell recognition (Dembic et al., 1987; Gabert et al., 1987). The strong correlation between CD8 expression and class I restriction of T cells suggested that CD8 might bind to non-polymorphic regions of class I proteins (Swain, 1983). Direct evidence of CD8 binding to class I was provided by studies examining purified CD8 proteins in artificial membrane vesicles (Rosenstein et al., 1989), and by demonstrating adhesion between class I-bearing B cells and CD8 α-transfected CHO cells (Norment et al., 1988).

A large body of evidences show that CD8 serves as both an adhesion molecule and a co-receptor which, in concert with the TCR, delivers activation signals to the T cells.

Adhesion Function of CD8:

Two series of experiments have directly supported the hypothesis that CD8 binds to class I MHC molecules on target cells and contribute to the enhancement of T cell-target cell adhesion. First, using an intercellular adhesion assay, Salter et al (1990) have found that human CD8αα dimers can bind an exposed loop (residues 222-229) in the α3 domain of class I MHC molecules. Importantly, these data also indicated that the TCR and CD8 molecules bind distinct sites on class I, and under these assay conditions the interaction between CD8 and class I MHC molecules may occur in the absence of the TCR. Second, Sanders et al (1991) have shown that mutations in the V-like domain of the CD8α chain interfere with the ability of $CD8^+$ cells to adhere to class I MHC bearing cells. Furthermore, by introducing CD8 molecule into T cell hybridomas in which they cannot bind to the same MHC ligand as the TCR has allowed for independent assessment of CD8-ligand and TCR-ligand interactions (Ratnofsky et al., 1987). These experiments demonstrated that CD8 can increase T cell response by binding independently of the TCR, presumbably by acting as the adhesion molecule to strengthen the overall avidity of the T cell for the APC.

Although direct binding assays demonstrated basal affinity of CD8 for class I, these systems employed high, non-physiological surface densities of CD8 and/or class I proteins. During attempts to examine the adhesion of cloned murine $CD8^+$ CTL to affinity-purified, immobilized class I proteins, it was demonstrated that there was no significant binding of $CD8^+$ CTL to irrelevant (non-antigenic) class I proteins. However, adhesion became readily detectable when the CTL were stimulated by addition of fluid-phase anti-TCR mAb (O'Rourke et al., 1990). Like the anti-TCR mAb, engagement of the TCR by an Ag can similarly up-regulate CD8-mediated adhesion of cloned CTL lines (O'Rourke et al., 1993). Thus basal affinity of CD8 for class I protein is insufficient to mediate detectable adhesion when it is expressed at the levels present on normal T cells, but engagement of the TCR activates CD8 such that the affinity of the interaction is increased. The mechanism by which TCR engagement evokes this change is not yet known, but does not appear to involve changes in the level of CD8 surface expression. Other explanations include a signaled conformational change in CD8 to alter its affinity or an avidity modulation mediated by localized clustering and/or cytoskeletal attachment of CD8, either directly or possibly via the associated $p56^{lck}$. However the signaled conformational change in CD8 is not generally accepted (Leahy, 1995).

The cosignalling role of CD8 in activating responses

A large and convincing body of literature have shown that CD8 performs a cosignalling function by acting in parallel with the TCR-CD3 complex, thus CD8 is viewed as a "co-receptor" rather than as an autonomous accessory molecule. When acting as a coreceptor, there is a physical association between CD8 and the TCR, and CD8 may primarily function complexed with the TCR. This was first suggested by observations that mAb-mediated co-clustering of the TCR with CD8 was likely synergistic for T cell activation compared with separate ligation, suggesting that proximity of CD8 with the TCR complex favored increased signaling. Therefore, signaling by TCR-CD3 complexes associated with CD8 appears far more efficient for inducing T cell activation than the signaling mediated by the TCR-CD3 complexes alone. Strong support for this came from the discovery that the cytoplasmic tail of CD8α is associated with $p56^{lck}$. Transfection of

hybridomas with full-length CD8 molecules that can associate with $p56^{lck}$, results in greater augmentation of TCR-mediated responses than does transfection with CD8 molecules that are truncated to prevent $p56^{lck}$ association.

Although it has been demonstrated that CD8 molecules can act as either adhesion or co-receptor molecule, the relative contributions of the adhesion and co-receptor roles of CD8 to generate a response is difficult to evaluate. Increased adhesion alone may result in enhanced responses by promoting interaction between the cell surfaces, thus leading to higher TCR occupancy levels: this effect is difficult to distinguish from signalling mediated directly by engaged CD8 (O'Rourke and Mescher, 1993).

In addition to the adhesion and co-receptor roles of CD8, a recent study has shown that CD8 can modulate the TCR-ligand interactions on living cytotoxic T lymphocytes (Luescher et al., 1995). Using TCR photoaffinity labeling with a soluble, monomeric photoreactive H-$2K^d$-peptide derivative complex, the authors reported that the avidity of TCR-ligand interactions on cloned cytotoxic T cells is very greatly strengthened by CD8. This enhancement can be inhibited by CD8-specific antibodies. The CD8 modulation of TCR-ligand binding can be primarily explained by coordinate binding of ligand molecules by CD8 and the TCR, because substitution on class I MHC of the CD8-binding residue Asp 227 of K^d with lysine severely impaired the TCR-ligand binding on $CD8^+$ but not on $CD8^-$ cells. Kinetic studies on $CD8^+$ and $CD8^-$ cells further showed that CD8 imposes distinct dynamics and a remarkable temperature dependence on TCR-ligand interactions. Therefore the ability of CD8 to act as coreceptor can be modulated by CD8-TCR interaction. This study is novel in that CD4 and CD8 have so far been reported not to contribute significantly to TCR-ligand binding (Matsui et al., 1991; Sykulev et al., 1994). However, similar function for CD4 in TCR-ligand binding has not been demonstrated.

In this study, a difference between CD8αα and CD8αβ in enhancing the TCR-ligand interaction has also been revealed. As the anti-CD8 α Ab barely affect the TCR-ligand binding, the anti-CD8 β Ab strongly inhibit this interaction. It seems that CD8 αβ

can support more the TCR-ligand binding than the CD8 αα. So it is tempting to speculate that CD8-class I interactions relevant for CD8 coreceptor function may involve, at least in part CD8 β. This may explain the importance of CD8 β in T cell development (which will be discussed in details in the next section) and in antigen recognition, even though CD8 β, unlike CD8 α, according to current knowledge, is not associated with any tyrosine kinase or directly involved in signaling.

Structural Features of CD8/class I MHC Interaction

Although the actual nature of the molecular interaction between CD8 and class I molecules is unclear, some important features are beginning to emerge. Mutational analyses on class I MHC have identified a conserved seven amino acid loop (residues 222-229) within the membrane proximal α3 domain as a principal CD8 binding site (Potter et al., 1989; Salter et al., 1990). On the CD8 side, Sanders et al (1991) have shown that mutations in the CDR1-like and CDR2-like loops of CD8 had the greatest effect on class I binding in a cell adhesion assay.

Now the crystal structure provides further evidence for the involvement of the CDR-like loops through analysis of electrostatic potential on CD8 surfaces (Leahy, 1992). As the CD8-binding site on class I MHC has been identified as a region of highly negative charge, a surface on CD8 with a complementary positive charge was sought. From the crystal of CD8 it is known that the only surface with a uniformly positive charge is the CDR-like loops. Involvement of this surface in interactions with class I MHC is possible, since it is oriented away from the cell membrane thus would be directed toward a ligand on another cell. In fact, employment of this surface to interact with a ligand matches the role of the homologous surfaces on Igs. Based on these assumptions, more extensive mutations in CD8 were made after the crystal structure became available. These mutations suggested that in addition to this positively charged CDR loops, an additional surface of CD8 composed primarily of residues from the A strand, the B strand was involved in binding to class I (Giblin et al., 1994). Therefore a model concerning the interaction between CD8 and class I MHC has been proposed. In this model the surface of CD8 containing the

CDR-like loops interacts with the negatively charged loop on the α3 domain of class I, whereas the A/B strand surface binds to a region on the α2 domain of class I (though the functional data suggesting a direct involvement of the α2 domain of class I in binding to CD8 is still lacking). Furthermore, this model also suggests that it is feasible for a single CD8 dimer to interact with two MHC class I molecules and raises the possibility that CD8-mediated cross-linking of MHC molecules may send signal to the antigen presenting cells (Giblin et al., 1994).

3.43 Role of CD8 in Thymic Selection

CD8 acts as a coreceptor for the mature T cell recognition of class I MHC antigens (Janeway, 1992). It also plays an important role in the thymic positive and negative selection, as indicated by antibody-blocking experiments (Zuniga-Pflucker et al., 1990) and by observations in knockout mice in which the CD8 gene is eliminated (Fung-Leung et al., 1991). Administration of anti-CD8 monoclonal antibodies *in vivo* has been shown to block the development of the CD8 single positive thymic subset (Zuniga-Pflucker et al., 1990). In addition mice homozygous for mutant CD8 α chain genes ($CD8^{-/-}$) lack cytotoxic T cells and make poor class I-restricted T cell responses (Fung-Leung et al., 1991). This is seen both at the bulk population level and in TCR transgenic systems (Fung-Leung et al, 1991; Schonrich et al., 1993; Fung-Leung et al., 1993). Crossing of transgenic TCRs into $CD8^{-/-}$ mice provided evidences that CD8 is obligatory for positive selection of MHC class I-restricted TCRs, but is differentially required for negative selection, depending on the antigen specificity of the TCR (Schonrich et al., 1993; Fung-Leung et al., 1993). Moreover, evidences have suggested that CD8 must bind to the same MHC allele as does the TCR to drive positive selection (Aldrich et al., 1991; Ingold et al., 1991), however, similar result for CD4 is still lacking though a comparable role has been postulated.

The expressions level of CD8 on cell surface also influences the thymic selection. Increasing surface expression of CD8 on thymocytes in transgenic mice has been shown to affect the outcome of thymic selection differently, depending on the T cell affinity (Robey

et al., 1991; 1992; Lee et al., 1992). A twofold increase in CD8 expression was shown to enhance positive selection of thymocytes expressing a class I-restricted TCR (Robey et al., 1991). In contrast, the same level of CD8 expression results in deletion of another different class I-restricted TCR (Robey E et al., 1992; Lee et al., 1992). The differences observed in selection of those transgenic TCRs in the presence of increased CD8 expression may be attributed to different increments in TCR avidity. This interpretation is consistent with the affinity model of thymic selection.

The cytoplasmic tail of CD8 α is associated with the tyrosine kinase $p56^{lck}$. It is believed that this association is important for the function of CD8. However, reconstitution of the CD8$^-$/ mice with a cytoplasmically truncated form of CD8 α (CD8 $\alpha^{tailless}$) demonstrated that the cytoplasmic domain is not absolutely necessary for the thymic maturation of class I-restricted T cells, but it dramatically enhances the efficiency of positive selection (Fung-Leung et al., 1993).

As there are two types of CD8 on the cell surface, CD8αα and CD8αβ, the role of the CD8β chain is less clear and somewhat more complicated than the CD8α chain. Recently, the CD8β$^-$/ mice have been generated which showed a severe deficiency in CD8$^+$ T cell positive selection, as only 20-30% of normal number of CD8$^+$ T cells developed (Nakayama et al., 1994; Crooks et al., 1994; Fung-Leung et al., 1994). These T cells do have normal cytotoxic functions. This is different from the CD8 α$^-$/ mice which lack cytotoxic T cells and make poor class I restricted T cell response. This may suggest that the CD8 β chain is important but not absolutely essential for the thymic selection and for the cytotoxic function of the CD8$^+$ T cells. However, by crossing over the CD8 β$^-$/ mice with transgenic mice expressing a class I-restricted TCR, a significant role of the CD8 β chain in both positive and negative selection has been demonstrated (Itano et al., 1994). This discrepancy in CD8 β chain dependency in thymic selection may be attributed to the TCR affinity. Though the functional role of the cytoplasmic tail of CD8 β is still unclear, transgenic mice expressing a truncated CD8 β chain have shown it can act as a

dominant negative mutant to suppress the expression of the wild-type endogenous CD8 β chain (Itano et al., 1994). When crossing over of the CD8 β tailless transgene with different class I-restricted TCR transgenes, CD8 β was shown to play a role in thymic development, and different TCRs differ in their dependence on the cytoplasmic tail of CD8 β. Up to now, no $p56^{lck}$ and other known intracellular proteins have been identified to be associated with the tail of CD8 β, however, it is reasonable to speculate that it may function through an interaction with some intracellular proteins which may have a role in signal transduction.

4. Important Role of the CD4 Molecule

4.1 The CD4 Molecule: Cellular Expression and Structural Features

The CD4 molecule is a 55 kD cell surface glycoprotein which is a member of the immunoglobulin gene superfamily. It is expressed on most thymocytes and on a subset of T lymphocytes (Reinherz et al., 1980; Biddison et al., 1982; Krensky et al., 1982; Meuer et al., 1982). It can be detected at low levels on monocytes, granulocytes, eosinophils and dendritic cells. CD4 interacts with its physiological ligand- the class II major histocompatibility complex (MHC) molecules which are expressed on the antigen presenting cells (APC). Interaction between CD4/class II MHC may transmit signal for thymocytes differentiation (Kruisbeek et al., 1985) and for T cell activation (Eichmann et al., 1987). Human CD4 is also the cellular receptor for human immunodeficiency virus (HIV) (Dalgleish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986).

The CD4 molecule consists of an extracellular portion (residue 1-372), a single transmembrane segment (373-393) and a cytoplasmic tail (394-433) at the C-terminal (Figure 5). The extracellular portion contains four distinct domains namely D1 to D4 joined in an extended rod-like structure (Maddon et al., 1985; Wang et al., 1990; Ryu et al., 1990; Brady et al., 1993). The N-terminal D1 shares extensive structural and sequence

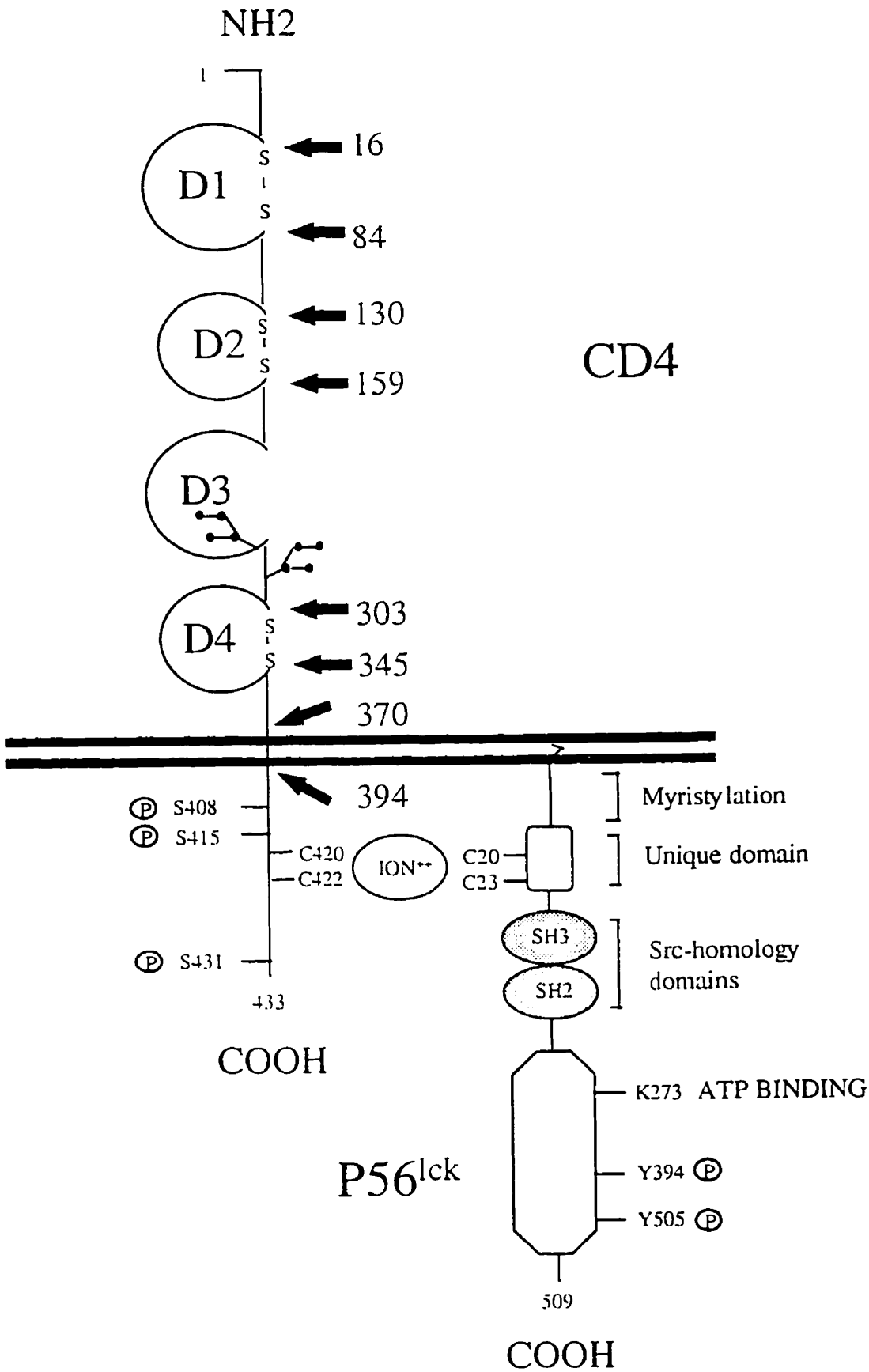

Figure 5. The Schematic Diagram of CD4 and its Association with Lck.

homology to the variable region of immunoglobulin (Ig) light chains. The other three domains are less closely related to Ig molecules at the level of primary structure but fold similarly to Ig-like domains, confirming that CD4 is a member of the Ig gene superfamily. Post-translational modifications of CD4 include the formation of disulfide bonds which stabilize the D1, D2 and D4 domains, and the addition of two N-liked glycans in D3 and D4. The intracellular portions of CD4 are extensively conserved among CD4 from different species. CD4 noncovalently associates with the tyrosine kinase $p56^{lck}$ through the double cysteine at residues 420 and 422 (Veillette et al., 1988). Association of CD4 with a class II MHC/TCR complex appears to bring this kinase into the TCR signaling pathway in the context of T cell activation.

The CD4 gene expression is under the control of a promoter and an enhancer which does not discriminate between the T cell lineages ($CD4^+$ and $CD8^+$) (Sawada et al., 1993; Killeen et al., 1993; Blum et al., 1993). This differentiation towards the $CD4^+$ T cell lineage is rather regulated by a cell type specific transcriptional silencer (Sawada et al., 1994).

4.2 Crystal Structure of CD4

When the cDNA sequence of CD4 became available, a region on the NH_2 terminus was immediately recognized as homologous to Ig variable domains (Maddon et al., 1985). Later authors detected three additional Ig-like domains in the sequence of the remaining three-quarters of the extracellular region of CD4 (Clark et al., 1987), but this observation was not universally embraced. Many laboratories began attempts to produce diffraction-quality crystals of the extracellular portion of CD4, spurred on by the finding that CD4 is the receptor for HIV. Producing diffraction-quality crystals of the entire extracellular portion of CD4 proved to be difficult, however, leading to the suspicion that a flexible structure of this molecule exists between D2 and D3 domains (Kwong et al., 1990; Davis et al., 1990). Proteolytic analyses showed that CD4 could be cleaved into stable fragments, and these fragments proved much more amenable to crystallographic studies (Hendrickson

et al., 1992; Fleury et al., 1991). In 1990, two groups, Ryu et al and Wang et al, simultaneously reported the crystal structure of the NH2-terminal half D1 and D2 domains (174 amino acids) of the human CD4 molecule (Ryu et al., 1990; Wang et al., 1990). In 1993, the crystal structure of the domains 3 and 4 of rat CD4 was reported (Brady et al., 1993).

The crystal structure of the NH2-terminal domains 1 and 2 of human CD4 revealed a larger domain D1 in close association with a smaller domain D2 (Figure 4). D1 has nine β strands, forming two β sheets. One sheet contains strands AGFCC'C'', and the other sheet contains strands BED. Although unmistakably homologous to Ig-variable region, D1 also shows some unique features. Compared to Ig, the loops connecting the CC' and FG strands (correspond to the CDR3 region of Ig) in CD4 are shortened by several residues. Importantly, in Ig and CD8 the CC' and FG loops help to make up the interface of a dimer of two Ig-like domains. However, the truncation of these loops in CD4 suggests that dimerization through these two loops are unlikely. It may also reflect the fact that under normal conditions CD4 exists as a monomer on the cell surface. In contrast, the C'C'' loop (homologous to the CDR2 region of Ig) in D1 of CD4 is unusually prominent and extended by several residues. An extended C'C'' loop is also found in CD8 (Leahy et al., 1992). Therefore it has been speculated that this loop may be involved in a function common to CD4 and CD8 but not to Igs, such as interaction with MHC molecules. However such a function has not been firmly established (Leahy, 1995). It has been known that the C'C'' loop of human CD4 is crucial for the high affinity binding of HIV glycoprotein gp120 (Capon et al., 1991), especially the prominently exposed side chain of Phe-43 just at the end of the C'C'' loop.

The D1 and D2 domains are intimately connected. The G strand of D1 (the last strand of D1) extends directly into the A strand of D2 (the first strand of D2), and a substantial interface is buried between these two domains, thus explaining the rigidity of this D1D2 fragment and its diffraction at high resolution. D1 and D2 are related by an approximate two fold screw axis, and the same relative disposition of D1 and D2 is found

in differently packed crystal forms of CD4 (Ryu et al., 1994), indicating that the D1D2 fragment is likely to form a rigid rod with little interdomain flexibility, and the dimension of this fragment is half the length estimated for soluble CD4.

The D2 domain can be described as a truncated variable domain with a number of unique features. First, D2 is smaller with only 75 residues compared to 100 or more residues in Ig domains. There are seven strands: GFCC' in one sheet and ABE in the other. Comparing to Ig, the C" and D strands are absent, and the C' strand makes a "shortcut" from C to E.

From the crystal structure of the D3D4 fragment of rat CD4, we have known that this fragment has a striking similarity to the D1D2 fragment of human CD4 (Brady et al., 1993; Lange et al 1994) (Figure 4). First, the larger D3 domain is structurally homologous to D1, and the smaller D4 domain is homologous to D2. This leads to the suggestion that the four-domain CD4 arose from the duplication of a two-domain precursor (Williams et al., 1989). In the D3 domain, there are nine β strands forming two sheets, one is AGFCC'C", the other is BED. In D4 domain, there are seven β strands forming two sheets, ABE and GFCC'. As in D1D2, the G strand of D3 extends directly into the A strand of D4, and a substantial interface is buried between D3 and D4. However, the relative orientation of D4 to D3 differs approximately 25° from the relative orientation of D2 to D1. Unlike in the case of D1, the CC' and FG loops of D3 are not shortened and are of comparable length to the homologous loops in Ig. However there are two glycosylation sites in the D3D4 fragment. One glycosylation site lies on the F strand of D3- Asn 270 which is conserved in CD4 from all species sequenced so far except from dog. Another glycosylation site is located in the AB loop of D4 which is observed in CD4 sequence from human, mouse, dog , monkey and cat, but not in rat CD4. In rat CD4, an additional glycosylation site is found to lie close to the membrane (position 367, after G strand), which is spatially similar to the site in the AB loop of CD4 from other species. The presence of the conserved N-linked glycosylation on the F strand of D3 and AB loop of D2 would spatially interfere with dimer formation or other interactions of CD4 through

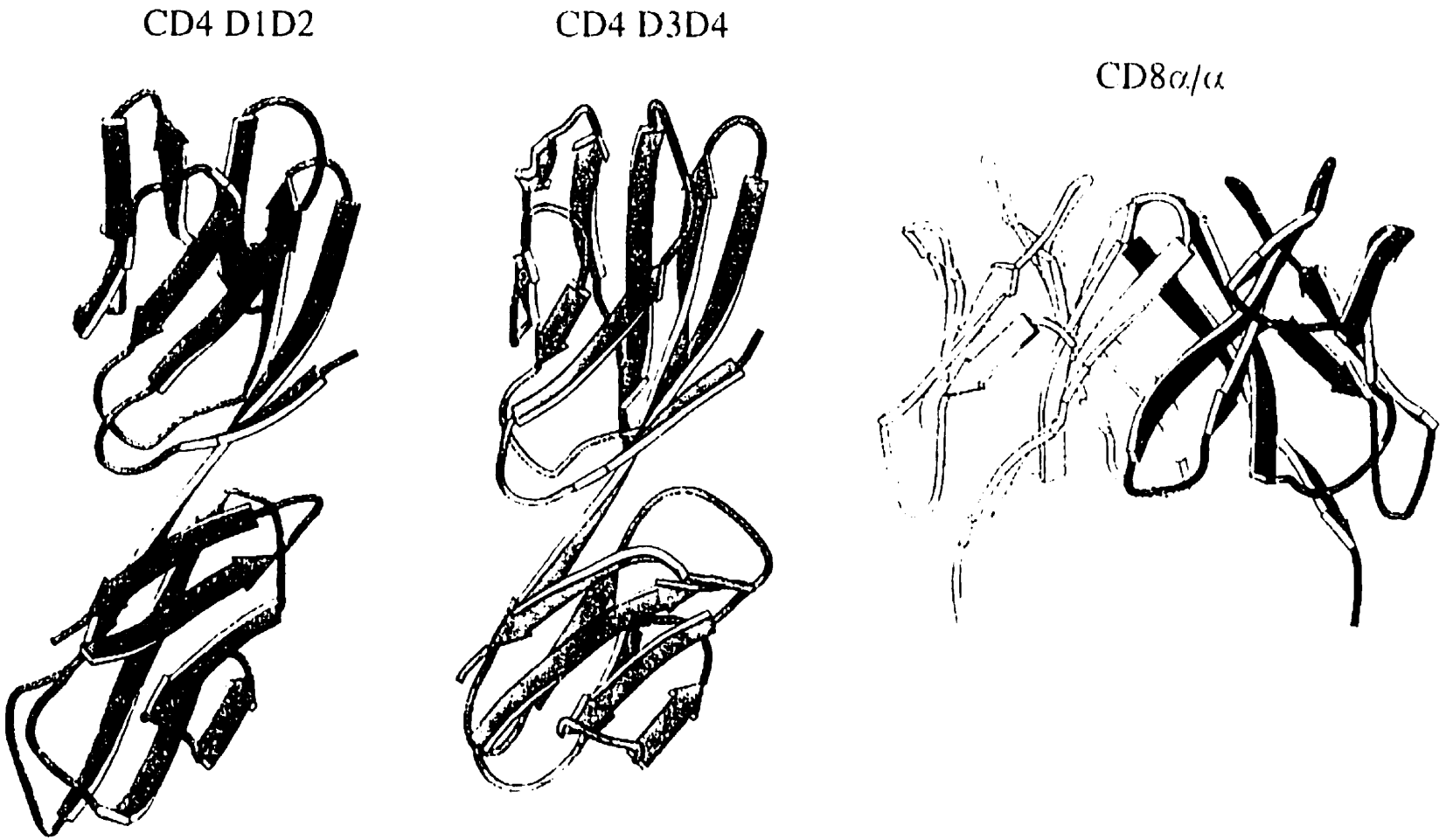

Figure 4. The Ribbon Diagram of the CD8αα and CD4 D1D2 and D3D4 (from Leahy 1995).

this face of D3. Therefore, the CC' and FG loops are unlikely to mediate dimerization of CD4 as in Igs and CD8. However, the face of CD4 free of any carbohydrates could be involved in interaction with other proteins, i. e., MHC class II and TCR.

The D1D2 and D3D4 fragments of CD4 have been crystallized independently. When putting the two crystal structures together, only three residues of unknown structure were missing which intervene between the known structure of D1D2 and D3D4 fragments. A simple model of the entire extracellular region of CD4 (sCD4) can therefore be constructed by the juxtaposition of the two structures. Evidence from hydrodynamic studies, electron micrographs, and crystallization parameters all suggest that the extracellular portion of CD4 is rod-like and extends to 120 Å in length (Kwong et al., 1990; Lange et al., 1994; Davis et al., 1990). This dimension corresponds to approximately the sum of the long axes of the D1D2 and D3D4 and suggests that CD4 may adopt an extended structure. Such an extended structure would be required for CD4 to span the length of a TCR and a class II MHC molecule to interact with its binding site on the membrane proximal α2 and β2 domain of class II MHC molecules (Figure 3). Lange et al (1994) have proposed a model in which the three residues (179-181) intervening between D1D2 and D3D4 fragments have been assumed to continue as β strand from D2 to D3.

The extracellular portion of human CD4, consisting of 370 amino acid residues and two N-linked glycosylations, can be expressed in soluble form by secretion from CHO cells. This recombinant protein could be crystallized into several forms (Kwong et al., 1990). All crystals of this intact soluble CD4 are poorly ordered with limited diffraction. The cause of poor diffraction is not understood, but it may well be associated with internal flexibility of the molecule. The juncture between D2 and D3 is particularly sensitive to proteolytic cleavage, further suggesting this juncture as a major point of segmental flexibility. Whether such a flexibility is functionally significant is still to be studied. The intersegmental flexibility is in accordant with the model proposed by Leahy (Laehy, 1995) and others (Kwong et al., 1990; Hendrickson et al., 1992), which suggests that the

interface between D2 and D3 is relatively small, consistent with the structural features of the hinge region, as that in Igs and CD8.

The study on the soluble four-domain CD4 has suggested that CD4, like CD8, might exert its biological function on all the cell surface as a dimer (Kwong et al., 1990; Ryu et al., 1990). At the high protein concentrations CD4 oligomerizes. All five distinctive forms of CD4 that were characterized have multiples of two molecules per asymmetric unit. This suggests that the extracellular part of CD4 could dimerize, although hydrodynamic studies have shown no evidence for such interactions unless they are of lower affinity than $10^5 M^{-1}$. In Igs, CC' and FG loops are normally involved in dimerization. However, in D1 domain CC' and FG loops bear a truncation, and the D1D2 fragment crystallizes as a monomer. In D3 domain, the presence of the conserved glycosylation on the F strand would spatially interfere with dimer formation of CD4 through this face of D3, thus CC' and FG loops here may not play a similar role as in the Igs and CD8. In the recently refined D3D4 crystal dimer formation through CC' and FG loops is not seen (Lange et al., 1994), though a dimeric interaction generated by the crystal lattice has previously been described (Brady et al., 1993).

Although no dimer formation in the crystal of either D1D2 or D3D4 fragments have been detected, the recurrent association of sCD4 in different crystals suggests CD4 may have a natural tendency to oligomerize. It may also imply that the hinge region, by giving flexibility to the CD4 molecule, has an effect on the self-association of CD4. It is possible that the association between CD4 occurs through interaction of the transmembrane and cytoplasmic domains or via other proteins it interacts with. This may be the reason that the dimerization is not seen in studies on the recombinant extracellular domains (Brady and Barclay, 1996). Up to now, the possible dimerization interface of CD4 is not clear from crystallization studies. However, it is probably mediated by the membrane-proximal D3 portion, as suggested by a recent study which has implicated the face opposite to the OKT4 binding site Arg 240 in D3 domain, that is, the BED face (Sakihama et al., 1995).

The propensity for CD4 to associate into dimers/oligomers could be important in transmembrane signaling (Ullrich and Schlessinger, 1990; Hendrickson et al., 1992). The physiological ligand of CD4- the class II MHC molecules DR1 has been crystallized as a dimer. Many cell surface molecules involved in receptor-ligand interaction and signal transduction are dimers in structure, or would be induced to dimerize following ligand binding. The resulted dimerization have an important impact on enhancing the signal transduction.

4.3 Functional Role of CD4 in T Cell Activation: Co-ligand versus Co-receptor

The CD4 molecule can play at least two functions during T cell activation and development: as signaling and as adhesion molecules.

Early evidence for a role of CD4 in T cell function came from studies demonstrating that TCR-mediated responses to class II-restricted antigens could be blocked by antibodies directed against CD4. Additional studies demonstrated that anti-CD4 blocked the formation of conjugates between class II-restricted T lymphocytes and their target cells. These results, combined with the correlation between CD4 expression and the MHC restriction of T cells, led to the hypothesis that CD4 may be involved in augmenting T cell recognition by binding to the monomorphic determinant on class II MHC molecules. This has been shown that CD4 binds directly to the non-polymorphic region on class II, and expression of CD4 in non-lymphoid cells rendered these cells capable of binding to cells expressing class II molecules (Gay et al 1988; Doyle et al 1987; Cammarota et al., 1992; Konig et al., 1992). Therefore, it has been postulated that CD4 provides the "accessory molecule" function of increasing the avidity of a T cell for its APC. Accordingly, mature T cells of exceptionally high affinity for antigen would be relatively independent of CD4 function; whereas lower affinity T cells would be more dependent on CD4 function to enhance the T cell response.

The expression of CD4 in T cell hybridomas where CD4 binds to an MHC ligand not directly involved in antigenic restrictions, has allowed for independent assessment of CD4-ligand and TCR-ligand interactions (Gay et al 1987; Gay et al., 1988). These experiments clearly demonstrated that CD4 can potentate the antigen specific IL-2 production by binding independently of the TCR to MHC proteins on the APC, presumably by acting as adhesion molecules to strengthen the overall avidity of the T cell for its APC.

Moreover, a growing body of evidence have suggested that there is a physical association between the TCR and CD4 (Gallagher et al., 1989; Anderson et al., 1988; Rojo et al., 1989), and that CD4 may primarily function by complexing with the TCR. When using sub-optimal stimuli to activate T cells, cross-linking of CD4 to the TCR greatly enhance activation of T cells by anti-TCR antibodies. Moreover, when specific antigen recognition occurs, conjugates of T cells and target cells will be formed. Direct inspection of such conjugates shows that CD4 and the TCR concentrated in the site of cell interaction. This phenomenon is called co-capping. Interestingly, co-capping does not occur in the absence of specific antigen recognition, even though the class II molecule is present on the APC. Additionally, it has been suggested that CD4 may play an active role in T cell signaling. Support for this idea comes from findings that the T cell-specific tyrosine kinase $p56^{lck}$ is bound to the cytoplasmic tail of CD4, that stimulation of CD4 with anti-CD4 monoclonal antibody increases the *in vitro* kinase activity of the CD4 associated $p56^{lck}$, and that cross-linking of the TCR and CD4 enhances the degree of TCR-mediated tyrosine phosphorylation and the level of lymphokine response. Therefore they have been referred to as coreceptors (reviewed in Janeway, 1992).

Figure 6 shows the distinction between the two major models for CD4 function, the accessory molecule (also called co-ligand) and the co-receptor. The co-receptor model is clearly distinct in two ways. First, CD4 and the TCR bind the same class II MHC molecule on the APC surface. Second, the cytoplasmic domains of CD4 and the TCR/CD3 complex are brought together by this event. This allows the CD4-associated protein

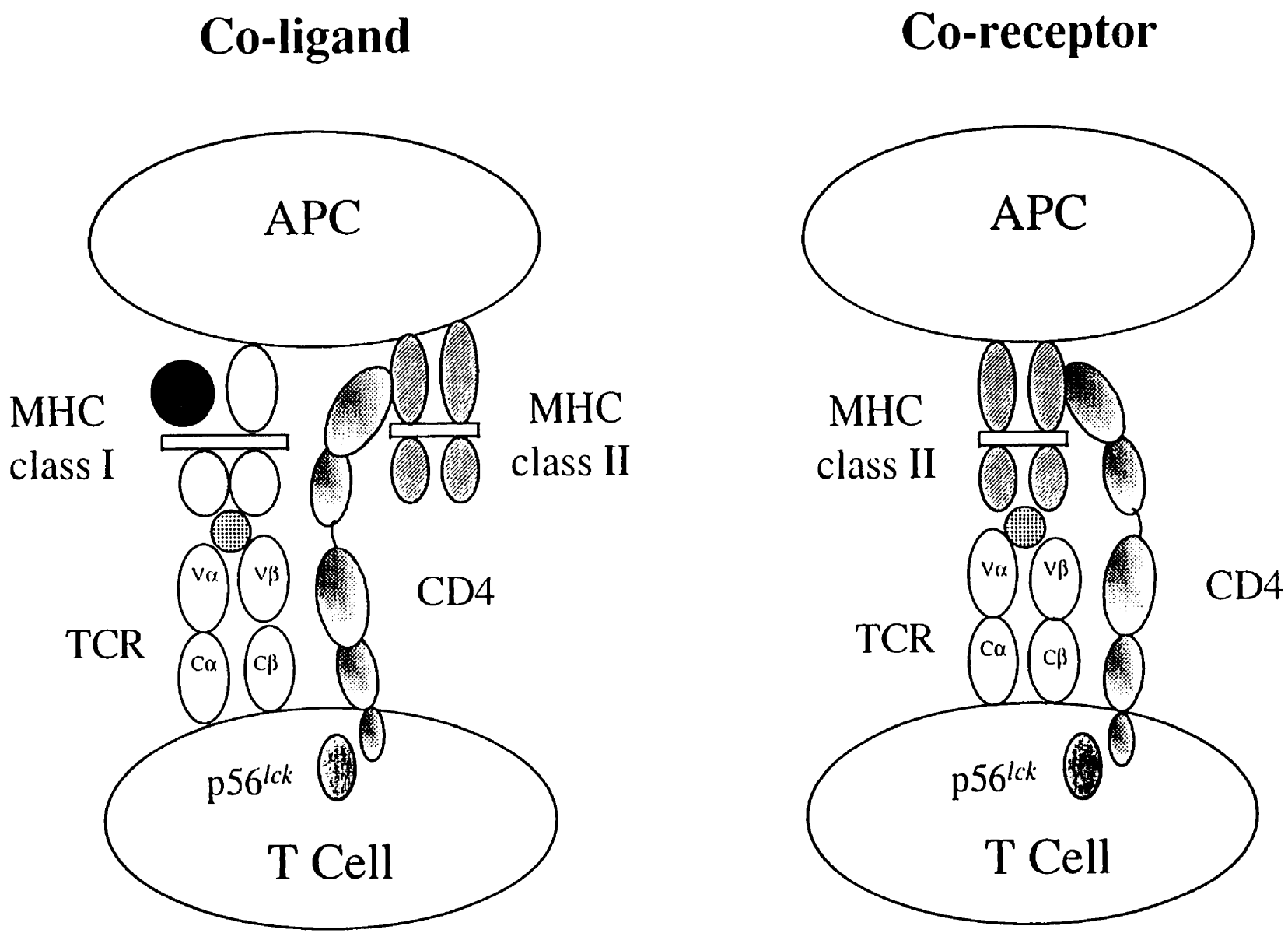

Figure 6. Co-ligand vs Co-receptor Model.

tyrosine kinase $p56^{lck}$ to phosphorylate elements of the cytoplasmic tails of the CD3:TCR complex on tyrosine. This model postulates that these events greatly potentate signalling through the T cell receptor. The evidence favoring the coreceptor model are the following.

4.4 Interaction of CD4 with Its ligand- Class II MHC Molecules and Residues on CD4 Implicated

The interaction of CD4 with MHC class II molecules during both T cell development and antigenic response are central to T cell function. The molecular nature of these interactions and signals generated are thus of great interest. As crystal structure of both CD4 and class II MHC molecules have become available, it is possible to model this interaction. An individual interaction of CD4/class II is relatively weak, in comparison to the tight association between CD4 and HIV gp120. Therefore it is difficult to provide a direct physical demonstration of such an interaction. However, at high concentrations, the *in vitro* binding of soluble human class II molecule HLA-DR4 to immobilized soluble CD4 has been successfully shown (Cammarota et al., 1992). Less direct but still compelling evidence were obtained from cell biology experiments. The CD4/class II interaction has been studied by cell adhesion assays measuring rosette formation between COS cells transfected with CD4 and the class II MHC^+ B lymphocytes (Doyle and Strominger, 1987; Clayton et al., 1989), and in assays of interleukin-2 (IL-2) production upon mixing of APCs with T cells transfected with mutated CD4.

The mouse T cell hybridoma 3DT52.5, which expresses L3T4 (mouse CD4), in conjunction with a TCR specific for mouse class I MHC molecules, has enabled the distinction between interaction of CD4/MHC and TCR/MHC, since the TCR and CD4 bind to different classes of MHC molecules. Activation of 3DT52.5, as measured by IL-2 production, was enhanced when target cells expressing class I antigen were further transfected with class II molecules. When human CD4 cDNA became available, this system was used to detect the association of human CD4 and class II molecules. Transfectants expressing human CD4 gene into the $L3T4^-$ variant of 3DT52.5, the

3DT52.5.8, enhanced the ability of this cell line to produce IL-2, in response to the class I cognate antigen when human class II molecules are co-expressed on APCs. Later, this spontaneous variant 52.5.8 cells were used to study the CD4/class II interaction, by expressing different mutated forms of CD4 in this cell line.

By using different cellular assays, interaction sites on both the class II MHC and CD4 molecules have been revealed. On class II molecules, the exposed region containing the residues 137-143 in the β2 domain is implicated in binding to CD4 (Konig et al., 1992; Cammarota et al., 1992). More recent studies have suggested that the α2 domain of class II may also play a role in interaction with CD4 (Konig et al., 1995). On the CD4 molecule, corresponding sites on the NH2-terminal Ig-like domains have been identified (Fleury et al., 1991; Moebius et al., 1992).

Most of the initial studies of class II binding sites on CD4 involved insertions and deletions of CD4 residues which can have indirect effects (Lamarre et al., 1989; Clayton et al., 1989; Lamarre et al., 1989). A later study by Fleury et al (Fleury et al., 1991) involved point mutations and was analyzed in light of the D1D2 crystal structure. It implicated a face of the CD4 molecule bounded by Ser19 and Glu89 from D1 and Gln165 from D2, but somewhat puzzling effects were also found at Thr45 and Gly47 which are in the CDR2-like region of D1 on an opposite face. These were discounted as essential determinants because a deletion mutation D43-49 showed no effects in either the IL-2 production or rosette formation. Subsequent work by Moebius et al (Moebius et al., 1992) using a cell adhesion assay involving the $CD4^+$ COS cells and B^+ lymphocytes, however, has implicated many of the same residues as identified by Fleury et al, plus some residues from the gp120 binding site. Furthermore, additional mutations analyzed by the Sekaly group (Hendrickson et al., 1992), showed further effects of mutations at exposed residues on strands A, B and G, from the face bounded by residues 19, 89 and 165. Fortunately, there is some evidence for convergence; Moebius et al (Moebius et al., 1993) have also found effects from many of the same mutations on the ABG face, including the upper parts of D2.

Up to now, the picture resulted from mutational tests of the CD4-MHC interaction is still rather confusing. It is difficult to draw any solid conclusion about the binding interaction between CD4 and class II. Apparently, differences in the assay system lead to the detection of somewhat different interactions. Quite possibly, interactions differ depending on whether the MHC molecule is engaged only with CD4 or is also in a complex with the TCR. Dimerization of CD4 molecules (Kwong et al., 1990), or dimerization of engaged class II molecules (Brown et al., 1993), for which there is evidence from crystal structures, might also play a significant role in the CD4-MHC interactions. Nevertheless, it is difficult in CD4-class II modeling studies to rationalize binding sites on opposite faces of D1. Inter-CD4 interactions might offer an explanation, although the lack of appropriate self association by D1D2 fragments (Ryu et al., 1994) would argue against dimerization mediated by this portion of CD4.

4.5 Interaction of CD4 with TCR

The initial evidences for the interaction of CD4 with the TCR on T cells were provided by following studies: using suboptimal stimuli to activate T cells, and cross-linking of CD4 to the TCR greatly potentates activation of T cells by anti-TCR antibodies (Eichmann et al., 1987; Ledbetter et al., 1987). These experiments provided dramatic and direct evidence that when CD4 is cross-linked together with the TCR, stronger signals for activation are developed, however, they did not show that CD4 and the TCR do associate during T cell activation.

When T cells recognize antigen on the surface of an antigen presenting cell, the two cells form a conjugate. Conjugate formation appears to be initiated by adhesive interactions between LFA-1 and its ligands ICAM-1 and ICAM-2, and by CD2 binding to its ligands LFA-3. However, these interactions do not lead to T cell activation and the cells soon dissociate. Only when specific antigen recognition occurs, the conjugates are stabilized. Double-immunofluorescence experiments confirmed that while the TCR and

CD4 remained uniformly distributed at the surface of non-specifically stimulated cloned murine helper T cells, specific antigen presentation induced a co-clustering of CD4 and the TCR at the T cell-APC contact site (Kupfer et al., 1987). Interestingly, CD4 does not migrate to the site of cell interaction when specific antigen recognition does not occur, even though the class II MHC ligand of CD4 is present on the APC.

An increase in CD4-TCR complex formation can be induced during T cell activation under certain circumstances. Anti-TCR antibodies directed against different regions of the TCR on the T cell clone D10 have been shown to induce different levels of CD4-TCR complex formation. The ability of these anti-TCR Abs to induce complex formation correlates well with their ability to stimulate lymphokine production. In agreement with these findings, fluorescence energy transfer studies have shown that binding of the mouse CD3/TCR complex by anti-CD3 antibodies induce its redistribution proximal to cell surface CD4. Such redistribution was not observed in hybridomas expressing a truncated human CD4 instead of a full length CD4, thus implicating the cytoplasmic tail in the CD4/TCR complex formation. Studies in which CD4 was artificially aggregated with the TCR using either CD4/TCR heteroconjugate monoclonal antibodies or Abs bound to beads, demonstrating that TCR signaling and TCR mediated lymphokine production are enhanced with CD4-TCR aggregation. Taken together, these data suggest that TCR-CD4 complex formation augments TCR activation (reviewed in Janeway, 1992).

4.6 Association of CD4 with Tyrosine Kinase $p56^{lck}$

The support for the role of CD4 as the co-receptor for T cell activation came from the finding that the tyrosine kinase $p56^{lck}$ is specifically associated with the cytoplasmic tail of CD4 (Veillette et al., 1988). The association of CD4 with Lck has suggested a potential role in signaling transduction for the coreceptor. Ab-mediated cross-linking of CD4 to CD3 enhances TCR signalling. The significance of the association of CD4/Lck has been demonstrated using a CD4-dependent Ag-specific murine T cell that lacks endogenous

CD4 (Glaichenhaus et al., 1991). Restoration of TCR function occurred solely after the introduction of CD4 molecules that are capable of associating with Lck.

$p56^{lck}$ is a member of the Src oncogene family of internal membrane tyrosine kinase, and have been implicated in T cell activation and differentiation. Indeed, dysregulation and over-expression of $p56^{lck}$ have been shown to promote tumorigenesis in both human and mouse cells (Abraham et al., 1991). Figure 4 shows structural features important for Lck function which include: (I) an amino-terminal myristylation required for membrane association.; (ii) a Src-homology domain 3 (SH3) apparently required for association with the cytoskeleton; (iii) a catalytic domain containing the tyrosine 394 (tyr-394) residue involved in *in vitro* autophosphorylation and positive regulation of Lck activity; (iv) a carboxyl-terminal regulatory domain containing the Tyr 505, phosphorylation of which inhibits Lck activity in vivo; (v) a SH2 domain whose affinity for phosphotyrosine protein presumably modulates the interaction of Lck with its specific substrates.

The SH2 domain is also involved in intramolecular negative regulation of Lck, whereby phosphorylation of Tyr 505 induces its interaction with the phosphotyrosine-binding SH2 domain of the same molecule (Sieh et al., 1993). The inferred conformational change might inactivate Lck by either masking the catalytic domain or impairing interaction with specific substrates containing phosphotyrosine amino acids (Amrein et al., 1992). Another mode of regulation of $p56^{lck}$ following T cell stimulation involves the phosphorylation of serine residues. Although the exact role of serine phosphorylation in the regulation of $p56^{lck}$ activity is unknown, the serine residue at position 59 has been shown to be phosphorylated following TCR cross-linking or phorbol ester stimulation (Watts et al., 1993).

The amino acids involved in Lck interaction with the cytoplasmic tail of CD4 have been mapped to the 30 N-terminal residues (Shaw et al., 1989). Site-directed mutagenesis performed on the N-terminal sequence of Lck revealed the essential contribution of two

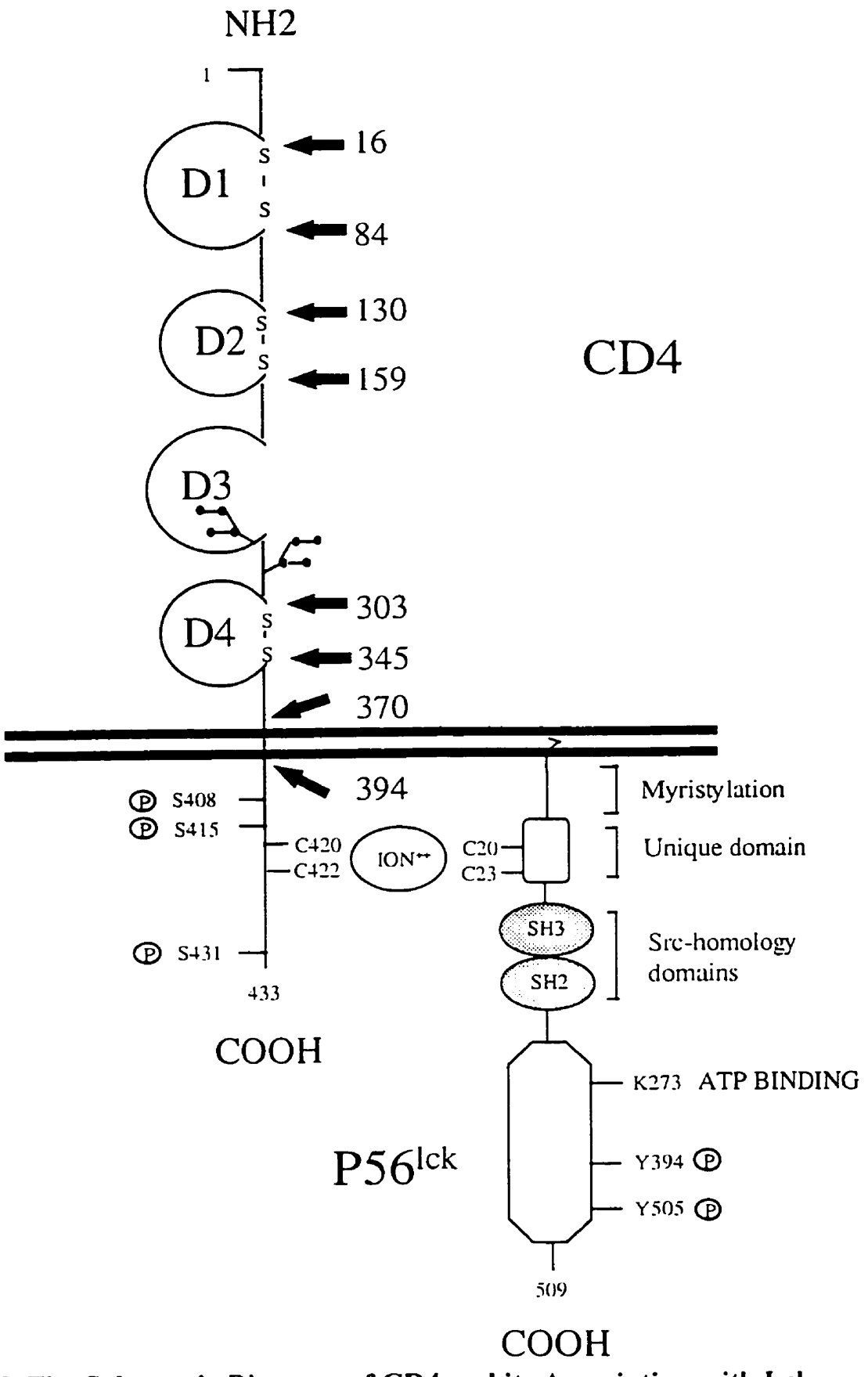

Figure 5. The Schematic Diagram of CD4 and its Association with Lck.

cysteines residues at positions 20 and 23 for binding to CD4 (Turner et al., 1990). These two amino acids interact with a similar motif contributed by cysteine residue 420 and 422 in the cytoplasmic tail of CD4 (Turner et al., 1990). Since the cysteine residues are not involved in intermolecular disulfied bonds, the interaction between CD4 and lck is postulated to involve the sharing of a metal ion by the cysteine motifs of each molecule, probably a zinc ion (Turner et al., 1990).

The lck was shown to be expressed in both murine and human T cells but not B cells or monocytic cell lines (Rudd et al., 1988). Consistent with the tissue distribution, the zeta chain of mouse TCR was shown to be a putative substrate of lck phosphorylation (Barber et al., 1989). Antigen presentation by APC, cross-linking of CD4, and cross-linking of CD4 with the TCR were each shown to induce T cell activation with the resulting phosphorylation of the zeta subunit of TCR (Abraham et al., 1991; Dianzani et al., 1992). Since this process was dependent on the association of lck with the cytoplasmic tail of CD4, it was concluded that lck was the intracellular component responsible for CD4-positive signal transduction (Glaichenhaus et al., 1991). CD4 molecules which cannot bind to lck fail to enhance anti-TCR stimulation, this is due to impaired association of CD4 with the TCR/CD3 complex (Collins et al., 1992). In addition, when Lck is not closely associated with the TCR, stimulation through TCR$\alpha\beta$ (not CD3) will be inhibited (Haughn et al., 1992). Thus Lck appears to be essential for both CD4 subcellular localization and initiation of signal transduction events leading to T cell activation following MHC class II-restricted antigen presentation.

The ζ chain may not be the only substrate of Lck, as alternative substrates of Lck that may be phosphorylated after T-cell activation include a group of mitogen-activated proteins, the Ras GTPase-activating protein, and phospholipidaseC-γ1 (Amrein et al., 1992; Ettehadieh et al., 1992; Weber et al., 1992). Lck interactions with different substrates may occur at different stages of T-cell activation or development.

CD45, a specific surface antigen of hematopoietic cells that exhibit protein tyrosine phosphotase activity, has been involved in regulation of Lck activity (Koretzky, 1993). Activation of $p56^{lck}$ was reduced in $CD45^-$ T lymphoma cell lines compared with the $CD45^+$ counterpart (Mustelin et al., 1989). This correlates with the increased phosphorylation of Lck at the inhibitory Tyr-505 residue and inactivation of the enzyme by intramolecular interactions between Tyr-505 and SH2 domain (Sieh et al., 1993). Cross-linking of CD45 and CD4 confirmed that CD45 could directly dephosphorylate the Lck tyr-505, leading to increased kinase activity (Ostergaard et al., 1990).

The counterpart of CD45 appears to be a cytoplasmic protein tyrosine kinase, $p50^{csk}$, which down-regulates Lck catalytic activity by specific phosphorylation of the Tyr-505 residue. Since $p50^{csk}$ possesses a phosphotyrosine-binding SH2 domain but no myristylation signal, it may be recruited by the phosphorylated Try-394 residue of activated Lck. Although the $p56^{lck}$ and $p50^{csk}$ interaction remains to be clarified, the role of Csk in the negative regulation of Src family kinases and T-cell activation is now well established (Chow et al., 1993; Nada et al., 1993; Superti-Furga et al., 1993).

4.7 CD4 Can Transduce Inhibitory Signals to T Cells

CD4 can transduce positive signal to T cells when cross-linked with the TCR/CD3 complex. However, TCR-independent ligation of CD4, by cross-linked antibodies, was shown to inhibit the proliferation and interleukin-2 production signals delivered by mitogenic lectins. In addition, the use of bivalent and monovalent Fab fragments of anti-CD4 mAbs provided a system which enables to distinguish whether the inhibitory effect of anti-CD4 mAbs was due to physical cross-linking of CD4 or due to induction of steric hindrance blocking the association of CD4 with TCR/CD3. Fab fragment of anti-CD4 mAbs, unable to cross-link CD4, were devoid of ability to inhibit lectin-induced proliferation but retained the ability to block T cell activation mediated by anti-CD3 antibody. In contrast, bivalent anti-CD4 Abs were able to deliver a negative proliferation signal to T cells (Janeway, 1992).

Cross-linking of CD4 can deliver both positive and negative signal to T cells. The transduction pathway may involve activation of $p56^{lck}$, since cross-linking of CD4 but not TCR (generating a negative-signaling responses) enhanced the catalytic activity of Lck despite an apparent increase in the phosphorylation of the negative regulatory Tyr-505 residue. Thus delivery of a proliferative rather than an inhibitory signal will depend on the physical approximation of the CD4-Lck activated complex with the relevant substrates within the TCR/CD3 complex. This could involve either the migration of CD4-Lck close to the activated TCR, or , more probably, conformational changes in preformed CD4-TCR complexes that would allow the interactions of Lck with the CD3-ζ chain substrate (Suzuki et al., 1992; Veillette et al., 1989). Failure to induce a conformational change within the TCR would impair Lck association with specific substrate or alternatively, expose an inhibitory substrate within the TCR, leading to negative signaling.

4.8 Role of CD4 in Thymic Selection and T cell Maturation

CD4 acts as a coreceptor for class II MHC antigen in T cell activation. It also plays an important role in thymic development of T cells, as suggested by antibody-blocking experiments and by gene knockout mice in which CD4 is eliminated.

Administration of anti-CD4 monoclonal antibodies *in vivo* has been shown to block the development of the $CD4^+$ subsets (Zuniga-Pflucker et al., 1990). Mice homozygous for CD4 gene ($CD4^-/-$) show a huge deficiency in class II-restricted T cells (Rahemtulla et al., 1991; Killeen et al., 1993). However, some class II-restricted T cells can be detected (Locksley et al., 1993). These cells appear as a small population of DN mature T cells that behave as if they were CD4 cells: they are class II-restricted, can help B cells, make typical CD4 lymphokines, and their maturation is dependent on class II expression. Thus these cells mature and survive functionally without the need of a CD4 coreceptor. It is not clear, however, whether this indicates that another cell surface receptor can substitute for CD4,

and/or whether CD4-independent, class II-restricted T cells come from a distinct lineage of thymocytes.

In the CD4$^{-/-}$ mice, introduction of a transgene human CD4 molecule has been shown to rescue the defect of the CD4 lineage, indicating that human CD4 can substitute for its murine counterpart (Killeen et al., 1993). Interestingly, the presence of the CD4 molecule is differentially required for negative selection of class II-associated superantigens. Although in Mls-1a (MMTV-7) positive CD4$^{-/-}$ animals, Vβ6-, Vβ8.1- and Vβ9 expressing thymocytes were clonally deleted, this was not the case for Vβ7^{+} cells (Wallace et al., 1992). In vitro, Vβ7^{+} T cells have a lower avidity for Mls-1a than the other Vβs. Thus these cells might require the CD4 coreceptor to stabilize the binding or mediate signaling for clonal deletion.

In MHC class II deficient mice, unexpectedly, a substantial amount of CD4^{+} thymocytes (17-33% of wild type) are observed; however, the complete maturation of these cells seemed to be blocked, as indicated by the expression of heat stable antigen (HSA), low levels of CD8, and intermediate amounts of TCR. In addition, these CD4 cells are smaller than normal CD4 SP thymocytes and reside predominantly in the cortex of the thymus (Cosgrove et al., 1991; Grusby et al., 1991). In invariant chain deficient mice (which show reduced levels of class II complexes on the cell surface resulting from an aberrant cytoplasmic compartmentalization of class II MHC), only a diminished number of CD4^{+} thymocytes are detected which coexpress low amounts of CD8 and are CD3 positive (Viville et al., 1993; Bikoff et al., 1993). DP thymocytes in these mice have elevated amounts of CD4 and CD3. Negative selection of potentially self-reactive T cells seems to be impaired as indicated by the presence of Vβ11^{+} cells in I-E positive animals (Viville S et al., 1993). Thus, functional class II molecules appear to be required for the effective selection of CD4 SP thymocytes. In contrast, maturation of DP thymocytes to CD8 SP cells is not dependent on class II molecules. (reviewed in Pfeffer and Mak, 1994)

4.9 CD4 as the Cellular Receptor for Human immunodeficiency Virus Glycoprotein gp120 and the Region Implicated in Binding to gp120

CD4 is the principal receptor for the human immunodeficiency virus (HIV) (Dalgleish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986). The interaction between CD4 and gp120, the external envelop glycoprotein of HIV, facilitates the binding and penetration of viral particles, and is central to cytopathic processes mediated by the HIV envelop glycoprotein (Lifson et al., 1986a; 1986b; Sodroski et al., 1986).

As mice do not support HIV infection, and the mouse CD4 molecule, L3T4, does not bind gp120, attempts have been made to identify residues involved in binding to gp120 by producing mutant CD4. These mutant CD4 molecules had residues substituted from mouse CD4 for the human counterpart at comparable positions (Peterson and Seed, 1988; Clayton et al., 1988). These experiments, along with other studies using anti-CD4 monoclonal antibodies and site-directed mutagenesis, have implicated a region of CD4 that is roughly homologous to the CDR2 region of immunoglobulin as being involved in specific interactions with gp120. Recombinant CD4 proteins with mutations in this region, specifically between positions 40-53, have greatly diminished apparent binding affinity for gp120, and for certain anti-CD4 mAbs that had previously been shown to inhibit gp120 binding and HIV infection (Arthos et al., 1989; Berger, et al., 1988; Peterson and Seed, 1988; Brodsky et al., 1990; Clayton et al., 1988; Ashkenazi et al., 1990; Sweet et al., 1991.)

While these studies clearly identified the CDR2-like region of CD4 as important in interacting with gp120, they were typically based on assays involving the interaction of soluble CD4 constructs with soluble gp120 as a putative model for virus interaction with cell-associated CD4, an approach now considered to have significance limitations (Moore et al., 1992). In a different approach, carrier protein-linked synthetic peptides derived from the CDR2-like domain of CD4 were shown to bind to gp120. Among the residues in the CDR2 region, the exposed Phe-43 is the most critical determinant.

Surprisingly, in view of the mutagenesis data pointing to the CDR2-like region as the "high affinity binding site for gp120" (Arthos et al., 1989), other peptides derived from the CDR3-like region of CD4 (residues 81-92), blocked HIV infectivity, viral-induced cell fusion and gp120 binding, with the precise sequence specificity. Side chain derivation was required for anti-HIV activity of the CD4 81-92 peptides; derivation may constrain the conformation of the peptides to approximately that found in this region of the CD4. Alanine scanning mutagenesis which carefully controlled for conformational effects also indicated that the CDR3-like domain of CD4 is critical for gp120 binding (Ashkenazi et al., 1990), although these results remain controversial (Sweet et al., 1991).

Both site-directed mutagenesis experiments and synthetic peptide studies have also implicated the CDR3-like domain of CD4 in the membrane fusion events that may occur as a consequence of the binding of HIV envelop glycoproteins to CD4 (Lifson et al., 1991; Camerini et al., 1990). In addition, anti-CD4 mAbs directed against the CDR3 region have also been shown to inhibit HIV infection, HIV-induced cell fusion (Truneh et al., 1991) and gp120 binding (Eiden and Lifson, 1992).

Based on all these findings, it has been postulated that the CDR2 and CDR3 region of CD4 together contribute to the multipoint attachment required for high-affinity binding of gp120 to CD4. The discontinuous nature of the CDR2 and CDR3 binding domains renders the CD4-gp120-gp41 interaction susceptible to conformational modulation through regions not otherwise directly Involved in binding (Eiden and Lifson, 1992).

5. Rationale:

The CD4 molecule plays an important functional role in T cell development and T cell activation. It is expressed on a subset of T lymphocytes, the T helper cell population, whose TCR is restricted to the MHC class II molecules. CD4 binds to the non-polymorphic regions $\alpha 2$ and $\beta 2$ of the MHC class II molecules, by doing so it can mediate

adhesion, and thus strengthen the affinity of the TCR to the Ag/MHC complex. As CD4 is intra-cellularly associated with the protein tyrosine kinase $p56^{lck}$, it is believed that CD4 has also a signaling function, upon interacting with the class II/TCR complex and this will bring this kinase into the T cell signaling pathway.

In addition to binding to its physiological ligand class II MHC molecules, CD4 has been shown to interact with other cell surface molecules on the T cell, such as the TCR, and even with CD4 itself. It has been known that virtually all transmembrane receptors that rely on the protein tyrosine kinase function to initiate intracellular signal transduction depend on the ligand-induced dimerization of receptor subunits to initiate the second messenger generation. There are considerable evidences that antigen receptors such as membrane Ig and TCRs, must undergo multimerization for signaling to occur. The most provoking evidences came from the class II HLA-DR1 and the myelin basic protein peptide-specific TCR Vα crystals which have both been crystallized as dimers. Furthermore, results from functional assays suggesting oligomerization of CD4 allow us to put forward a model in which the organized oligomerization event for the TCR/CD4/class II MHC must occur in order to initiate T cell activation. The mechanism of self-association of CD4 and the possible interface involved in this oligomerization is far from clear. Furthermore, apart from its physiological role, CD4 has been found as the cellular receptor for HIV virus, serving as its port of entry into the immune system. The high affinity interaction between the envelop glycoprotein gp120 of HIV and CD4 is a central molecular event in AIDS pathogenesis. It is therefore of great interest to better understand the functional interaction of CD4 with its physiological ligand class II MHC molecules at the molecular level. This could be helpful for the modulation of the immune responses.

The thesis work presented here focuses on three aspects of the CD4/class II MHC molecule interaction.

Firstly, although CD4 can bind to different alleles and isotypes of class II molecules from the same species through the non-polymorphic regions, controversy still remains with

respect to the efficacy of inter-species interactions of human and mouse CD4 to murine and human class II MHC molecules respectively, despite the fact that these two molecules share more than 60% homology. We tried to answer the question regarding the inter-species interaction of CD4 and class II MHC by using the 3DT T cell hybridoma system. 3DT cells expressing either human or mouse CD4 molecule were co-cultured with APCs expressing different class II alleles and isotypes from either human or mouse, together with the cognate antigen or superantigen. The interaction of CD4 with class II will result in an enhanced T cell response. Furthermore, in order to investigate if residues implicated in the human/class II interaction have a role in the cross-species interaction between human CD4 and murine class II molecules, we have used a panel of CD4 mutants which had been shown previously to be impaired in their interaction with human class II molecules. We have also tried to study if these residues play a role in binding to class II from a different species, thus we have mapped the functional domains important for this inter-species interaction of CD4/class II.

Secondly, CD4 has been shown to bind to the $\alpha 2$ and $\beta 2$ regions of class II; however, the contact sites on CD4 with class II are less clear, and rather controversial regarding the involvement of the CDR2 region of D1 domain of CD4 in this CD4/class II interaction. Here we used the 3DT system which allows us to distinguish the function of CD4 as the co-ligand (accessory molecule) from the co-receptor in one cellular system. To further characterize the class II MHC binding sites on CD4 at the molecular level and to compare residues implicated in the co-ligand assay with those in the co-receptor assay, a large panel of mutants throughout D1 and the FG loop of D2 were generated. These mutants involved highly exposed residues according to the available crystal structure of CD4. Moreover, the functional importance of the hinge region, a region between D2 and D3 domains which has been proposed from crystallographic and proteolytic studies, was studied for the first time by mutagenesis, serology and functional assays.

Thirdly, crystallographic studies have at time provided evidences for the dimerization/oligomerization of CD4. Recent functional studies bringing more supports for

this oligomerization events of CD4/class II/TCR interaction. Moreover, the class II molecule DR1 has been shown as a superdimer in crystal, so does the TCR Vα chain specific for the myelin basic protein peptide. In order to study if CD4 oligomerization is essential for its function as either the co-ligand or co-receptor, we have co-expressed both human and mouse CD4 on the 3DT cells. As human and mouse CD4 shares more than 60% homology and there is an inter-species interaction between CD4 and class II from human and mouse origins, we tried to investigate if these two molecules can oligomerize together or not. Furthermore, by co-expressing either human or mouse CD4 wild-type with CD4 mutants which have been shown to be involved in CD4/class II interaction, we explore this oligomerization event, its mechanism and the possible interface involved in this.

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Chapter 2

Analysis Of The Contact Sites On The CD4 Molecule With Class II MHC Molecule: Co-Ligand Vs Co-Receptor Function

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Preface

The CD4 molecule, by binding to the non-polymorphic regions in class II MHC molecules on PACs, plays an important role in T cell activation. Two functional roles of CD4 have been suggested: the adhesion molecule and the co-receptor. Previous structural and functional analyses on CD4/class II MHC interaction have implicated residues from the CDR regions of D1 and the FG loop of D2. However, controversy still remains with respect to the involvement of the CDR2 region in class II binding. It seems that residues mapped on CD4 vary with the cellular systems. Here, our effort is to further define the class II binding sites, to address the controversy over the CDR2 region, and to determine the functional importance of the region between D2 and D3 domains of CD4. Crystal structure informed site-directed mutagenesis have been carried out. The 3DT cellular system which provides assays for the two functions of CD4 was used.

Abstract

The CD4 molecule interacts with the α2 and β2 domains of the major histocompatibility complex (MHC) class II molecules. However, the class II contact sites on CD4 are less clear. Involvement of different regions throughout D1, D2 and D3 domains have been suggested. To further delineate the class II MHC contact sites on CD4, a crystal structure informed mutagenesis was performed. Alanine scan mutants were generated for exposed residues located throughout D1 and the FG loop of D2, and in the "hinge-like" region, a short and flexible region between D2 and D3. Mutants were tested in a co-ligand (D^d stimulation) and a co-receptor (SEB stimulation) assay. In the co-ligand assay TCR and CD4 interact with two distinct ligands (D^d or HLA-DR) while in the co-receptor assay both molecules interact with the same ligand, namely HLA-DR. Results show that residues from both lateral faces of D1 and the FG loop of D2 are implicated in interaction with class II, although a bigger surface of CD4 was involved in co-receptor compared to co-ligand function. The potential involvement of residues on both the top and two side faces of CD4 is consistent with a bivalent model, which involves the interaction between a single CD4 and two class II molecules. Alternatively, our results can be interpreted with a model of a specifically organized CD4 and/or class II oligomerization event. Finally, results from the hinge region mutants revealed a very important role in maintaining the overall structural integrity of CD4, its topology and function.

Introduction

The CD4 molecule is a cell surface glycoprotein with a molecular weight of 55 kDa. It is a member of the immunoglobulin (Ig) gene superfamily. It is a monomer with four extracellular Ig-like domains, a transmembrane portion and a cytoplasmic tail (1). The cytoplasmic tail of CD4 is non-covalently associated with the tyrosine kinase $p56^{lck}$ (2). CD4 is expressed on a subset of T lymphocytes and thymocytes. The T cell receptors (TCR) of these $CD4^+$ cells recognize antigens presented by class II major histocompatibility complex (MHC) molecules. The CD4 molecule binds to its ligand-class II MHC molecules to enhance the T cell response, either as an adhesion molecule (co-ligand) or in a ternary complex with the TCR as a part of the antigen recognition process and which is directly involved in the resulted signal transduction (co-receptor) (3). The CD4/class II interaction is critical for thymic selection during T cell development, and it is as well important for the activation of mature T cells. The signaling functions of CD4 are mediated by a Src family tyrosine kinase $p56^{lck}$ which is associated non-covalently with the cytoplasmic tail of CD4 (2). Association of CD4 with a class II/TCR complex appears to bring this kinase into the TCR signaling pathway. CD4 has also been characterized as the primary cellular receptor for the human immunodeficiency virus (HIV) glycoprotein gp120 (4, 5, 6).

The CD4 binding site on class II molecules has been shown to be located in the $\alpha 2$ and $\beta 2$ domain of class II (residue 134-140) (7, 8, 9). However, the class II contact sites on CD4 are still controversial. Results from our laboratory using a co-ligand assay (D^d), whereby CD4 and TCR interact with two different MHC molecules have demonstrated that residues in the CDR1 and CDR3 regions of D1, and the FG loop of D2, all located on the same face of CD4 which is opposite to the CDR2, are implicated in binding to class II (10). Interestingly, the CDR2 deletion mutant (deletion of residues 43-49) can still interact with different murine and human class II alleles (11). As studies have shown that the CDR2

region is quite divergent among species (12), therefore, it is less likely for the CDR2 region to bind to a highly conserved non-polymorphic region in class II. On the other hand, results from adhesion assays suggested that in addition to the CDR1, CDR3 regions of D1 and the FG loop of D2, an extended CDR2 region is involved in binding to class II (13, 14, 15). Thus, the major question remains: should the extended CDR2 region be implicated in interaction with class II or in another function of CD4 itself, such as CD4 dimerization/oligomerizaton or association with the TCR/CD3 complex.

The full length CD4 molecule has not been successfully crystallized with high resolution. Crystal structures obtained so far are the D1D2 fragment of the human CD4 (16, 17) and the D3D4 fragment of the rat CD4 molecule (18). The link between the two fragments has been suggested as a short and flexible "hinge-like" region (18, 19). This has been supported by crystallographic and proteolytic studies (20-23). When residue 180 or residues 180-181 were deleted, the CD4 molecule turned into a straightened rod-like structure instead of a bent structure, as observed under the electronic micrographs (personal communication with Dr. Leo Brady). The hinge region has been proposed to play an important role in maintaining the overall conformation of CD4, and in ligand binding induced conformational modification or formation of dimer/oligomer, in normal signal transduction, and in membrane fusion upon HIV binding (19, 23-26). Experimental evidence for the functional importance of this hinge region has yet to be obtained.

In order to further delineate the class II binding site on CD4 and to understand the role of the CDR2 region in the class II interaction, a large panel of CD4 mutants encompassing residues located throughout both lateral faces of D1 and the FG loop of D2 domain of CD4 was generated. Moreover, in order to study the role of the hinge region, alanine scan and deletion mutants of the junctional region between the D2 and D3 domains were generated. All the mutated CD4 molecules were expressed in the 3DT52.5.8 murine T

cell hybridoma which is $CD4^-CD8^-$, and whose TCR (Vβ8) is specific for the cognate alloantigen H-$2D^d$. This cell line is dependent on CD4 for an efficient allo-response. Mutants were studied in the co-ligand (D^d) assay described previously (10), and in the co-receptor assay which involves the CD4/TCR interaction with class II HLA-DR molecules in the presence of the bacterial superantigen SEB. Results presented in this report reveal the functional importance of these different domains of CD4 in the two assays.

Materials and Methods:

Plasmids: Full length human mutant CD4 cDNAs were obtained from different sources (10) or generated in our laboratory by PCR overlap extension method (27). For D1 and D2 mutants, the mutated CD4 cDNAs were prepared as blunt-ended *Bam* HI or *Hind* III-*Not* I fragments and subcloned into the pMNC-stuffer retroviral vector. The amphotropic helper cell line DAMP (28) was transfected with the different pMNC-mutant CD4 constructs (pMNC-mCD4), and G418 resistant DAMP cells were selected and used as the producer cell lines of the recombinant retrovirus to transfect the 3DT52.5.8 cell line. The proviral pMNC-mCD4 DNA confers resistance to the selection agent G418 and allows stable long-term expression of the mutated CD4 molecules in infected cells. For mutants in the hinge region, the mutated CD4 cDNAs were generated by PCR overlap extension (see Mutagenesis section).

Mutagenesis: The hinge region mutants were generated by the PCR overlap extension method. Briefly, two complementary oligonucleotides bearing specific mutations were used to generate two fragments with overlapping ends. Subsequent PCR reaction was carried out on the mixture of these fragments where the 3' overlap of each strand serves as primer for the 3' extension of the complementary strand. The amplified fragments were then used to replace their homologues in the CD4 WT. Mutagenesis was confirmed by DNA sequencing. The full length cDNAs coding for the mutant CD4 molecules were prepared as fragments and subcloned at the *Xba* I site of the eucaryotic expression vector SRα (29). Then they were used for electroporation.

Transfection: D1 and D2 mutants were generated as described (10). Briefly, $CD4^+$ DAMP cells were obtained by the calcium phosphate co-precipitation technique (30) as previously described (31). The DAMP cells transfected with the various pMNC-mCD4 constructs were enriched by FACS sorting with OKT4 antibody. These DAMP $CD4^+$ cells

were used to infect the 3DT52.5.8 hybridomas as described (10). G418-resistant 3DT 52.5.8 cells were then analyzed for the expression of the mutated CD4 molecule by flow cytometry. Populations of cells expressing homologous levels of TCR and CD4 were generated by aseptic cell sorting.

For the hinge region mutants, electroporation of 3DT52.5.8 cells with the SRα neo-mutated CD4 constructs was carried out. For each electroporation, 5 X 10^6 3DT cells were resuspended in 500 µl of complete RPMI supplemented with 10% FCS. To this cell suspension 10 µg of DNA was added, and electroporation was carried out at a voltage of 270 mV. Forty-eight hrs later, cells were subjected to G418 selection. 40,000 cells were resuspended in 2 ml of complete RPMI with 10% FCS. G418 at a concentration of 1.5 mg/ml was added. The medium was changed every three days, G418-resistant 3DT 52.5.8 cells were analyzed for the expression of the mutated CD4 molecule by flow cytometry.

Cytofluorometric Analysis of cells: Cells (5 X 10^5) were incubated for 60 min at 4°C with 100 µl of the appropriate mAb (10 µg/ml) diluted in complete medium. Following washes in PBS, cells were incubated with fluorescein-coupled goat-anti-mouse Ig (Becton Dickinson) for 30 min at 4°C. Cells were then washed, resuspended in PBS containing 2% FCS and analyzed for fluorescence using Lysis II flow cytometry. Dead cells were excluded by propidium iodide (0.5 µg/ml) gating, and 10,000 cells were acquired for the analysis on a four decade logarithmic scale. As a control, cells were stained only with the fluorescein-coupled goat-anti-mouse Ig.

Stimulation of Effector Cells and IL-2 Assay :

Co-ligand Assay: This assay has been described previously (10, 11). Briefly, 7.5 X 10^4 3DT52.5.8 cells expressing the mutated CD4 molecules were cocultured with 7.5 X 10^4

DAP-3 cells expressing H-2D^d or H-2D^d DR4. After the co-culture, supernatants were harvested for IL-2 production assay. The IL-2 ratio is calculated by comparing the IL-2 production for a given CD4 mutant upon stimulation with H-2D^d + DR4 over the IL-2 value obtained with H-2D^d alone. The responses of this co-ligand assay are displayed as such (in Figure 2): on the X-axis, the positions of the mutated residues are given; while the IL-2 ratios are displayed on the Y-axis. Furthermore, when we compare the IL-2 ratio of the mutants to that of the CD4 WT$^+$ cells, we obtain the IL-2 ratio in the co-ligand assay of the mutants as compared to the wild-type (IL-2 ration- D^d mt/wt) (as shown in Table V).

Co-receptor Assay: 7.5 X 10^4 3DT 52.5.8 cells expressing the mutated CD4 molecules were cocultured with 2 x 10^4 DAP-3 cells expressing human class II molecule DR1 in 200 µl complete medium for 20 hr at 37°C in 96-well flat-bottom culture plates, in the presence of 2-fold serial dilutions of SEB ranging between 10^5 to 10^2 pg/ml (a kind gift from Kappler and Marrack). Titration of SEB was carried out both with 3DT52.5.8 and 3DT52.5.8 transfected with the human CD4 WT. After an overnight co-culture supernatants were harvested for IL-2 measurement. The responses of the T cell hybridomas expressing different CD4 mutants are displayed as such: on the X-axis, the log concentrations of the bacterial superantigen SEB are given; while on the Y-axis, the IL-2 units produced by each mutant upon SEB stimulation are displayed (as shown in Figure 3).

Anti-TCR stimulation : The TCR-specific antibodies KJ12 (recognizes the TCR association of the α chain specific for D^d alloantigen and the Vβ8.1) and F23.1 (recognizes all the Vβ8s) were used to stimulate the cells. A wide range of concentrations (3, 1, 0.3, 0.1, 0.03, 0.01, 0.003 µg/ml) of the purified mAb were coated at 4°C in 96-well flat-bottom plates overnight. To these wells, 7.5 X 10^4 3DT 52.5.8 cells expressing different mutated CD4 molecules were added in 200 µl of complete RPMI medium and incubated for 20 hr at 37°C. The supernatants were harvested for the IL-2 production assay later.

EC 50 Calculation: EC 50 is defined as the concentration of a given stimulus (antigen, superantigen or anti-TCR antibody) required to obtain a half-maximal response for a cell line. For all the stimulation assays such as the SEB and the anti-TCR assays, the results were plotted as log concentrations of the stimulus on the X axis against the IL-2 values on the Y axis. Curve-fitting was done with Sigma Plot program. The EC50 value was determined from the curve for each CD4 mutant, by reading out the X axis value such that the Y value equals to the half of the peak IL-2 value on the curve.

The EC 50 values of all the CD4 mutants were compared to that of the wild type CD4 expressing cells, which gives the EC50 ratio (as shown in Table IV). In addition, for the SEB assay, an IL-2 ratio for each mutant was calculated as the following: at the EC50 of SEB determined from the WT $CD4^+$ cells, the IL-2 production of the mutants tested at the same conditions were calculated from the curve; and these IL-2 values were compared with that of the WT $CD4^+$ cells, this gives the IL-2 ratio of SEB mt/wt (as displayed in Table IV).

Molecular Modeling: The CD4 images were generated using the Quanta software (Polygen Corp., Waltham, MA) on a Silicon Graphics workstation (Mountain View, CA).

RESULTS

Description and expression of CD4 mutants

D1 and D2 Mutants:

In order to further define the molecular interactions between MHC class II and CD4, a detailed mutational analysis of CD4 was undertaken using the coordinates of the available crystal structure. We examined three major issues concerning the CD4-class II interaction: (i) the potential contribution of other residues from the CDR1, CDR3 regions of D1 and the FG loop of D2, (ii) the involvement of other regions on the same face but outside the CDR-like regions in the D1 domain, (iii) the role of the CDR2 region in class II binding. For mutagenesis, we chose amino acids which are exposed on the surface and whose side chains are highly solvent accessible, reasoning that these residues are most likely to make contact with MHC class II molecules, and that changes at these positions were least likely to result in global structural alterations. We made 16 substitutions in D1 and 5 in D2. Single amino acid substitution to alanine were introduced into CD4 by site-directed mutagenesis. Mutants are described by a single-letter code for the amino acid involved and the position of the mutated residue.

Hinge Region Mutants:

In order to investigate the possible role of the hinge region in T cell activation, mutants were generated, including alanine scan for V175, V176, L177, Q180, QK180-181, and deletion mutants Deletion 175-177, Deletion 180-181. Among these residues, V176 and L177 are conserved among species including human, mouse, rat, rabbit and cat; and residue K181 is conserved among human, rabbit and cat.

Serological Analysis of the CD4 mutants:

A detailed serological analysis was undertaken to determine the structural impact of the mutations on the conformation of the CD4 molecule. All the antibodies and their

epitopes are summarized in Table I. The results of the serological studies for D1 and D2 mutants are summarized in Table II, while results for the hinge region mutants are summarized in Table III.

All 17 mutants from the D1 and D2 domains were stained with a panel of 7 different mAbs that map to different regions in D1 or D1 + D2. MAb OKT4 recognizes a region on the membrane-proximal portion of CD4, and all the CD4 mutants express the OKT4 epitope. None of the D1 or D2 mutations resulted in the loss of epitopes tested. However, some mutants had limited effects such as reduced binding for one or two epitopes. Results illustrated in Table II show that mutants K7A and Q20A had reduced binding for only one mAb; mutant T17A had reduced binding for two mAbs. These results clearly showed that there are no overall conformational changes induced by the mutations; the other regions should still be intact and able to interact with other surface molecules such as class II MHC molecules and the T cell receptor.

For the hinge region mutants, a panel of 17 monoclonal antibodies which map from the D1 through the D4 domain of CD4 were used. All the CD4 mutants retained the epitope for OKT4. However the epitopes for the other anti-CD4 mAbs were differentially affected by these mutations. The deletion mutant DEL 175-177 had the most drastic effects on the conformation of the CD4 molecule, as shown by the loss of four out of 17 epitopes and decreased binding for another ten antibodies. Only two mAbs out of 17 still bind to this mutant at levels comparable to that of CD4 WT. Besides, the V175A mutant and the deletion mutant DEL 180-181 have lost one epitope and show several epitopes being affected. The other mutants have several epitopes being affected but lost no epitopes (Table III). From these results it appears that the epitopes affected by the various hinge region mutations occur throughout D1-D4, suggesting that this region is very important for the conformation of CD4. Among all the mutants, the deletion DEL 175-177 mutant had the

most drastic effects, indicating that the conserved residues V176 and L177 are especially critical in maintaining the overall structure of CD4. Deletion may shorten the hinge region, resulting in the close association of D2 and D3 domains leading to the formation of a rod structure. This mutation may also change the orientation of the membrane-distal domain of CD4.

Functional Analysis of the CD4-Class II MHC Interactions:

All the D1, D2 and hinge region mutants expressed in the 3DT52.5.8 hybridoma cells were examined for their ability to interact with the class II MHC molecule in two functional assay systems: the co-ligand and the co-receptor assay (the two assay systems are illustrated in Figure 1). Introduction of CD4 in both assays results in an enhanced production of IL-2. A minimum of three hybridoma populations expresssing each CD4 mutant and comparable levels of CD4 and the TCR were used in the two assays. The responses of each one of the mutants to the TCR-specific mAbs (KJ12 and F23.1) coated on plates were comparable. Finally each mutant was tested in at least three independent experiments.

Co-ligand Stimulation

D1 and D2 Mutants

T cell populations which were selected for functional assays showed comparable EC50s in their responses to the TCR-specific Ab KJ12, as shown in Table IV (EC50 ratios of the KJ12 stimulation range from 0.8 to 1.4). Although all the D1 mutants produce comparable levels of IL-2 upon KJ12 stimulation, their responses in the co-ligand assay were variable. Results presented in Table IV show that mutations K1A, K2A, K7A, T15A, S19Y, Q20A, K46A, E77A, E87A, D88A, Q89L and Q89A affect the binding to class II, while other mutations such as T17A, S19A, K21A, S23A, DEL 43-49 and K90A did not

show any effect. Interestingly, S19A can still interact with class II, although an abrogation of the interaction by substitution of this residue to tyrosine was previously reported (10).

While the CDR2 deletion mutant DEL 43-49 can still interact with different human and mouse class II alleles (11), single mutations within the CDR2 region-K46A, and mutation of a residue on the same face as the CDR2, i.e. E77A, can no longer enhance the response. Though this is different from our prediction that the CDR2 region is not involved in binding to class II, it is consistent with our previous study in which substitutions in this region, such as T45P, K46N/G47V and G47R, did have an effect on the interaction with class II (10). It is possible that the deletion mutant adopts a different conformation which somehow, restores its binding to class II, maybe through a different contact interface. Alternatively it is possible, as recently suggested, that this domain of CD4 is not directly involved in CD4/class II interaction but rather involved in CD4 oligomerization (32).

All the alanine scan mutants for residues from the FG loop of D2 were studied. Their responses in the co-ligand assay were variable. Mutants N164A and Q167A can still enhance the D^d response, showing a phenotype comparable to that of the $CD4^+$ cells; in contrast, mutants Q163A, Q165A and K166A behave like the $CD4^-$ cells and the previously shown mutant 165[SR] (10). They do not produce increased levels of IL-2 in the presence of class II^+ cells (Figure 2, Table IV).

Hinge Region Mutants

The hinge region mutants were also expressed in the 3DT52.5.8 cells and studied in the co-ligand and KJ12 stimulation assays. Although all the CD4 mutants gave similar responses after anti-TCR stimulation when compared to the WT $CD4^+$ cells, only mutants Q180A and QK180-181A showed a comparable response to D^d as reflected by IL-2 production ratios. The other mutants, including those which did not lose any epitopes,

responded to D^d with a magnitude similar to the $CD4^-$ cells (Figure 2). The latter result clearly suggests that this region of CD4 could play an important role in the structural integrity of CD4 and in its interaction with class II.

Co-receptor Stimulation

In the present study, we confirm that the presence of the CD4 molecule in the 3DT52.5.8 T cell hybridomas can enhance the T cell response to the sub-optimal concentrations of SEB (11). Cells expressing CD4 require about 10 fold less SEB (700 pg/ml) for a half maximal stimulation when compared to the $CD4^-$ cells (7000 pg/ml). Moreover, the IL-2 production of the $CD4^-$ cells (4000 U/ml) represents only 10% of that of the WT $CD4^+$ cells (48,000 U/ml) (data not shown and Table IV). This enhancement has been demonstrated in I1B3 (human CD4 WT^+) (11), and two other independently transfected cell lines expressing the human CD4 WT- B57 and E10 (Figure 3-Panel A).

D1 Mutants

Mutants S19Y, Q89L and 165[SR] which were already shown in the co-ligand assay as no longer being able to enhance the 3DT response to D^d (10) were also studied in the SEB assay. These mutants gave a significantly reduced response to SEB, since they reproducibly required 3-7 times more SEB for a half maximal response (n=3); moreover at the EC50 of the $CD4^+$ cells (700 pg/ml) IL-2 production by the CD4 mutants reach about 4-20% of the WT $CD4^+$ cells (Figure 3-panel C, F; Table IV). Thus, these CD4 mutants display a phenotype similar to that of the $CD4^-$ cells. These results indicate that at least for these residues mutations which affect the CD4/class II interaction in a co-ligand assay also affect it in the co-receptor assay, further suggesting that the same contact residues were shared in the two functional assays. Surprisingly, when we tested the CDR2 deletion mutant (residues 43-49 of D1), we found that it could no longer enhance the T cell response to SEB. Indeed cells expressing this mutant needed at least 7 times more SEB for a half

maximal response. Moreover their IL-2 production was only 7% at the EC50 (700 pg/ml) of the WT $CD4^+$ cells (Figure 3-panel D and Table IV). This is different from the co-ligand assay results which showed that the CDR2 deletion mutant could still interact with different human and mouse class II alleles (10, 11). This result indicates that the contact sites on CD4 for class II in a co-ligand binding may not always be consistent with those involved in a co-receptor binding.

All the D1 alanine scan mutants except one (K90A) studied in the co-receptor assay, responded to SEB with a magnitude similar to that of the $CD4^-$ cells. These CD4 mutants require 3-10 fold more SEB for a half maximal response, while the IL-2 ratios are only 4-20% of the CD4 WT^+ cells (Table IV). This suggests that a relative large surface on the CD4 molecule is involved in the co-receptor contact with class II. This surface includes residues from both lateral faces of CD4. On one face it consists of residue K1, K2, K7, T15, T17, S19, Q20, K21, S23, D88 and Q89, which are from the A strand, the B strand, the CDR1 and CDR3 regions of D1. On the other face, it is composed of residues from the CDR2 or outside of the CDR2 region, i.e., residue K46 and E77. Comparison of the CD4 contact residues mapped from the co-receptor assay with those from the co-ligand assay reveals some discrepancies. Residues T17A, S19A, K21A and S23A are implicated only in the co-receptor assay but not in the co-ligand assay. The only mutant which did not show any effect in both assays was K90A (Figure 3-panel F), suggesting that this residue is not a direct contact residue for class II.

D2 Mutants

All the alanine scan mutants for residues located in the FG loop of D2, were studied in the co-receptor assay (Table IV and Figure 3-panel G, H, I, J). The Q165A mutation affects the CD4/class II interaction in a similar way to 165[SR], further confirming the involvement of residue 165 in the interaction with class II. The other mutations which affect

the class II binding are Q163A, K166A and K167A. These mutants require 4-6 fold more SEB for the half maximal response, and the IL-2 ratios are 10-40% of the WT $CD4^+$ cells. However, mutant N164A can still enhance the SEB response to the level comparable to CD4 WT (Table IV). Furthermore, residue K167 appears to be differentially required in the two assays. Mutant K167A enhances the D^d stimulation as well as CD4 WT^+ cells, as shown by an IL-2 ratio of 1.09 between the mutant and the wild type. However, this mutant does not enhance the SEB response. It requires about 5.8 fold more SEB to reach the half maximal response and IL-2 ratio is only 20% in the SEB assay (Table IV).

Hinge Region Mutants

All the mutations we generated in the hinge region showed an effect on the SEB response. Mutants V175A, V176A, L177A, VVL175-177A, Q180A, QK180-181A and Deletion 180-181 showed a reduced response to SEB (Figure 1C-panel K, L, M, N; Table IV). At least 3-10 times more SEB were required to obtain the half maximal response for cells expressing these mutant CD4 molecules. Moreover, the IL-2 ratios of the mutants are between 1-20% when compared to the CD4 WT. However, the implication of mutants Q180A and QK180-181A in the co-receptor assay differs from the co-ligand assay. They affect the CD4/class II interaction in the co-receptor assay, although they have no effect in the co-ligand assay. Moreover, the deletion mutant DEL 175-177 responded poorly to SEB when compared to the CD4 WT (Figure 3-panel K). In fact, this mutation has affected most of the antibody epitopes (Table III). This weak response might be due to a poor interaction between CD4 and class II molecules; alternatively it could be due to the interference of CD4 interaction with other cell surface molecules on T cells, such as TCR or CD45.

Discussion

Based on the assumption that CD4 might use different structural components to interact with class II molecules, depending on its functional role as co-ligand vs co-receptor, it is therefore worthwhile to investigate this issue by using the 3DT system. The advantage of the 3DT system is that the TCR has a dual specificity. Indeed this system allows us to study the two functional roles of CD4, co-ligand and co-receptor. It is of great interest to investigate and compare the molecular nature of the potentially different modes of CD4/class II interaction using the same cellular system.

Results from the two assays have involved residues from both the lateral faces of D1 and from the top part of D2 in interaction with class II molecules. Interestingly, as most residues implicated in both functional assay systems are quite concordant, differences do exist. The discordant residues lie mainly in the CDR1 region of the D1 domain, such as residues T17, S19, K21 and S23, which are implicated in the co-receptor assay, but not in the co-ligand assay. The same pattern was noticed for residues K167 in the FG loop of D2. Computer modeling of the CD4 crystal structure containing the D1 and D2 domains were generated (Figure 4). They reveal that those residues from the D1 and D2 domains, i.e., K1, K2, K7, T15, E87, D88, Q89 from D1, and residues Q163, Q165 and K166 from D2, which when mutated have an effect in both the co-ligand and co-receptor assay, are located on the same face of the CD4 molecule (Figure 4A). Furthermore, a side view of the crystal (Figure 4B) shows that most of those residues cluster together, thus their implication in forming a binding site for class II is likely.

The finding that more residues are implicated in the co-receptor assay than in the co-ligand assay, suggests that a wider surface of CD4 is implicated in the co-receptor than in the co-ligand function. The exact nature for this is not known, however, it may be attributed

to the differences between the two functions of CD4. In the co-receptor function of CD4, TCR may bind to the same class II molecule as CD4 binds to. Co-clustering of CD4 and TCR occurring on the T cell surface has been shown during antigen recognition (33, 34). This interaction is essential for T cell activation. Thus, it is possible that some of the mutations in CD4 may induce conformational changes that will affect the docking of CD4/TCR, rather than affect the CD4/class II interaction directly.

Various approaches have been used to investigate the role of the CDR2 region of CD4 in its interaction with class II MHC molecules. However, different systems provided controversial results (10, 11, 13, 14, 35). In the present study some very interesting results were revealed regarding the CDR2 region of CD4 and its implication in binding to class II. First, the deletion mutant DEL 43-49, which still interacts with different class II alleles from both human and mouse in a co-ligand assay (11), can no longer enhance the IL-2 response in a co-receptor assay, suggesting that the CD4 molecule may use distinct structural components to bind to class II in either function. Moreover, single mutants K46A and E77A affect the binding to class II in both co-ligand and co-receptor function of CD4. This result is consistent with our data from rosette formation and co-ligand assay for mutants T45P, K46N/G47V and G47R which also seem to affect the CD4/class II interaction (10). From this study, it seems that the CDR2 region is mainly implicated in the co-receptor function, rather than its co-ligand function. A recent study using synthetic aromatically modified exocyclic analogs of CD4 supports our finding (36). These CD4 analogs were tested in a competition binding assay for their ability to inhibit the binding of recombinant soluble ^{125}I-labeled human class II DR4 to immobilized recombinant soluble CD4. They were also tested for their ability to inhibit T cell activation. Results show that a CDR3 analog is able to bind to the β2 domain residues 134-148 of class II. However, CDR2 analogs only show slight effect in inhibiting the CD4/class II binding and T cell activation. Thus, a direct involvement

of the CDR2 region in class II contact, at least for the known β2 region sequence of class II, is not supported.

In the co-ligand assay, CD4 and the TCR bind to their respective MHC ligands, whereas in the co-receptor assay both the TCR and CD4 bind to the same class II MHC molecule. The difference between these two functions of CD4 thus can be attributed to the physical proximity of CD4 and the TCR in one function (co-receptor) but not the other (co-ligand). Therefore, the CDR2 region could be a docking site for the TCR during the co-receptor function of CD4. This could explain why the CDR2 deletion mutant affects only the co-receptor function but not the co-ligand function. However, results of single mutants showing their implication in both functions of CD4, and results from adhesion assay in which the binding of CD4/class II is TCR-independent, do not favor such a model (Figure 5-A).

The recently solved crystal structure of the human class II DR1 molecule showed it as a dimer of the αβ heterodimer (37). If this dimer is formed on the cell surface upon antigen stimulation of T cells, it would be logical to assume that CD4 could interact with a class II dimer using both faces. As suggested in a recent paper (9), that class II molecule DR1 could use both the α2 and β2 domains to interact with CD4, however, both the α2 and β2 domains could not bind to a single CD4 because they are 180° away. Thus, one possibility is that one face of CD4 is involved in binding to class II α2 domain, another face is involved in binding to class II β2 domain. This assumption can be tested in complementation studies using both CD4 mutants and class II mutants (Figure 5-B).

Alternatively, it has been suggested that CD4 itself could dimerize (20, 32), the implication of two faces of CD4 in the above described functional assays can thus be interpreted as one face being involved in the actual contact site with class II, while the opposite one is involved in dimerization of CD4 itself. Based on the structural and functional

distinction between the two assays, we can propose the following: in the co-ligand assay, dimerization or oligomerization of CD4 might not be required since CD4 and TCR binds to different MHC molecules, there might not be any specifically organized CD4 and/or class II dimerization or oligomerization event; however, in the co-receptor assay, such a specifically organized CD4 and/or class II dimerization or oligomerization event could be induced to happen, since CD4 and TCR bind to the same class II molecule. Whether this assumption is the reflection of the physiological situation needs further studies (Figure 5-C).

It is clear that the contact sites for class II involve multiple faces of CD4, and that a bigger region is implicated in the co-receptor than in the co-ligand function of CD4. At least three molecular interactions are involved in the former function: CD4, TCR and class II, which could explain the fact that they are more sensitive to any structural changes. It would not be surprising to find out that some of the residues mapped are the contact residues, while others are just docking residues. However, this can only be solved by the co-crystallization of CD4 and class II. It could also be possible for a specifically organized oligomerization of the three molecules to occur in the co-receptor assay, but, not in the co-ligand assay. Furthermore, and consistent with our results, studies on CD8$\alpha\alpha$ mutants revealed a multiple faces involved in interaction with class I MHC molecules, suggesting that CD4 and CD8 use several faces of the same structural unit to interact with their respective ligands (38).

The hinge region of CD4 and its functional importance in the light of class II interaction was studied here for the first time. Due to lack of crystallographic data of the hinge region, the elucidation of the structure relies mostly on the antibody epitope mapping. Serological studies on hinge region mutants have shown that many epitopes throughout D1-D4 domains are affected by mutagenesis in this region. Furthermore, this study shows that mutations in the hinge region also affect the CD4/class II interaction drastically, regardless

of the co-ligand or co-receptor function of CD4. The abrogation of class II binding following by mutations, especially single amino acid substitutions, indicates that these residues are closely involved in maintaining a structure essential for class II interaction. These hinge residues may not be the directly contact residues for class II, as overall conformational changes occur as the result of mutations. Rather, it could suggest that loss of CD4 function may be a consequence of interfering the interaction of CD4 with other surface molecules on the T cells, such as the TCR, or CD45; alternatively, these perturbations could interfere with the possible dimerization /oligomerization of CD4. At this stage we do not know the exact mechanism for such a drastic functional effect by the mutations. Nevertheless, this result indicates a very important role for the hinge region in maintaining the overall structure of CD4, and that such a conformation is essential for CD4 to fulfill its function.

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4. Abbreviations used in this paper:

Ag: Antigen
APC: Antigen presenting cells
CDR1: Complementary Determining Region 1
CDR2: Complementary Determining Region 2
CDR3: Complementary Determining Region 3
D1: Domain 1
D2: Domain 2
D3: Domain 3
D4: Domain 4
Del: deletion

HIV: Human immunodeficiency virus

Ig: Immunoglobulin

IL-2: Interleukin 2

mAb: Monoclonal antibody

MHC: Major Histocompatibility Complex

SEB: Staphylococcal enterotoxin B

TCR: T cell receptor

WT: wild type

Table I. Summary of Antibodies and The Epitopes

mAb Name (a)	Epitope Domain of CD4 (b)	Epitope Amino Acid (c)
OKT4	D3	
OKT4C	D1, D3, D4.	20/27, 57, 89, (277-280), (328-330), (349-356), (363-369).
OKT4D	D1, D4	20, 47, 55, 57, 89, (349-356).
OKT4E	D4	(363-369).
OKT4F	D1, D4	48, 55, 57, (349-356).
B66.6.1	D1, D2	20/27, 23/24/27, 38, 40, 45, 47, 46/47, 42-49, 57, (99-105).
L34	D1	26-83.
L110	D1	26-83.
L71	D1	83-92.
L83	D1, D2	26-83, 120-202.
L116	D1, D2	1-25, 120-202.
MT425	D1	
MT407	D1, D2, D3	19, 20, 46/47, 47, 55, 57, 60, 165, (39-43), (48-52), (99-105), (121-123), (277-280).
Q425	D3	D strand of D3, 230-235.
MT151	D1, D2, D4	38, 94, 165, (328-330), (349-356), (363-369).
IF3	D1	gp120 binding site.
Leu3a	D1	Del 42-49, 57.

(a) Names of antibodies and (b) the domains of CD4 which bind to. (c) The epitopes were characterized from previous mutagenesis studies and are displayed as the position of amino acid residues which when mutated will affect the binding of the antibody. The number represents the position of the residue on the human CD4 molecule, and the number in bracket represents residues that when substituted with the murine counterparts affect the epitope.

Table II. Serological Analysis of D1 and D2 Mutants

	Antibody (b)						
Cells (a)	OKT4	L34	L110	L71	L83	L116	MT425
CD4 WT	+	+	+	+	+	+	+
D1 MUTANTS							
K1A	+	+	+	+	+	+	+
K2A	+	+	+	+	+	+	+
K7A	+	+	+	+	+/-	+	+
T15A	+	+	+	+	+	+	+
T17A	+	+/-	+	+	+	+/-	+
S19A	+	+	+	+	+	+	+
Q20A	+	+	+	+/-	+	+	+
K21A	+	+	+	+	+	+	+
S23A	+	+	+	+	+	+	+
K46A	+	+	+	+	+	+	+
E77A	+	+	+	+	+	+	+
D88A	+	+	+	+	+	+	+
Q89A	+	+	+	+	+	+	+
K90A	+	+	+	+	+	+	+
D2 MUTANTS							
Q163A	+	+	+	+	+	+	+
N164A	+	+	+	+	+	+	+
Q165A	+	+	+	+	+	+	+
K166A	+	+	+	+	+	+	+
K167A	+	+	+	+	+	+	+

(a) Mutants are designed by the name of the residue (one letter amino acid code), its position on the sequence, followed by the name of the substituting residue. Del stands for deletion mutation.

(b) Murine T cell hybridoma 3DT52.5.8 expressing human CD4 or mutant CD4 were subjected to indirect immunofluorescent staining with all the antibodies listed here at concentration saturating for the CD4 WT. Analysis were carried out with FACScan flow cytometer. Results were calculated as follows: {[mt FL (mAb) - mt FL (neg)] / [mt FL (OKT4) - mt FL (neg)]} / {[WT FL (mAb) -WT FL (neg)] / [WT FL (OKT4) - WT FL (neg)]}. FL stands for mean fluorescence value, mt stands for a given CD4 mutant, WT stands for the wild type CD4, neg stands for the negative control. Symbols for binding: +: > 50%; +/-: 30-50%; -: < 30%.

Table III. Serological Analysis of The Hinge Region Mutants

Cells (a)	Antibody (b)																
	OKT4	OKT4C	OKT4D	OKT4E	OKT4F	B66.6.1	L34	L110	L71	L83	L116	MT425	MT407	Q425	MT151	IF3	LEU3A
CD4 WT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
V175A	+	+/-	+/-	+	+/-	+	+	+	+	+/-	+	+	+	-	+	+	+
V176A	+	+	+	+	+	+	+	+/-	+	+	+/-	+	+	+	+	+/-	+
L177A	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DEL 175-177	+	+/-	+/-	+	+/-	+/-	+/-	+/-	+/-	+/-	-	+/-	-	-	+	-	+/-
Q180A	+	+	+	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+
QK180-181A	+	+	+/-	+	+	+	+	+/-	+	+	+/-	+	+	+/-	+	+	+
DEL 180-181	+	+	+/-	+	+/-	+	+	+	+	+	+/-	+	-	+	+	+	+

(a) name of the mutant.

(b) Fluorescent analysis. Calculations and symbols are the same as described in Table II.

Table IV. Summary of The CD4 Mutants

Cells (a)	Expression level (mt/WT) (b)			Stimulation (EC50 Ratio: mt/wt) (c)			IL-2 Ratio-SEB mt/wt (d)	IL-2 Ratio-Dd mt/wt (e)
	OKT4	KJ12	F23.1	KJ12	F23.1	SEB		
WT	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$CD4^-$	-	0.8	0.9	0.6	1.1	4.6	0.1	0.2
D1								
K1A	0.6	1.9	1.1	0.9	1.4	3.0	0.1	0.3
K2A	0.9	0.6	1.0	1.2	1.3	3.0	0.1	0.2
K7A	0.5	0.7	0.7	1.1	1.3	6.7	0.1	0.2
T15A	1.0	0.9	0.9	ND	1.6	4.9	0.1	0.2
T17A	0.9	1.0	0.9	1.2	0.6	>10	0.1	1.2
S19A	1.0	1.3	0.8	1.3	1.5	5.1	0.2	1.5
S19Y	0.5	2.0	1.5	ND	2.0	6.0	0.1	0.3
Q20A	0.8	0.8	0.6	1.2	2.5	6.3	0.1	0.3
K21A	0.7	0.8	0.8	ND	ND	4.4	0.1	0.9
S23A	0.7	0.8	0.8	ND	ND	5.1	0.2	1.0
D 43-49	1.1	1.4	1.6	1.1	1.2	5.0	0.1	1.1
K46A	1.1	0.8	0.8	1.3	1.1	5.0	0.1	0.1
E77A	1.5	2.6	2.5	0.8	0.9	5.0	0.1	0.1
E87A	0.5	0.8	0.6	1.4	1.4	6.0	0.1	0.2
D88A	1.0	1.0	1.0	1.1	1.4	8.5	0.1	0.2
Q89A	0.9	0.8	1.0	0.8	ND	3.0	0.1	0.2
Q89L	0.8	0.9	0.9	1.1	1	7.0	0.04	0.2
K90A	1.1	2.0	1.1	1.4	1.1	1.3	0.8	1.5
D2								
Q163A	0.7	0.7	0.7	1.2	1.0	4.3	0.1	0.1
N164A	0.8	1.3	1.0	1.4	1.2	1.2	0.8	1.4
Q165A	0.5	0.9	0.6	1.2	1.3	3.6	0.2	0.2
165[SR]	0.5	0.9	1.1	1.2	0.8	3.0	0.2	0.1
K166A	1.0	1.7	1.7	1.0	0.9	4.5	0.4	0.3
K167A	1.0	1.6	0.9	0.7	1.4	5.8	0.2	1.1
Hinge								
V175A	0.4	0.7	0.7	0.9	0.9	3.3	0.1	0.1
V176A	2.2	1.3	1.3	ND	1.3	4.3	0.1	0.1
L177A	1.2	0.7	0.7	1.6	1.3	6.7	0.1	0.1
VVL175-7A	0.7	0.9	1.0	ND	1.6	4.9	0.1	0.2
D 175-7	0.5	0.8	0.9	1.2	0.6	>10	0.01	0.1
Q180A	0.9	1.5	1.6	ND	0.6	3.5	0.2	2.6
QK180-1A	0.8	1.6	1.7	ND	1.5	4.0	0.1	0.9
D 180-1	0.7	1.9	1.7	ND	1.6	2.8	0.1	0.2

(a) Names of the mutant.

(b) Expression levels for CD4 (OKT4), TCR (KJ12 and F23.1) are defined as the mean fluorescence value of a given mutant divided by that value of the WT^+ cells.

(c) Stimulations by anti-TCR mAbs (KJ12 and F23.1) and SEB were defined as the EC50 ratios. The obtaining of EC50 value and calculation of EC50 ratios are described in M & M.

(d) and (e) The IL-2 ratio-SEB mt/WT and IL-2 ratio-D^d mt/WT are also defined in M & M.

Figure Legends:

Figure 1. Schematic representation of the two functions of CD4 in the 3DT cell system: co-ligand vs co-receptor. In the co-ligand system, CD4 and TCR bind to different classes of MHC molecules, that is, the TCR recognizes the class I cognate alloantigen H-2D^d, while CD4 interacts with the class II MHC molecules DR4. In the co-receptor system, the TCR and CD4 can both bind to the class II molecule DR1 in the presence of the bacterial superantigen SEB. The interaction of CD4 with the class II molecules can enhance the T cell response in both cases.

Figure 2. Quantitative analysis of the interaction between the CD4 mutants and the class II MHC molecules in the co-ligand D^d assay. The 3DT52.5.8 T cell hybridomas expressing the WT CD4 or different CD4 mutants in D1, D2 and hinge region were analyzed for their ability to produce IL-2, when co-cultured with DAP-3 cells expressing either D^d and D^d DR4. Results were presented as such: the Y axis is expressed as the IL-2 ratio (D^d DR4/D^d), as described in the materials and methods section. On the X axis, positions of the mutated residues are indicated. Unless specifically indicated, mutants shown here are alanine substitution mutants.

Figure 3. Quantitative analysis of the interaction between the CD4 mutants and the class II MHC molecules in the co-receptor SEB assay. D1, D2 and hinge region mutants were stimulated with the bacterial superantigen SEB presented by human class II molecules DR1 expressed on the DAP-3 cells. The SEB concentration displayed on X-axis are: 10^5, 2.5 X 10^4, 1.25 X 10^4, 6.25 X 10^3, 3.13 X 10^3, 1.56 X 10^3, 7.8 X 10^2, 3.9 X 10^2, 1.95 X 10^2, 0.98 X 10^2 pg/ml. On the Y-axis the IL-2 units are presented. A. Panel A to F: D1 mutants; B. Panel G to J: D2 mutants; C. Panel K to N: the hinge region mutants.

Figure 4. Computer model. A. On the crystal of the D1D2 fragment, the residues which are implicated in both the co-ligand and co-receptor assays are displayed. On one face it shows residues K1, K2, K7, T15,

E87, D88, Q89 from the D1 domain, and residues Q163, Q165 and K166 from D2; while on the opposite face E77 is presented. B. The side view of the crystal of the D1D2 fragment. Most of the residues implicated in class II binding from both assays are shown here to form a cluster on the surface.

Figure 5. Proposed models of CD4/class II interaction. A. The CD4 molecule uses its residues from the face composing of the CDR1 and CDR3 region to interact with the β2 region of the class II MHC molecule, while the CDR2 region is involved in the interaction with the T cell receptor. B. CD4 uses both faces to interact with a class II superdimer which contains two class II αβ dimers. On one face it contains residues from the CDR1 and CDR3 regions, on the opposite face it contains the CDR2 region. C. CD4 dimerizes through the face containing the CDR2 region, while the opposite face containing the CDR1 and CDR3 regions are involved in binding to the β2 domain of class II.

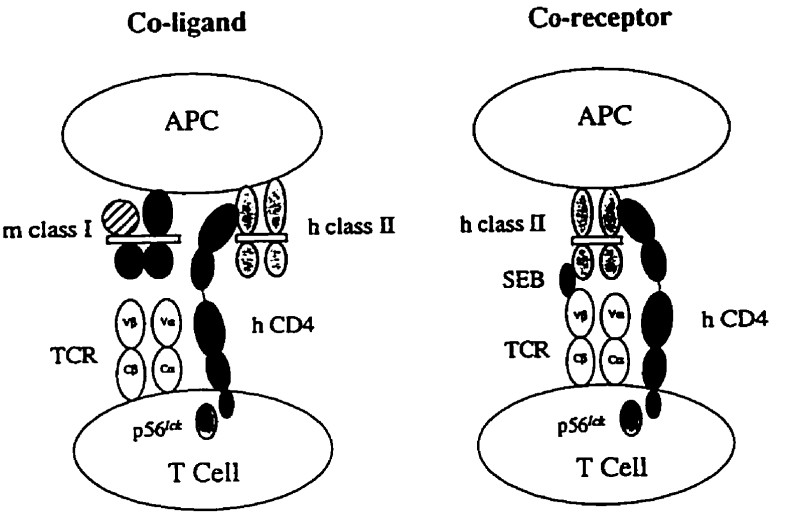
Co-ligand
APC
m class I
h class II
TCR
h CD4
p56lck
T Cell
Co-receptor
APC
h class II
SEB
TCR
h CD4
p56lck
T Cell

Figure 1

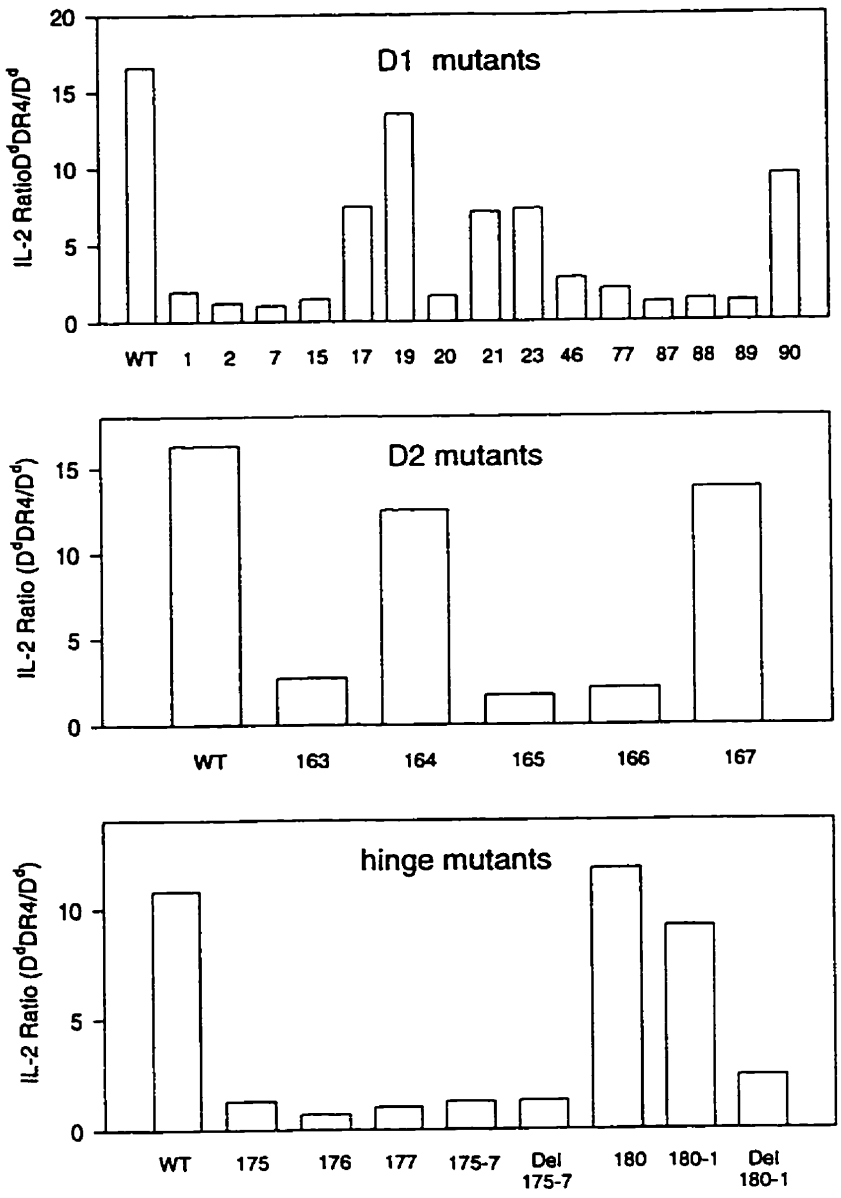
D1 mutants
IL-2 RatioD^{d}DR4/D^d
20
15
10
5
0
WT 1 2 7 15 17 19 20 21 23 46 77 87 88 89 90
D2 mutants
IL-2 Ratio (D^{d}DR4/D^d)
15
10
5
0
WT 163 164 165 166 167
hinge mutants
IL-2 Ratio (D^{d}DR4/D^d)
10
5
0
WT 175 176 177 175-7 Del 175-7 180 180-1 Del 180-1

Figure 2

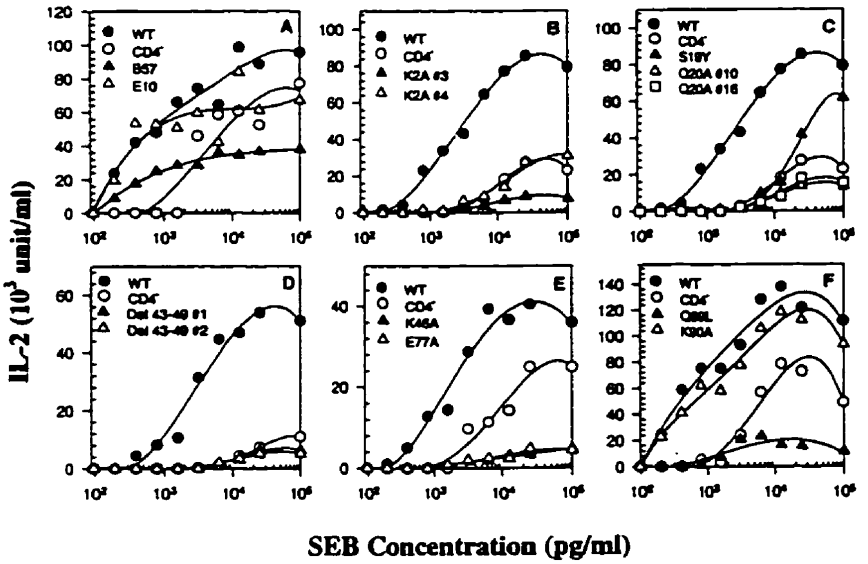
IL-2 (10^3 unit/ml)
SEB Concentration (pg/ml)
A
WT
CD4-
B57
E10
B
WT
CD4-
K2A #3
K2A #4
C
WT
CD4-
S18Y
Q20A #10
Q20A #16
D
WT
CD4-
Del 43-49 #1
Del 43-49 #2
E
WT
CD4-
K46A
E77A
F
WT
CD4-
Q99L
K30A

Figure 3A

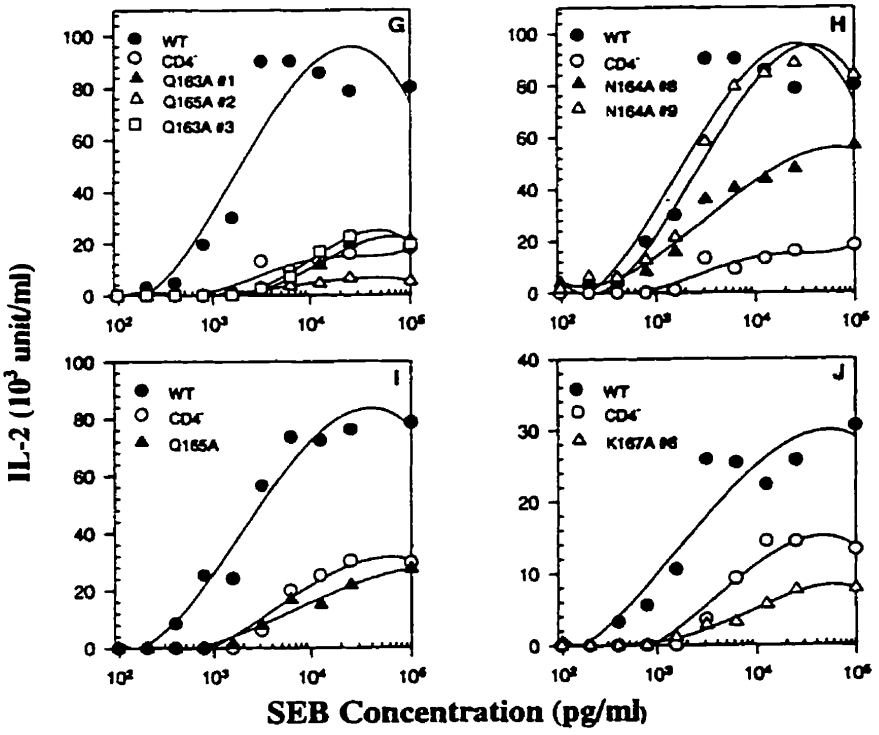
G
WT
CD4-
Q163A #1
Q165A #2
Q163A #3
H
WT
CD4-
N164A #8
N164A #9
I
WT
CD4-
Q165A
J
WT
CD4-
K167A #6
IL-2 (10³ unit/ml)
SEB Concentration (pg/ml)

Figure 3B

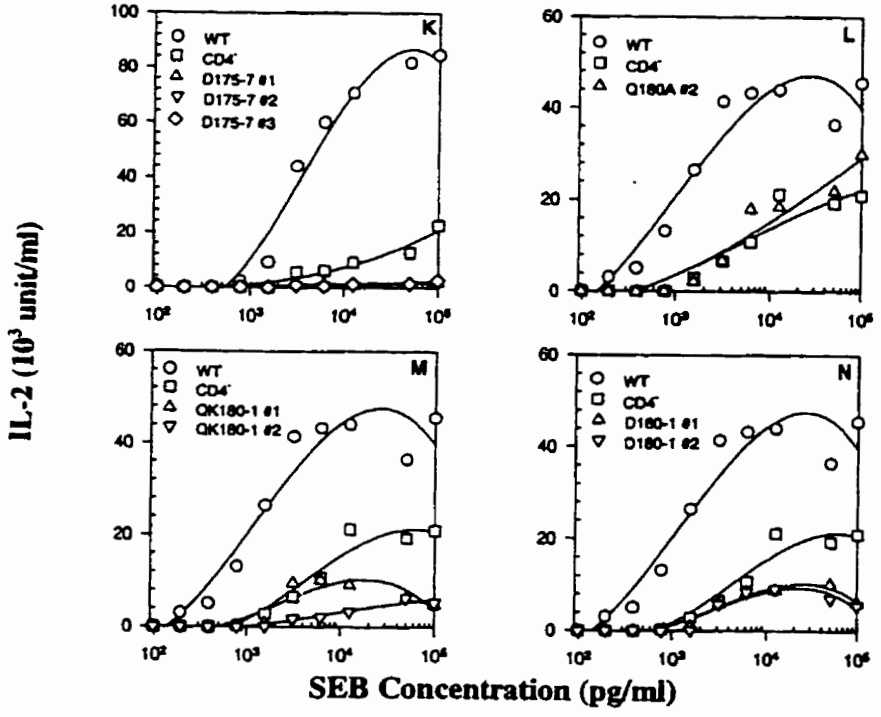
IL-2 (10^3 unit/ml)
K
WT
CD4-
D175-7 #1
D175-7 #2
D175-7 #3
L
WT
CD4-
Q180A #2
M
WT
CD4-
QK180-1 #1
QK180-1 #2
N
WT
CD4-
D180-1 #1
D180-1 #2
SEB Concentration (pg/ml)

Figure 3C

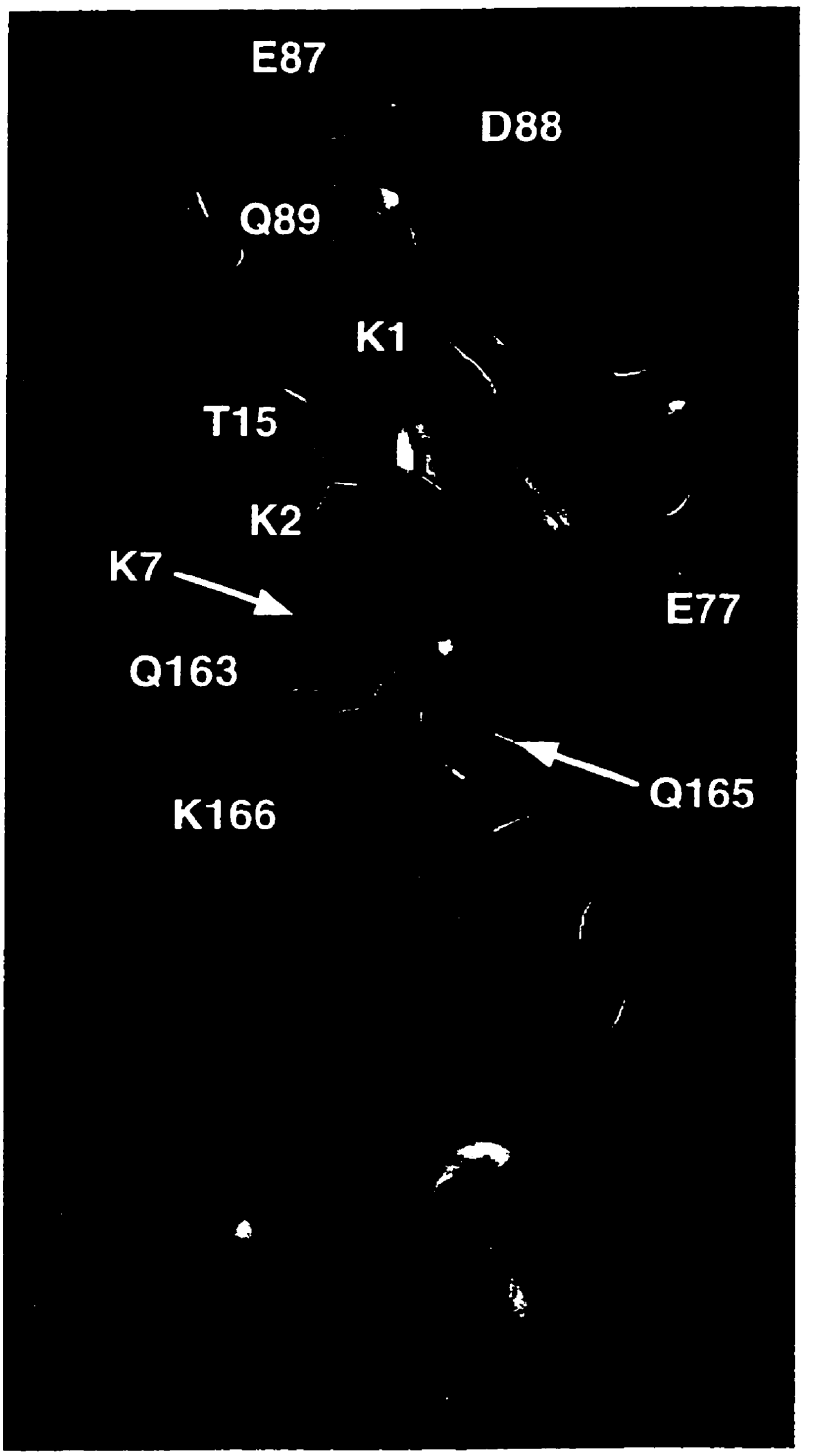

Figure 4A

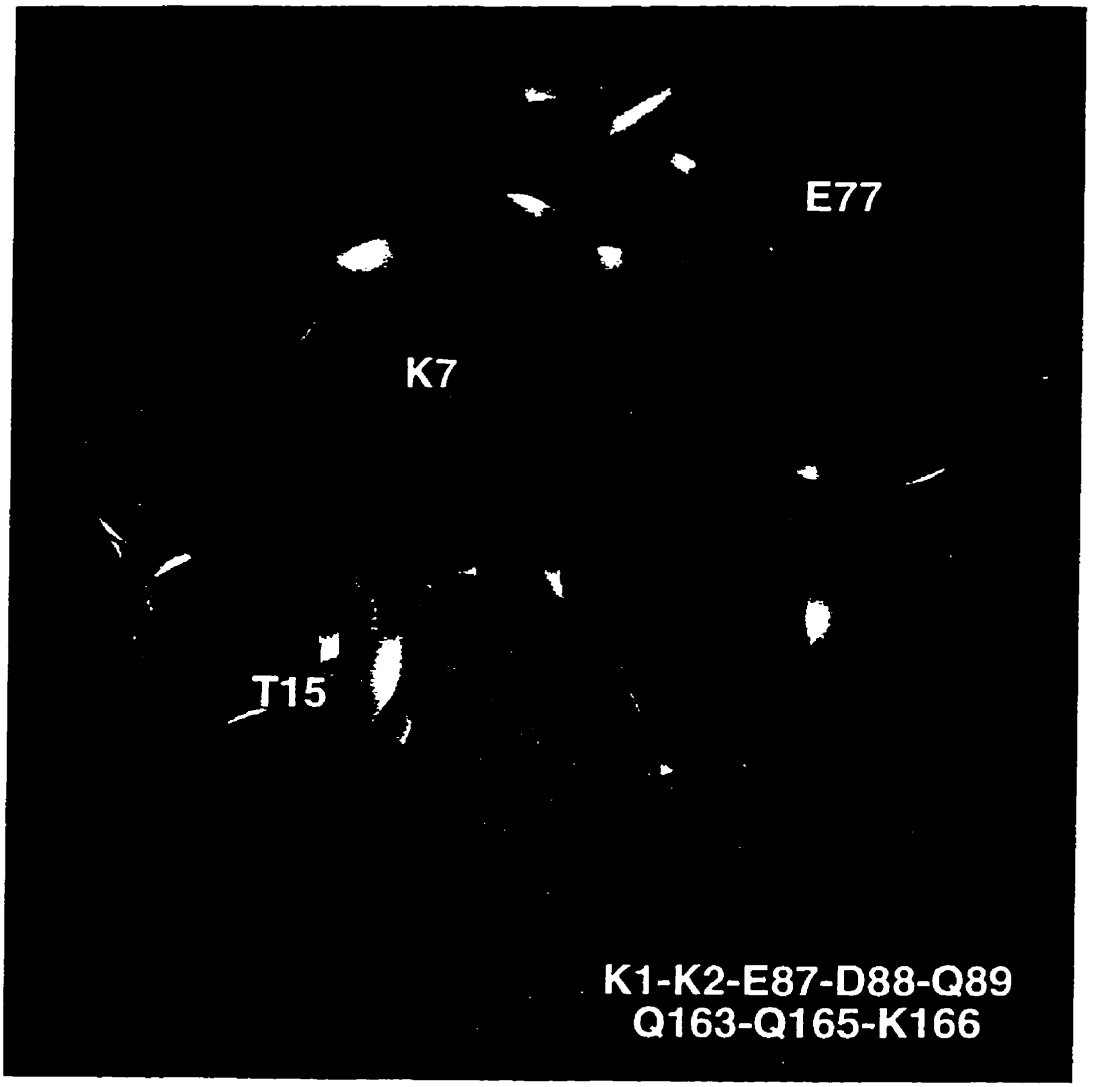

Figure 4B

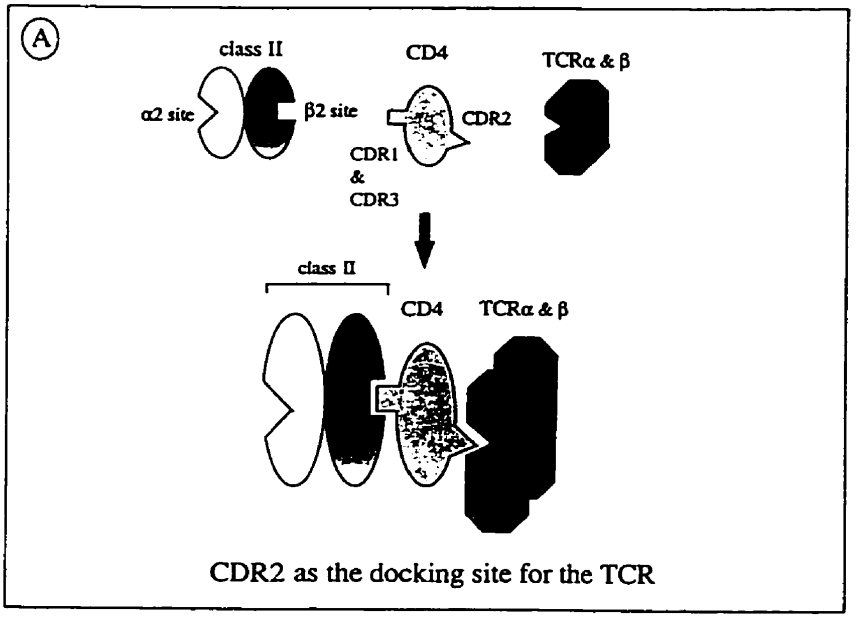
A
class II
CD4
TCRα & β
α2 site
β2 site
CDR2
CDR1
&
CDR3
class II
CD4
TCRα & β
CDR2 as the docking site for the TCR

Figure 5A

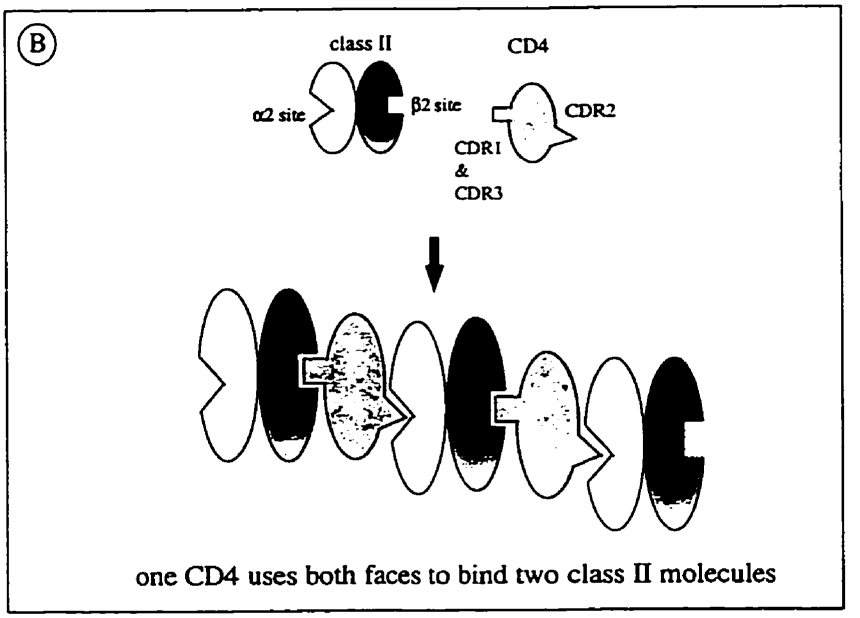
B
class II
CD4
α2 site
β2 site
CDR2
CDR1
&
CDR3
one CD4 uses both faces to bind two class II molecules

Figure 5B

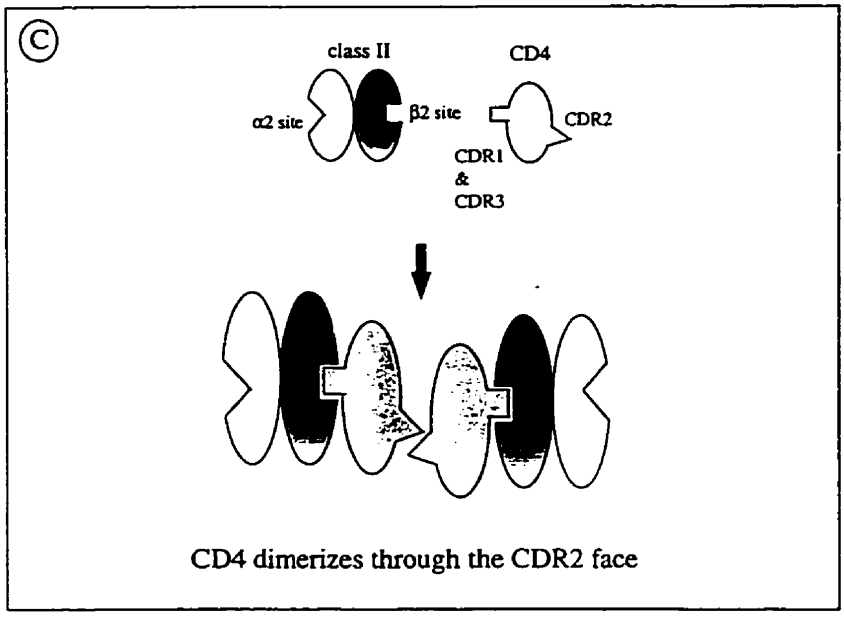
©
class II
CD4
α2 site
β2 site
CDR2
CDR1
&
CDR3
CD4 dimerizes through the CDR2 face

Figure 5C

Chapter 3

Mutations in Human CD4 impair the functional interaction with different human and mouse class II isotypes and alleles

Sylvain Fleury, Bei Huang, Anne Zerbib, Cilbert Croteau, Eric O. Long, and Rafick-Pierre Sekaly

Preface

Previous studies using certain cellular systems have showed that murine CD4 can not interact with human class II efficiently. However, other in vivo and in vitro experiments results have also shown that such a cross-species interaction does occur. Here we try to address this question of inter-species interaction between human and mouse CD4/class II from a structural and functional point of view. We investigate if such an inter-species interaction occurs between CD4 and different class II isotypes and alleles, and the structural basis of such an interaction.

Mutations in Human CD4 Impair the Functional Interaction with Different Human and Mouse Class II Isotypes and Alleles[1]

Sylvain Fleury,[2,3*†] Bei Huang,[2*‡] Anne Zerbib,[*§] Gilbert Croteau,[*] Eric O. Long,[‖] and Rafick-Pierre Sékaly[4*†‡]

The structure-function of the CD4-class II MHC interaction was investigated. Two functional assays were used to assess the responses of the 3DT52.5.8 murine T cell hybridoma expressing human CD4 (h-CD4) or murine CD4 (m-CD4). First, we determined the responses of the CD4$^+$ and CD4$^-$ effector cells toward DAP-3 cells co-expressing the cognate alloantigen H-2D^d together with several human (DRw52b, DR4-Dw4, DR2A, and DPw2) and murine (I-A^b, I-A^k, I-Aα^bI-Aβ^k and I-E^k) class II alleles and isotypes. We found that h-CD4 and m-CD4 strongly enhance the T cell response to H-2D^d, demonstrating that interspecies CD4/class II interactions occur efficiently. Furthermore, mutations in h-CD4 at positions 19, 89, and 165 markedly reduced the interaction with both human class II and mouse class II, indicating that the structural features of this cross-species interaction are strongly conserved. This was further supported by the finding that a h-CD4 deletion mutant (deletion F43-S49) interacted with both human and murine class II. Moreover, as 3DT cells express the responsive Vβ element for the bacterial superantigen staphylococcal enterotoxin B, a co-receptor assay was conducted. DAP-3 cells expressing only class II molecules were used as APCs to present staphylococcal enterotoxin B to h-CD4$^+$ and m-CD4$^+$ T cells. h-CD4 and m-CD4 were able to enhance the T cell response to staphylococcal enterotoxin B, further demonstrating the conservation of the CD4-class II MHC interaction. *The Journal of Immunology,* **1996, 156: 1848–1855.**

The CD4 molecule is a nonpolymorphic membrane glycoprotein of 55,000 m.w. that consists of four extracellular domains (D1 to D4)[5] that share sequence and structural homology with Ig domains (1). The TCR of CD4$^+$ T cells recognizes Ag only when associated with class II molecules of the MHC (2, 3). Functional studies and adhesion assays have been used to show that CD4 exerts its co-receptor function by binding to a conserved region of MHC class II molecules (4–8). The interaction between CD4 on T cells and class II MHC molecules on APC plays an important role in the selection of the TCR repertoire and activation of T cells in the periphery (9). The extracellular portion of the murine CD4 molecule shows 55% sequence homology with its human counterpart (h-CD4) (10). The D1D2 fragment of h-CD4 (11, 12) and D3D4 of rat CD4 (13) have been crystallized. Sequence comparisons among human, mouse (m-CD4), and rat CD4 showed that residues that are implicated in the overall conformation are conserved, suggesting that h-CD4 and m-CD4 will adopt similar 3D structures.

Although a highly conserved region between human and murine class II MHC (residues 137–143 of the β-chain) was identified to be critical for the interaction with h-CD4 and m-CD4, respectively (14, 15), controversy remains with respect to the efficacy of interspecies interactions of human and murine CD4 with murine or human class II MHC molecules. Two groups have reported that in vitro, human CD4 could substitute the murine counterpart in restoring the function of an Ag-specific, class II-restricted murine T cell hybridoma (16, 17). Using a different approach, Killeen et al. (18) clearly demonstrated that a h-CD4 transgene could phenotypically and functionally reconstitute the CD4$^-$ cell compartment in a m-CD4 knock-out mouse, suggesting that h-CD4 can interact efficiently with mouse class II molecules.

On the other hand, other studies have shown that xenoresponses between h-CD4$^+$ T cells and murine class II$^+$ cells, or vice versa, are very inefficient. These observations led to the suggestion that cross-species interactions between CD4 and class II MHC molecules are limited. This weak xenoresponse can be explained by different mechanisms. During T cell activation, accessory molecule interactions (CD2-LFA-3) between species can be inefficient (19). However, this explanation does not apply to systems using T cells and APCs from the same species. Second, during thymic selection, the TCR repertoire is biased toward the MHC of the species in which they develop, leading to the selection of TCRs with a restricted range of affinity for the host MHC alleles. These

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[5] Abbreviations used in this paper: D1, D2, D3, and D4, domains 1, 2, 3, and 4; CDR1, -2, and -3, complementary determining regions 1, 2, and 3; h-CD4, human CD4 molecule; m-CD4, mouse CD4 molecule; SEB, staphylococcal enterotoxin B; WT, wild type; GAM-FITC, fluoresceinated goat anti-mouse Ig.

0022-1767/96/$02.00

TCRs may have a weaker affinity for MHC products of other species. Finally, second signals mediated through receptors, such as cytokine receptors, that lead to T cell activation may work more efficiently in a particular species rather than between species (20). The last two hypotheses have not yet been directly tested. Several groups have tried to explain the weak T cell xenoresponses by studying accessory molecule functions. However, most of the experiments involved co-receptor assays that fail to discriminate between the interaction of CD4 and TCR with their respective ligands.

To study the interspecies interactions between h- and m-CD4 and class II molecules, we used the 3DT52.5.8 T cell hybridoma, which is $CD4^-CD8^-$ and specific for alloantigen H-$2D^d$. This system has been widely used by other investigators (6–8, 21). The advantage of this cellular system is that it provides a co-ligand assay system in which the CD4-class II interaction can be discriminated from the TCR-MHC/Ag recognition event, since TCR and CD4 recognizes different classes of MHC product. Therefore, it become possible to use this 3DT co-ligand assay to investigate whether different alleles and isotypes of class II from different species can fulfill the same function. Moreover, as the TCR of 3DT cells bears the responsive $V\beta$ element for the bacterial superantigen SEB, it is possible to use this T cell in a co-receptor assay, in which CD4 and TCR recognize the same MHC product. Results from both assays showed that h- and m-CD4 can function to a comparable extent with at least five different human class II MHC and four different murine class II MHC molecules. Mutations in h-CD4 that had been known to abolish or to reduce the interaction with the HLA-DP molecule (8) were also capable of abrogating or reducing the interaction with other human and murine class II MHC molecules.

Materials and Methods

Antibodies

The following Abs were used: mouse anti-Id TCR mAb KJ12 (kindly donated by J. Kappler and P. Marrack), mouse anti-h-CD4 mAb (OKT4), rat anti-mouse CD4 mAb (GK1.5), mouse anti-DRα mAb (L-243), mouse anti-DR and DP mAb (SG-465), mouse anti-I-Eα^k mAb (14.4.4), mouse anti-I-A^b (p77-7-7), and mouse anti I-Aβ^k (10.2.16). The other mAbs were all obtained from the American Type Culture Collection (Rockville, MD) and were used in purified form.

Cell culture

The mouse T cell hybridoma 3DT52.5.8 (6, 21) transfected with cDNA expression constructs encoding h-CD4, m-CD4, or various h-CD4 mutants were maintained in culture medium consisting of RPMI 1640 supplemented with 10% FCS, 10 mM 2-ME, 2 mM L-glutamine, 20 mg/ml gentamicin, and 500 mg/ml G-418 (Life Technologies, Grand Island, NY). The transfectants derived from the DAP-3 mouse fibroblast cells were maintained in DMEM supplemented with 10% FCS, 10 mM 2-ME, 2 mM L-glutamine, 20 mg/ml gentamicin, and the appropriate selective agents, as previously described (7).

Plasmids

Full-length h-CD4 wild-type (WT) or CD4 mutant cDNAs were obtained from different sources (22, 23). The m-CD4 WT cDNA was obtained from D. Littman (24). The h-CD4 cDNAs were prepared as blunt *Bam*HI, *Hin*dIII-*Bam*HI, or *Hin*dIII-*Not*I fragments and subcloned into the pMNC-stuffer retroviral vector. The m-CD4 cDNA was prepared as *Hin*dIII-*Not*I and subcloned into the pMNC-stuffer vector. The amphotropic helper cell line DAMP (5) was transfected with the different pMNC constructs and G-418-resistant DAMP cells were selected. The HLA-DR α-chain cDNA was previously described (7). The HLA-DR β-chain cDNAs corresponding to the different alleles of HLA-DR and HLA-DP-Dw2 $\alpha\beta$-chains were previously described (25–29).

DNA-mediated gene transfer

The $CD4^+$ DAMP cells and DAP-3 cells expressing H-$2D^d$ (D^d), D^d + DRw52b, D^d + DR4-Dw4, D^d + DR2A-Dw2, D^d + DRw11.1, D^d + DP-Dw2, D^d + I-A^b, D^d + I-A^k, D^d + I-Aα^bI-Aβ^k, D^d + I-E^k, DRw52b, DR4, DR1, and I-E^k were generated by the calcium phosphate co-precipitation technique (30) as previously described (7, 8). Homogeneous populations of transfectants expressing comparable levels of H-$2D^d$ and class II molecules were obtained by sorting. DAP cells expressing I-A^b (FT7.1C6), I-Aα^bI-Aβ^k (FT5.7H2C4C4), and I-E^k (DCEK) were obtained from R. N. Germain; I-A^k (Ca259) was obtained from B. Malissen. The different CD4 mutants have been previously described (8). For infection of the 3DT52.5.8 hybridoma, G-418-resistant populations of DAMP cells expressing the mutant CD4 molecules (>50% positive) were used for coculture with the 3DT52.5.8 cells (31). The transfected DAMP cells were used at 70% confluence, washed twice with PBS, and treated with 100 mg/ml of mitomycin C for 1 h at 37°C before infection. Cells were then washed three times with PBS containing 2% FCS and cocultured with 10^6 3DT52.5.8 cells in complete RPMI medium in the presence of 6 mg/ml of Polybrene for 24 h at 37°C. The 3DT52.5.8 cells were washed once with PBS and resuspended in complete RPMI medium for 24 h at 37°C. The cells were then plated at a density of 50,000 cells/well in 24-well plates in complete RPMI medium containing 1.5 mg/ml of G-418 (specific activity). Medium was changed every 3 days, and G-418-resistant 3DT52.5.8 cells were analyzed for expression of the mutated CD4 molecule by flow cytometry.

Cytofluorometric analysis of cells

Cells (5×10^5) were incubated for 30 min at 4°C with 100 ml of the appropriate mAb (2–10 mg/ml) diluted in complete medium. Following washes in PBS, cells were incubated with fluorescein-coupled goat anti-mouse Ig (Becton Dickinson Co., Mountain View, CA) for 30 min at 4°C. The cells were then washed, resuspended in PBS containing 2% FCS, and analyzed for fluorescence using a 4-decade logarithmic scale FACScan flow cytometer. Dead cells were excluded by propidium iodide (0.5 mg/ml) gating, and 10,000 cells were acquired for the analysis. As a control, cells were stained with the fluoresceinated goat anti-mouse Ig (GAM-FITC) only.

IL-2 assay and stimulation of effector cells

In the co-ligand assay, 3DT52.5.8 cells (7.5×10^4) expressing different CD4 molecules were cocultured overnight with 7.5×10^4 DAP-3 cells expressing D^d alone or D^d plus one of the different class II MHC molecules mentioned above. The responses of the $CD4^+$ T cell hybridomas to target cells expressing different human and murine class II molecules are displayed as such. On the *x*-axis, the names of different APCs are given; on the *y*-axis, scales are expressed either as the absolute IL-2 units produced by the effector cells or the IL-2 ratio calculated from the IL-2 value obtained in the presence of class II molecules (DAP D^d DRw52b, for example) over the value obtained in their absence (DAP D^d only). In the co-receptor assay, 7.5×10^4 effector cells expressing h-CD4, m-CD4, or CD4 mutant were cocultured with 2×10^4 DAP-3 cells expressing either human class II DR1 or DR4, or murine class II I-E^K in 200 ml of complete medium overnight at 37°C in 96-well flat-bottom culture plates in the presence of recombinant SEB (a gift from J. Kappler and P. Marrack). SEB concentrations presented by DR1 and DR4 were 10^5, 2.5×10^4, 1.25×10^4, 6.25×10^3, 3.13×10^3, 1.56×10^3, 7.8×10^2, 3.9×10^2, 1.95×10^2, and 0.98×10^2 pg/ml. SEB concentrations presented by I-E^k were 10^8, 5×10^7, 2.5×10^7, 10^7, 5×10^6, 2.5×10^6, 10^6, 5×10^5, 2.5×10^5, and 10^5 pg/ml. Anti-TCR stimulation was performed using the purified anti-Id mAb KJ12 at different concentrations (3, 1, 0.3, 0.1, 0.03, 0.01, and 0.003 mg/ml) previously coated for 3 h at 37°C in 96-well flat-bottom culture plates. To these wells, 7.5×10^4 3DT52.5.8 cells expressing different CD4 molecules were added in 200 ml of complete RPMI medium and incubated for 16 h at 37°C. Supernatants from the coculture were assayed for IL-2 production as previously described (8).

Results

Evaluation of the mouse-human CD4-class II MHC interspecies interaction

Previous experiments using h-CD4 mutants expressed in 3DT52.5.8 showed that residues S19 (CDR1 loop), Q89 (CDR3 loop), and Q165 (FG loop) were critical for the CD4-class II MHC interaction (8). The fact that residues 19, 89, and 165 as well as other residues on this face of CD4 are conserved between human and mouse has led us to postulate that h-CD4 and m-CD4 should be able to interact with both human and murine class II MHC molecules. To assess the ability of h-CD4 and m-CD4 to interact functionally with class II molecules from both species, human and

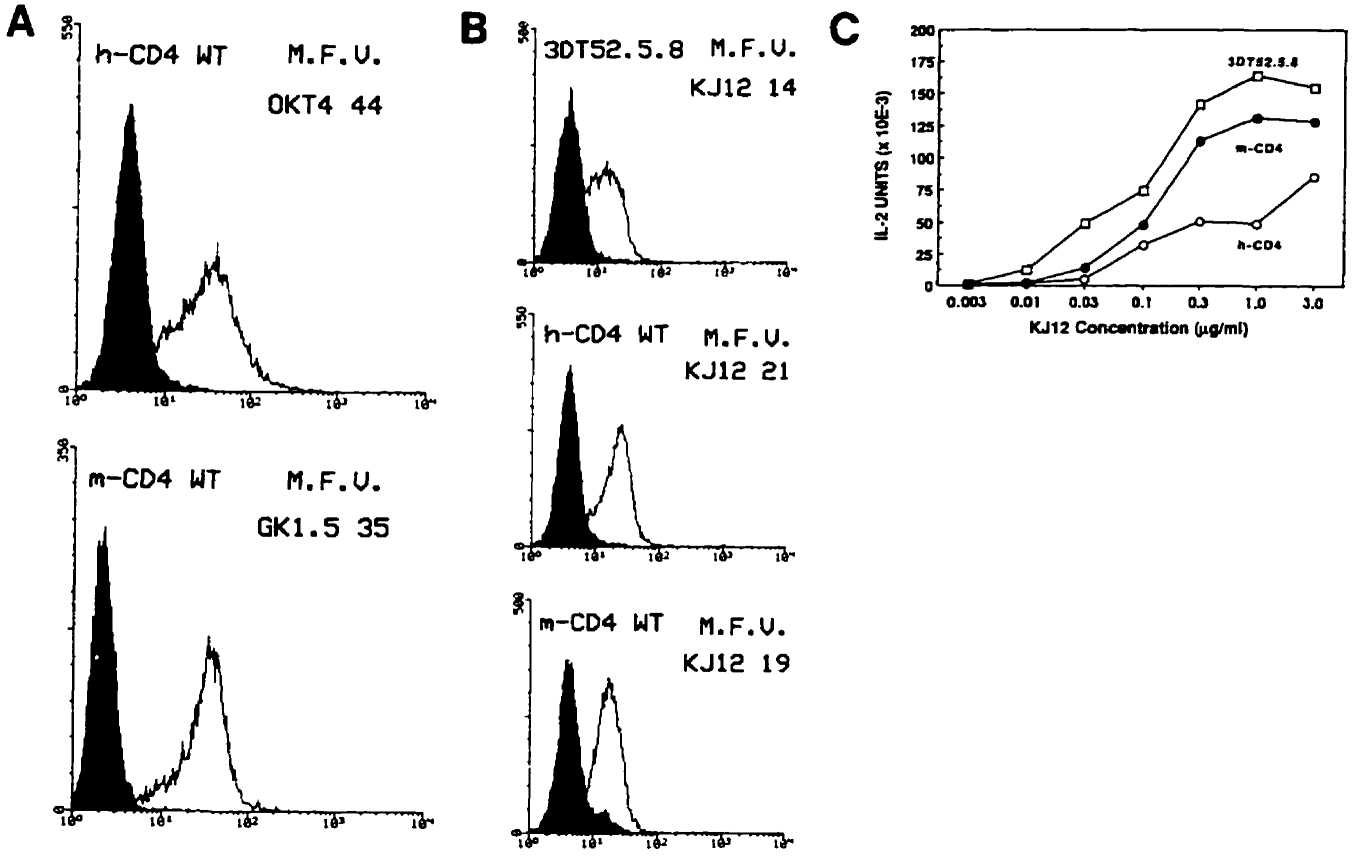

FIGURE 1. h-CD4, m-CD4, and TCR expression on the T hybridomas and the ability of the hybridomas to respond to an anti-TCR mAb. *A*, The h-CD4$^+$ and m-CD4$^+$ T hybridomas were stained with OKT4 or GK1.5, respectively. The shaded areas represent the control staining with the secondary Ab only (GAM/FITC or GAR/FITC). M.F.V., mean fluorescence value. *B*, 3DT52.5.8 cells and the CD4$^+$ derivatives were stained with the anti-idiotypic TCR Ab KJ12. The shaded areas represent the control staining with the secondary Ab only (GAM/FITC). *C*, 7.5×10^4 T cell hybridomas expressing the WT h-CD4 or the WT m-CD4 molecules were stimulated with plate-coated anti-TCR mAb KJ12 to evaluate their intrinsic capacity to secrete IL-2. The parental cell line 3DT52.5.8 CD4$^-$ CD8$^-$ was also stimulated.

murine CD4 cDNAs were introduced in the m-CD4$^-$CD8$^-$ T cell hybridoma 3DT52.5.8. The TCR of this hybridoma is allospecific for the murine class I Ag H-2D^d. In this study, we used the 3DT transfectants expressing good levels of h-CD4 or m-CD4 (Fig. 1*A*), displaying comparable levels of TCR (Fig. 1*B*), and producing high levels of IL-2 (less than a twofold difference) upon suboptimal stimulation with plate-bound anti-TCR mAb KJ12 (Fig. 1*C*). The cell surface expression of h-CD4 or m-CD4 on the T cell hybridoma 3DT is at least 20-fold lower than the CD4 expression level observed on PBL (data not shown). All DAP-3 transfectants expressed high levels of class II MHC and D^d molecules, as illustrated in Figure 2.

The ability of h-CD4$^+$ and m-CD4$^+$ T cell hybridomas to interact with several alleles and isotypes of class II molecules from both species on the APCs was determined in comparison with the CD4$^-$ 3DT52.5.8 cells. The results of a representative experiment are shown in Figure 3. 3DT52.5.8 cells produce comparable levels of IL-2 in the presence or the absence of class II molecules on the stimulator cells as shown; the difference is maximally twofold (Fig. 3*A*). T cell hybridomas expressing h-CD4 or m-CD4 produce low levels of IL-2 when cocultured with DAP-3 cells expressing only D^d Ag (no class II on APC; Fig. 3*B*). However, hybridoma cells expressing either h- or m-CD4 molecules can produce significantly higher levels of IL-2 when cocultured in the presence of cells expressing both D^d and class II molecules compared with cells expressing D^d molecules only. The h-CD4$^+$ T cell hybridomas show a 40- to 65-fold increase in the IL-2 ratio (see *Materials and Methods* for definition) when cocultured with DAP cells expressing D^d together with one of the human class II molecules, such as DR2A, DPw2, DR4-Dw4, or DRw52b (Fig. 3*B*). Similarly, the m-CD4$^+$ T cell hybridomas show a good reactivity to DAP transfectants expressing D^d together with one of the murine class II MHC molecules (I-Aα^bI-Aβ^k, I-A^k, I-E^k, or I-A^b). IL-2 levels were increased by 20- to 60-fold in the presence of the murine class II alleles and isotypes. In all cases, the enhancement of IL-2 production was inhibited by mAbs to CD4 (data not shown).

von Hoegen et al. (16) have previously shown in a murine CD4$^-$ T cell variant that expression of h- or m-CD4 could restore the class II-restricted Ag response with the same efficiency. Glaichenhaus et al. (17) obtained similar results showing a h-CD4/mouse class II interaction. These results led the authors to suggest that h-CD4 could interact with mouse class II MHC molecules. The results presented in Figure 3*B* also confirm the efficient interspecies CD4-class II interaction. Enhancement of IL-2 production (IL-2 values) was observed when h-CD4$^+$ T cells were stimulated with APC expressing several alleles or isotypes of mouse class II, and the enhancement was comparable to that in the case of human class II (Fig. 3*B*). Furthermore, m-CD4$^+$ T cells were quite efficient in strengthening the response to APCs expressing D^d and human class II molecules, as reflected by a 38- to 65-fold enhancement of the IL-2 ratio by m-CD4$^+$ T cells (data not shown). These results confirm the existence of efficient interspecies interactions

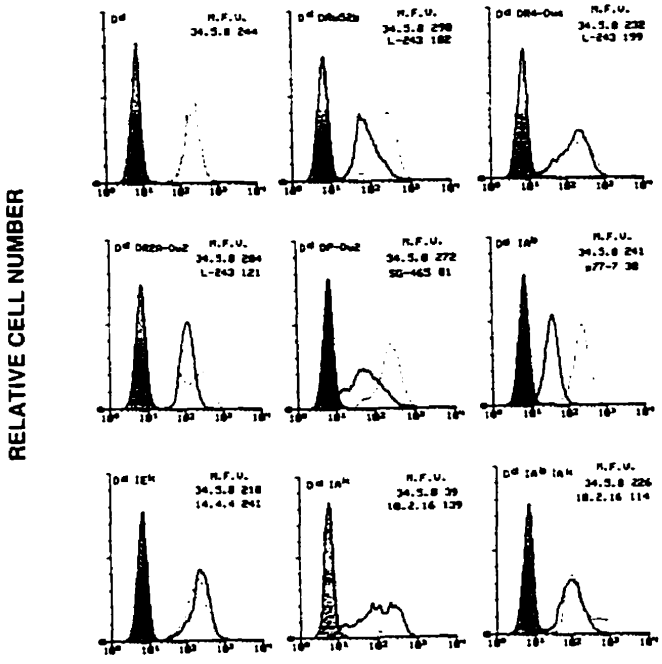

FIGURE 2. Flow cytometric profiles of the target cells. All murine DAP-3 transfectants were stained with a mouse class I anti-H-2D^d mAb (34.5.8: ·····), an anti-human class II mAb (L-243 or SG-465: ——), or an anti-mouse class II mAb (p77-7-7; 10.2.16; 14.4.4: ——). The shaded areas represent control staining with the secondary reagent only (GAM-FITC). M.F.V., mean fluorescence value. HLA-D nomenclature: DRw52b = DRB3 0201; DR4-Dw4 = DRB1 0401; DR2A-Dw2 = DRB5 0101; DP-Dw2 = DPB1 0201; and DRw11.1 = DRB1 1101. The class II molecule IAα^bIAβ^k corresponds to a mixed dimer resulting from co-expression of the α-chain IA^b with the β-chain Ia^k. In this figure, IAα^bIAβ^k is abbreviated as IA^{b}IA^k.

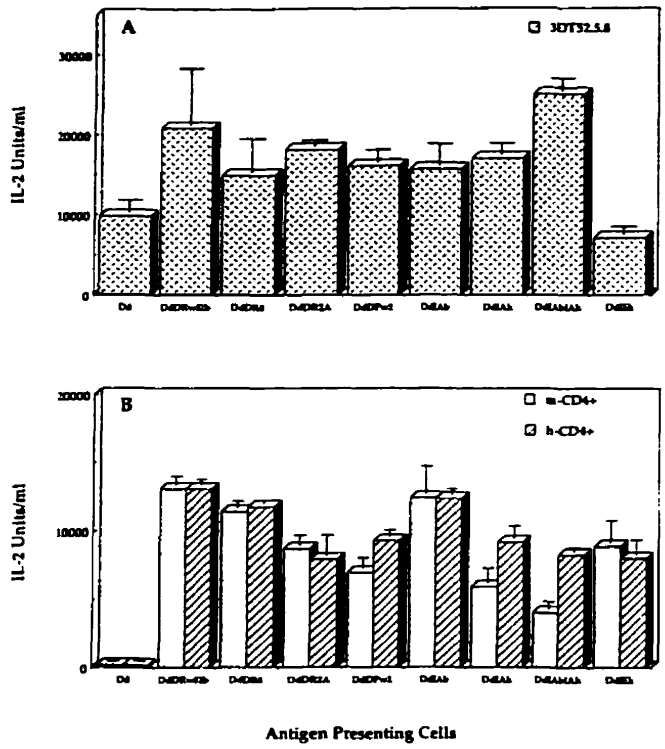

FIGURE 3. Responses of CD4$^+$ T cell hybridomas to target cells expressing different human or murine MHC class II molecules. *A*, IL-2 production of the 3DT52.5.8 parental cell line when cocultured overnight in a ratio of 1:1 (7.5×10^4 effectors:7.5×10^4 stimulators) with DAP-3 cells expressing H-2D^d alone or H-2D^d plus different class II alleles or isotypes. *B*, IL-2 production by T hybridomas expressing h-CD4 or m-CD4 molecules when cocultured overnight in a ratio of 1:1 (7.5×10^4 effectors:7.5×10^4 stimulators) with DAP-3 cells expressing H-2D^d alone or H-2D^d plus different class II alleles or isotypes is shown. On the *x*-axis, the target cells are listed; on the *y*-scale, IL-2 production is given in absolute values (units per milliliter), as described in *Materials and Methods*. The murine class II molecules I-Aα^bI-Aβ^k is abbreviated here as IA^{b}IA^k.

between CD4 and class II molecules. Interestingly, in the 3DT52.5.8 co-ligand assay system m-CD4 appears to interact better with human class II alleles than with murine class II molecules containing IAβ^k chain. We have previously shown that polymorphism in the HLA-DR β-chain influences the ability of the CD4 molecule to interact with class II MHC Ags (32). Based on these observations, it is possible to assume that some of the murine class II molecules are less efficient than others in their affinity for CD4 (such as I-Aβ^k). These interallelic differences could explain the more efficient interaction seen for h-CD4 and I-Aβ^k compared with m-CD4.

To verify that the increments in the IL-2 ratio of CD4$^+$ cells cocultured with D^d plus class II molecules over D^d alone are due to the CD4-class II interaction, but not due to variations in the expression levels of D^d molecules or other accessory molecules on the stimulator cells, the parental cell line 3DT52.5.8 (CD4$^-$) was cocultured with different APCs. 3DT52.5.8 cells produced a certain level of IL-2 in response to APCs expressing only its cognate Ag (H-2D^d). However, this level of IL-2 production was not significantly increased when 3DT cells were cocultured with APCs expressing both class I and class II molecules, as shown by an enhancement of IL-2 production by one- to twofold (Fig. 3*A*). This result confirms that the enhancement of IL-2 production observed in CD4$^+$ T cells was caused by the CD4-class II interaction rather than other factors, such as variations in the levels of expression of other accessory ligands on the different APCs.

Mutations in h-CD4 impair the interaction with both human and murine class II

Previous results have shown that h-CD4 mutants S19Y, Q89L, and 165[SR] interact poorly with HLA-DP molecules in the 3DT52.5.8 co-ligand system (8). However, if these mutations affect the binding of CD4 with all class II molecules or only with certain class II molecules, such as HLA-DP, is still not established. To determine whether these residues are involved in the interaction of CD4 with other class II alleles or isotypes, we tested these same CD4 mutants for their ability to interact with a series of human and murine class II alleles and isotypes. The results (Fig. 4*A*) show that these CD4 mutants perturb, albeit at different levels, the interaction with all human class II molecules tested (DRw52b, DR4, and DPw2). Indeed, mutant S19Y resulted in a constant low IL-2 ratio (five- to ninefold) in all class II molecules tested. Mutants Q89L and 165[SR] showed 17- and 18-fold increases in the IL-2 ratio, respectively, when cocultured with D^d plus DRw52b compared with a 67-fold enhancement by WT CD4. Lastly, mutations S19Y and Q89L more severely affected the interaction with DR4 than mutation 165[SR], although all three mutations abrogated the interaction with DP (8). The fact that the same mutations (Q89L or

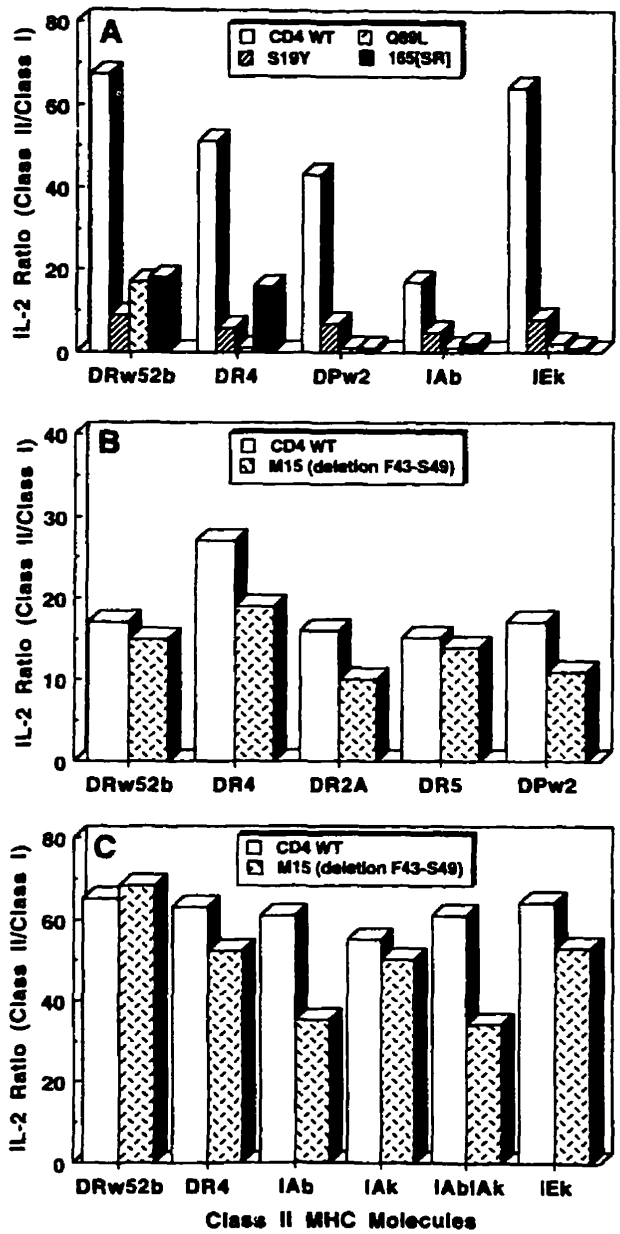

FIGURE 4. Responses of h-CD4 mutants to DAP transfectants expressing different class II molecules. *A*, The h-CD4 WT and three h-CD4 mutants, whose capacity to interact with HLA-DP class II molecules is greatly affected, were cocultured overnight at 37°C at a 1:1 ratio with stimulator cells. DAP-3 expressing H-2D^d and human or murine class II molecules that interact well with the h-CD4 WT were used as stimulator cells. *B*, 7.5 × 10^4 hybridomas expressing the h-CD4 WT or the mutated h-CD4 molecule (M15: deletion F43-S49) were cocultured overnight with 7.5 × 10^4 DAP-3 stimulator cells expressing mouse class I D^d and human class II Ags. *C*, CD4$^+$ hybridomas were cocultured with DAP-3 D^d cells expressing human or mouse class II molecules, using the same cell concentration as in *B*. SDs were not >10%. The *y* scale is expressed as the IL-2 ratio, as described in *Materials and Methods*. The murine class II molecules I-Aα^bI-Aβ^k is abbreviated as IA^{b}IA^k.

165[SR]) differently affected the class II interaction suggests that different class II alleles or isotypes may have distinct capacities or affinities for the CD4 molecule. Polymorphism in the β-chain of class II molecules affects CD4 binding (32), suggesting that CD4 might have a different relative affinity for class II. A lower affinity of CD4 for a class II molecule would result in facilitated perturbations of the CD4-class II interaction by a mutation, such as in the case of the h-CD4/DP interaction. In a protein-protein interaction such as the CD4/class II interaction (8), we know that several residues are involved. It is also possible that the introduction of a mutated amino acid into the CD4 molecule might not have altered the overall affinity for class II, but by locally disturbing the area affects the docking between CD4 and class II, thereby decreasing the binding efficiency. However, the affinity issue can only be addressed in assays involving soluble molecules.

These h-CD4 mutants were also tested for their capability to interact with murine class II molecules. Figure 4*A* shows that these mutations also affect the interaction with the mouse class II molecules tested (I-A^b and I-E^k). Functional interactions between h-CD4 mutants and DPw2, I-A^b, and I-E^k were almost absent, as shown by only a one- to eightfold increased IL-2 ratio in the mutants, compared with a 43- to 64-fold enhancement in the CD4 WT. In this series of experiments, we did not observe any hierarchy in the interactions of h-CD4 mutants with the murine class II molecules, since all mutants abrogated the CD4-dependent IL-2 response. These results strongly suggest that the same residues on the h-CD4 molecule are implicated in the binding to class II molecules from different species. They also suggest that h-CD4 might have a lower affinity for the murine class II molecules (in the case of I-A^b) than for some of the human class II molecules tested, since these mutations totally abrogate the interaction between CD4 and all murine class II molecules. However, we cannot rule out the possibility that these murine class II molecules tested in our assay are among the class II Ags that bind poorly to m-CD4 or h-CD4. Direct affinity measurements need to be made with soluble molecules to make a definitive statement.

We have shown previously in the 3DT52.5.8 D^d assay system that the CDR2 loop (F43 to S49) in the D1 domain of CD4, the primary site for gp120 binding, was not essential for the CD4/HLA-DP interaction (8). Results using class II alleles other than DP are illustrated in Figure 4*B*. They confirmed that the CDR2 loop is not directly required for an interaction with other isotypes of the class II molecules. T cell hybridomas expressing mutant M15 (deletion F43-S49) produced enhanced levels of IL-2 (10- to 20-fold enhanced IL-2 ratio) when cocultured with APCs expressing D^d and different human class II Ags (DRw52b, DR4-Dw4, DR2A-Dw2, DR5, and DPw2). The IL-2 ratio was comparable to that in the WT CD4 (15- to 27-fold increase). Furthermore, M15 shows a 34- to 53-fold increase in IL-2 ratio when cocultured with APCs co-expressing murine class II I-A^b, I-A^k, I-Aα^bI-Aβ^k, or I-E^k (Fig. 4*C*). The levels of IL-2 production are comparable to those obtained following stimulation of CD4$^+$ cells with human class II (DRw52b and DR4-Dw4). This clearly indicates that mutant M15 also interacts with murine class II molecules, suggesting that the CDR2 loop is not implicated in the interaction with murine class II molecules.

Cross-species interaction of CD4 with class II MHC is also conserved in the co-receptor assay

We reported previously that 3DT52.5.8 can recognize the bacterial superantigen SEB when presented by human class II MHC molecules (33). We also observed that the IL-2 response to SEB was significantly increased when CD4 was expressed on the T cell (33). It is widely believed that during superantigen recognition, TCR and CD4 molecules interact with the same class II MHC molecule, so CD4 acts as a co-receptor, as in Ag-specific recognition (co-receptor model) (9). This mode of interaction is different from the D^d alloreognition in the 3DT52.5.8 system, in which TCR and CD4 interact with different ligands (co-ligand). Here we used a co-receptor assay in which the responses to SEB by the CD4$^-$

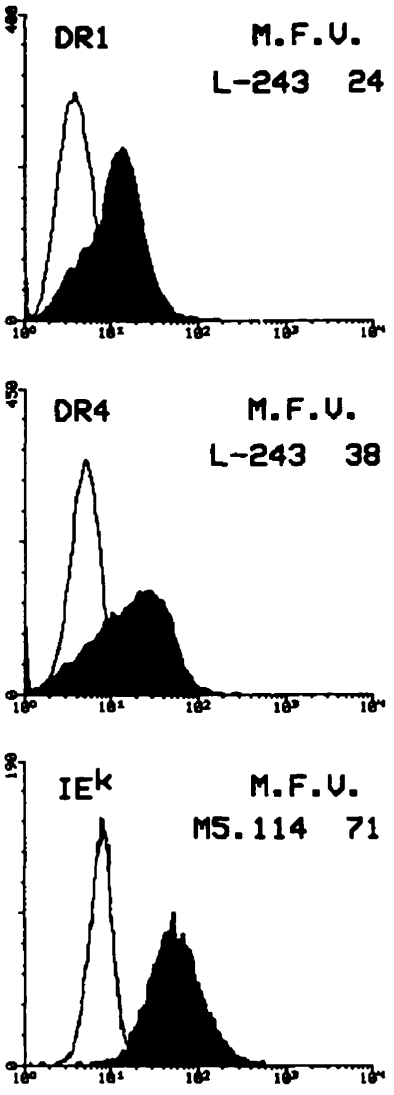

FIGURE 5. Flow cytometric profiles of the APCs used in the co-receptor assay. All DAP-3 transfectants were stained with anti-human class II mAb L-243 or anti-murine class II M5-114. Open areas represent the control staining with GAM/FITC only. The mean fluorescence values were 24 for the APC-DR1, 38 for DR4, and 71 for I-E^k.

3DT52.5.8 cells and its CD4$^+$ derivatives were compared, and the cross-species interactions of CD4 and class II were tested. All cells were stained with F23.1 mAb to ascertain that they express comparable levels of TCR Vβ8.1 (data not shown). T cells were incubated with APCs expressing human or murine class II molecules in the presence of a range of SEB concentrations. The human class II molecules DR1 and DR4 and the murine class II I-E^k were expressed at high levels on DAP-3 APCs (Fig. 5). Other murine class II alleles were not tested, since the conclusion of such assays could be made more difficult because of differences in the affinities of toxin for various class II alleles and isotypes.

Figure 6 shows the results of the co-receptor assay. 3DT52.5.8 produces low levels of IL-2 when stimulated with SEB presented by APCs expressing DR1 (Fig. 6*A*), DR4 (Fig. 6*B*), or the murine class II molecule I-E^k (Fig. 6*C*). Enhancement of the response to suboptimal SEB concentrations was achieved by introducing both human and murine CD4 molecules in the 3DT52.5.8 hybridoma. Moreover, higher concentrations of SEB (~100-fold) were required to trigger IL-2 production when SEB was presented by I-E^k

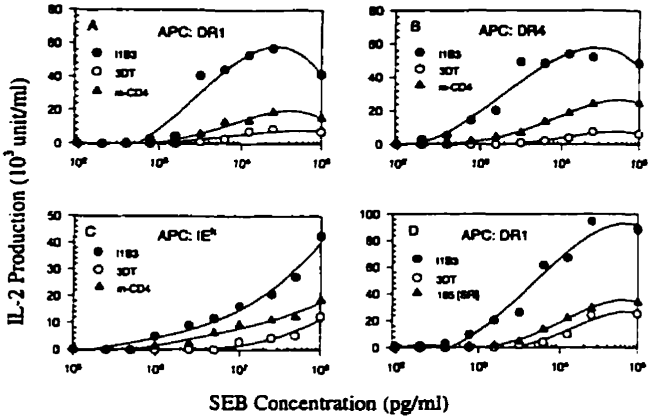

FIGURE 6. Both h-CD4 and m-CD4 molecules can enhance the T cell responses to SEB presented by APCs bearing human or mouse class II molecules. *A*, APC DR1; *B*, APC DR4; *C*, APC I-E^k; *D*, APC DR1. Cells from T cell hybridomas (7.5×10^4) expressing h-CD4 wt (11B3), m-CD4 wt, h-CD4 mutant 165[SR], or CD4$^-$ were cocultured overnight with 20×10^4 APCs in presence of SEB. The SEB concentrations in *A*, *B*, and *D* are 10^5, 2.5×10^4, 1.25×10^4, 6.25×10^3, 3.13×10^3, 1.56×10^3, 7.8×10^2, 3.9×10^2, 1.95×10^2, and 0.98×10^2 pg/ml. The SEB concentrations in *C* are 10^6, 5×10^7, 2.5×10^7, 10^7, 5×10^6, 2.5×10^6, 10^6, 5×10^5, 2.5×10^5, and 10^5 pg/ml.

compared with DR1, since the affinity of SEB for DR1 was at least 10-fold higher than that for I-E^k (34, 35).

The specificity of the SEB assay was further confirmed. CD4 mutant (165[SR]), which did not interact with class II in the co-ligand assay (8), failed to enhance IL-2 production (Fig. 6*D*), even though this mutant was able to produce comparable levels of IL-2 upon anti-TCR stimulation (data not shown). This result indicates that a region of CD4 that is involved in an interaction with class II MHC molecules in the co-ligand assay also has a functional role in the co-receptor assay. This suggests the existence of an overlap of the functional domains of CD4 in both types of assay. Recently, Jardetzky et al. (36) described the three-dimensional structure of the DR1/SEB complex. According to the crystal structure, class II MHC molecules that are already bound to SEB superantigen can still accommodate CD4 binding. Indeed, the SEB and CD4 binding sites on class II are distinct, as confirmed by mutagenesis and structural analysis. It is not known, however, whether the CD4 molecule in our co-receptor assay binds to a single heterodimer of class II or to the SEB/class II complex during the engagement of SEB/class II complex with the TCR.

Discussion

It is well established that xenoresponses between human and mouse are much weaker than alloresponses (37–39). Barzaga-Gilbert et al. (39) showed in human CD4 transgenic mice that while T cell responses to human class II-positive cell lines are enhanced up to 10 times, allogeneic responses remain unaltered and are much stronger than xenoresponses. Hence, despite the fact that the h-CD4 was overexpressed on murine T cells, responses to human class II molecules were much lower than those to murine MHC class II molecules. The enhancement of T cell stimulation might be due solely to the overexpression of the human CD4 molecule. The low xenogenic response suggested that the species-specific interactions between human class II molecules and human CD4 expressed on murine T cells are not sufficient. It is possible that some intrinsic differences in the murine TCR structure, which was originally selected on a mouse MHC background, prevents an efficient interaction with the human MHC class II molecules.

In two other studies, class II chimeras have been created by exchanging the CD4 binding site (β2 domain) between DR1 and I-E^k molecules (37, 40) or between I-Aα^bI-Aβ^k and DR molecules (38). These studies have shown that the alloresponse of mouse T cells against I-E^k (DR1β2) or I-A^b (DR1β2) chimeras was altered. Similar observations were obtained with stimulation of human T cells with DR chimeras containing the I-Aβ2 or I-Eβ2 domain. Moreover, no conformational alterations were detected by serology in the region of the class II molecule that interacts with the TCR. The main conclusion drawn from these experiments was that a partial species barrier occurred between murine and human CD4 and class II MHC molecules. However, our results, illustrated in Figure 3*B*, show that h-CD4 interacts efficiently with I-E^k, as shown by a similar level of IL-2 production when I-E^k is compared with human class II DRw52b and DR4. One explanation for the poor xenoresponses observed by the other groups may be due to the inability of the TCR to efficiently recognize the class II MHC molecules of other species, rather than to a weak interaction between CD4 and class II MHC molecules.

One peculiarity of the in vitro cellular systems used by these different groups is that both TCR and CD4 molecules associate with the same ligand. Since TCR and MHC molecules do not always belong to the same species, it becomes difficult to assess the relative contribution of TCR-MHC or CD4-MHC incompatibilities. In the 3DT52.5.8 cellular system, TCR and CD4 molecules recognize different MHC products, which allow us to better evaluate the respective contributions of the TCR and CD4 molecules in a CD4-dependent T cell response. More recently, two groups have shown a functional interaction between human class II and mouse CD4 molecules using transgenic mice (41, 42), further supporting our results.

In this report we have demonstrated that human and murine CD4 were capable of cross-species interaction with class II MHC molecules. These observations were obtained in two independent functional assays using the same T cell hybridoma. This experimental system avoids any problem of interpretation caused by the use of different TCRs. Our results from both assays (co-ligand and co-receptor assays) have shown that h- and m-CD4 can function with at least five different human MHC class II (DRw52b, DR4-Dw4, DR2A, DRw11.1, and DP-Dw2) and four different mouse MHC class II (I-A^b, I-A^k, I-Aα^bI-Aβ^k, and I-E^k) molecules. We have also demonstrated that mutations of highly conserved residues of the h-CD4 molecule reduce or abrogate the interaction with both human and mouse class II MHC molecules. Interestingly, mutations Q89L and 165[SR] showed differences in their interaction with the different class II molecules tested. This might suggest that the class II alleles or isotypes could have distinct affinities for the CD4 molecule. In fact, we have reported that some class II sequences outside the highly conserved CD4-binding QEEK motif can impair the CD4-class II interaction. We have shown that DRw53 interacts poorly with the CD4 molecule compared with DR4-Dw4 (32).

As mentioned previously, h-CD4 and m-CD4 share a certain structural homology. The fact that these CD4 molecules can interact with class II MHC from both species leads us to postulate that they interact with a conserved region on class II MHC molecules. Conversely, it is more likely that class II MHC molecules should also recognize the conserved regions on h-CD4 and m-CD4 molecules. Sequence alignment of the CDR1, CDR2, CDR3, and FG loops between h-CD4 and m-CD4 allowed us to determine which loops are the most conserved across species. The CDR1 and CDR3 regions show, respectively, 54% and 67% homology between h-CD4 and m-CD4, while the FG loop shows 39% homology. The CDR2 region shows the most divergence, with only 38% homology between h-CD4 and m-CD4 (8 residues of 21 are conserved). We have reported that the FG region is important in the interaction with class II because of its proximity to the CDR1 and CDR3 loops of the D1 domain. Moreover, Gustafsson et al. (43) reported recently the existence of an extensive allelic polymorphism in the CDR2-like region of the miniature swine CD4 molecule. In fact, three of the amino acids located within the CDR2 loop are polymorphic, including residue F43, which was identified in the h-CD4 molecule as a critical residue for gp120 binding (8, 20, 44, 45) and for class II binding (46–49). If the CDR2 region is involved in a critical function, such as binding to class II, evolutionary pressure should have preserved the integrity of this site within the miniature swine population, unless it is only a minor class II contact site. It is difficult to reconcile the direct involvement of the CDR2 loop of CD4 in the class II interaction with the polymorphism of the CDR2 reported in the swine CD4 molecule and the strong divergence in this portion of CD4 observed across different species. Recently, Sakihama et al. (50) have reported that when a CDR2 region mutant F43I was co-expressed with the wild-type CD4, the result was a dominant negative effect in class II binding, suggesting another function for this domain of CD4, such as interaction with other surface molecules.

We have shown previously (8) that CD4 interacts with class II through exposed residues located on the same face as residues S19, Q89, and Q165. Alanine scan mutagenesis was performed on CD4 residues that are conserved between h-CD4 and m-CD4 (e.g., K1, K2, K7, T15, T17, D88, etc.) (B. Huang, S. Fleury, A. Yachou, W. Hendrickson, and R.-P. Sékaly, manuscript in preparation). Our results confirm the involvement of this face of the CD4 molecule in the interaction with class II MHC molecules (51). Similar conclusions were reported by Moebius et al. (49). Our finding that h-CD4 and m-CD4 recognize class II MHC molecules of both species implies that CD4 interacts with a conserved region in the β2 domain or other conserved regions of class II MHC molecules. The CD4 binding site on class II molecules has been identified by two groups (14, 15). This region is highly conserved not only between human and mouse class II molecules, but also in class II molecules from other species. It was also shown that some mutations in the β2 domain of the murine class II I-A^d could affect m-CD4 function without disturbing the h-CD4 co-receptor function (15). These observations indicate that some of the class II MHC residues involved in the interaction with CD4 are shared by the h-CD4 and m-CD4 molecules, while others are specifically recognized by h-CD4 or m-CD4 molecules. Experimental approaches using class II MHC and m-CD4 mutants should provide more information.

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Chapter 4

The CDR2 Within The D1 Domain Of CD4 Participates In A Novel Function: Oligomerization Of CD4

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Preface

Cell surface receptors involved in signal transduction are general dimers in nature or are induced to form dimers upon ligand binding. Although CD4 is expressed as a monomer on cell surface, crystallographic and functional results have suggested that CD4 could dimerize upon interaction with its ligand -class II MHC molecules. In order to determine if CD4 Oligomerization does occur, and to define the possible oligomerization interface, we performed experiments present next.

Abstract

The CD4 molecule is a monomeric cell surface glycoprotein which interacts with the class II major histocompatibility complex (MHC) molecule. Crystallographic and functional evidences have at times suggested that CD4 might self-associate to form a dimer or oligomer upon interacting with its physiological ligand, and this multimer formation is essential for the signalling transduction and T cell activation. Here we tried to investigate if this oligomerization event of CD4 does occur on the T cell surface and if it is critical for CD4 to fulfill its function as either the accessory molecule (co-ligand) or the co-receptor. We have used the 3DT52.5.8 cellular system which provides both a co-ligand (D^d assay) and a co-receptor assay (SEB assay). In both assays the responses are enhanced by the presence of CD4 on T cells. We have co-expressed both the human and mouse CD4 wild-type (WT) molecules in 3DT52.5.8 cells. Moreover, we have co-expressed in the 3DT52.5.8 cells either the human or mouse CD4 WT molecule with a panel of CD4 mutants. These mutants include residues from the CDR1 (S19), CDR3 (Q89) regions of D1 and the FG loop (165) of D2, and have been previously mapped as important for interaction with class II molecules. Finally, human or mouse CD4 WT molecules have been co-expressed with mutants from the opposite face of CD4 which encompasses the CDR2 region. All these double transfectants were studied in both assays and similar results were obtained for each transfectant in both assays. Cells expressing both murine and human WT CD4 lost their capacity to produce enhanced level of IL-2 in the presence of class II MHC molecules on the APCs. This result suggested that murine CD4 (m-CD4) interferes with human CD4 (h-CD4) possibly by preventing their oligomerization. However, this inhibitory effect was not seen when the h-CD4 WT was co-expressed with mutants with residues from the face which encompasses the CDR2 of the D1 domain. Introducing CD4 WT into mutants from the CDR1, the CDR3 regions of D1 and the FG

loop of D2 did not rescue the class II MHC-dependent enhancement of IL-2 production, possibly because the mutants fail to interact with class II. Thus this study suggests that the dimer/oligomer formation of CD4 does occur and is essential for both function of CD4. The face encompassing the CDR2 region of D1 may plays a critical role in this event.

Introduction

The cell surface glycoprotein CD4 is expressed as a monomer on cells of the T lymphocyte lineage, and it is a member of the immunoglobulin (Ig) gene superfamily. CD4 interacts with its physiological ligand class II major histocompatibility complex (MHC) molecules on the antigen presenting cell (APC), either as an adhesion molecule (co-ligand) or a co-receptor (Janeway, 1992). Upon interaction with its ligand CD4 brings its cytoplasmically associated tyrosine kinase $p56^{lck}$ to the vicinity of the TCR. This is critical for the co-receptor function of CD4. Studies have shown that several sites throughout D1 and the upper part of D2 domains of CD4 are implicated in this interaction (Fleury et al., 1991; Moebius et al., 1992 & 1993; Huang et al., 1996). On the class II molecules the $\alpha 2$ and $\beta 2$ domains have been shown to encompass the binding sites for CD4 (König et al., 1992; Cammarota et al., 1992; König et al., 1995). Moreover, the class II MHC molecule HLA-DR1 has been crystallized as a superdimer (or dimer of the $\alpha\beta$ heterodimer) (Brown et al., 1993), though functional data for this class II dimerization is still lacking. On the other hand, studies have suggested that CD4 may exert its biological function by self-associating with each other to form a dimer or oligomer on the T cell surface, upon interacting with its ligand (Kwong et al., 1990; Hendrinkson et al., 1992; Sakihama et al., 1995).

Initial support for this CD4 dimerization/oligomerization hypothesis came from the crystallographic analysis of recombinant soluble CD4 molecules (sCD4, which contains only the extracellular four domains but lacks the transmembrane and cytoplasmic portions) (Kwong et al., 1990 PNAS). It appears that CD4 oligomerizes at high protein concentrations. All five distinctive forms of CD4 that were characterized include multiples of two molecules in the asymmetrical units. Though this sCD4 is monomeric in solution,

its recurrent association in different crystals suggests a natural tendency for CD4 to dimerize or oligomerize, this may be essential for CD4 to fulfill the signal transduction function.

In addition, results from different cellular assay systems have also led to the same suggestion. A recent study by Konig et al., has shown that regions from both the α2 and β2 domains of class II molecule are involved in interaction with CD4 (Konig et al., J. Exp. Med. 1995). Importantly, these two regions are situated 180° apart that the interaction of the class II MHC molecule with a single CD4 molecule simultaneously seems impossible. Therefore it indicates that specifically organized CD4 and/or MHC class II oligomers play a critical role in the co-receptor-dependent T cell activation.

Using a TCR-independent cell adhesion assay involving $CD4^+$ COS cells and class II MHC^+ B lymphocytes, Sakihama et al have suggested that CD4 oligomerization may be required for CD4/class II MHC interactions (Sakihama et al., 1995). Interestingly, when the CD4 WT and mutant F43I were co-expressed on COS cells, F43I could inhibit the function of the wild-type, showing a dominant negative phenotype. Based on these results the authors suggested that when the CD4 WT oligomerizes with a mutant which does not bind to class II, the mutant will perturb the oligomer structure rendering it inefficient in its interaction with class II. Moreover, these authors provided results indicating that the D3D4 domains are involved in oligomerization of CD4. The basis of this conclusion came from assays involving CD4 molecules containing D1D2 of CD4 and the D3D4 domains are replaced with either D1D2 of CD4 or D1D2 of CD2. These mutants were unable to mediate adhesion with class II. However, when these molecules were co-expressed with CD4 WT, the functional interaction with class II could be rescued. These results suggested, according to the authors, that chimerical molecules lost the oligomerization

sites, thus they could not form oligomer between themselves, nor between the WT and chimeras.

Up to now, the dimer interface of CD4 is largely unclear, though studies have pointed to the CDR3 region of D1 (Langedijk et al., 1993); or the face opposite to the OKT4 binding site Arg 240 in the D3D4 domains (Sakihama et al., 1995 PNAS). According to the crystal structure of CD4, the CC' and the FG loops in D1 whose homologues are involved in dimer formation in Ig and CD8, bear a truncation here, thus forming a dimer through these loops in D1D2 fragment seems unlikely (Ryu et al., 1990; Wang et al., 1990). In contrast, in D1 the C'C'' loop (homologous to the CDR2 region of Ig) is unusually prominent. This loop is crucial for the high affinity binding of HIV glycoprotein gp120, however, a function of this region in forming the dimer has not been firmly established (Leahy, 1995). From the crystal of the D3D4 fragment, the CC' and the FG loops are similar to that of Ig; however, a glycosylation site has been found on the F stand, which will prevent dimer formation via this region (Brady et al., 1993).

Here we would like to investigate if it is essential for CD4 to oligomerize with each other in order to exert its biological function as either the accessory molecule or the co-receptor; if there is any differences for the dependency of the CD4 oligomerization in either function of CD4. If this self-association is essential, then it becomes important to determine the sites on CD4 which are involved in this interface. To address these questions, we have used the 3DT52.5.8 T hybridoma cell line which can assay the two functions of CD4, namely the co-ligand and the co-receptor function. In the co-ligand assay the TCR and CD4 interact with different MHC ligands while in the co-receptor assay both CD4 and the TCR can interact with the same MHC class II molecule.

Materials and Methods

Plasmids: Full length human CD4 wild-type or CD4 mutants cDNAs were obtained from different sources (Peterson & Seed., 1988; Mizukami et al., 1988), or generated in our laboratory by PCR overlap extension method (Ho et al., 1989; Huang et al., 1996). The mouse CD4 (L3T4) wild-type cDNA was obtained from D. Littman. The h-CD4 mutant cDNA were prepared as blunt *Bam*HI, *Hind*III-*Bam*HI, or *Hind*III-*Not*I fragments and subcloned into the pMNC-stuffer vector. The amphotropic helper cell line DAMP cell (Slackman et al., 1988) was transfected with the different pMNC constructs and G418 resistant DAMP cells were selected and used as the producer cell lines of the recombinant retrovirus to infect 3DT52.5.8 T hybridoma cells. The proviral pMNC-mCD4 DNA confers resistance to the selection agent G418 and allows stable long-term expression of the mutated CD4 molecules in infected cells. The human CD4 cDNA was also prepared as *Hind*III-*Not*I fragments and subcloned into the vector SRα puromycin (Takebe, Y. 1988).

Transfection: CD4 mutants were generated as described (Fleury et al., 1991). Briefly, the $CD4^+$ DAMP cells were obtained by the calcium phosphate co-precipitation technique (Graham and Van de Eb, 1973) as previously described (Lamarre et al., 1989). The DAMP cells transfected with the various pMNC-mCD4 constructs were enriched for cells expressing high levels of membrane CD4 by FACS sorting with OKT4 antibody. These DAMP cells were used to infect the 3DT52.5.8 hybridomas as described. G418-resistant 3DT52.5.8 cells were then analyzed. Mutants S19Y, Q89L and 165[SR] were as descried (Fleury et al., 1991). Mutants K46A and E77A were described (Huang et al., 1996).

Co-transfection: For co-expression of both h-CD4 WT or L3T4 WT with CD4 mutants, CD4 mutants cells were electroporated with either plasmid containing the SRα

puromycin-h-CD4 WT construct, or plasmid containing pMNC-mCD4 WT plus plasmid SRα puromycin. For each electroporation, 5 X 10^6 3DT cells were resuspended in 500 µl of complete RPMI supplemented with 10% FCS. To this cell suspension 10 µg of SRα puromycin-h-CD4 WT, or 10 µg of pMNC-mCD4 WT plus 2 µg of SRα puromycin were added. Electroporation was proceeded at the voltage of 270 mV. Forty-eight hr later, cells were subjected to puromycin selection, and the expression of the mutant CD4 was maintained by G418. For selection, 40,000 cells were resuspended in 2 ml of complete RPMI with 10% FCS, to these cells, puromycin 3 µg/ml and G418 0.5 mg/ml were added. The medium was changed every three days, puromycin and G418-resistant 3DT 52.5.8 cells were analyzed for the expression of the wild-type and mutated CD4 molecule by flow cytometry.

Cytometrical Analysis of cells: In the preliminary experiments we have identified monoclonal antibodies which would discriminate between WT and mutant CD4 molecules. After introducing in 3DT52.5.8 cells both WT and mutated CD4 molecules which had lost an epitope recognized by a specific mAb, we screened for transfected cells. For staining, double-transfectants expressing both the h-CD4 WT and CD4 mutants were incubated with OKT4 and the respective antibody whose epitope is affected by this mutation. Briefly, 5 X 10^5 cells were incubated for 60 min at 4 °C with 100 µl of the appropriate mAb (10 µg/ml) diluted in complete medium. Following washes in PBS containing 2% FCS and 0.02% sodium azide, the cells were incubated with PE-coupled goat-anti-mouse Ig (Becton Dickinson) for 30 min at 4 °C. The cells were washed, incubated with OKT4/FITC for 30 min at 4 °C. After washing, cells were resuspended in PBS containing 2% FCS and analyzed for fluorescence using lysis II flow cytometry. Dead cells were excluded by propidium iodide (0.5 µg/ml) gating, and 10,000 cells were acquired for the

analysis. As the control, the cells were stained only with the fluorescein-coupled goat-anti-mouse Ig.

For double-transfectants expressing both the m-CD4 WT and CD4 mutants, cells were incubated with OKT4/FITC for 30 min at 4 °C. After washing with PBS, cells were incubated with GK1.5/PE. Then the cells were analyzed as above.

Stimulation of Effector Cells:

Co-ligand Assay: 7.5 X 10^4 3DT52.5.8 cells expressing CD4 molecules (either WT, CD4 mutant, WT with mutant, or h-CD4 with m-CD4) were cocultured with 7.5 X 10^4 DAP-3 cells expressing H-2D^d or H-2D^d DR4 in 200 µl of complete medium without selective agent for 20 hr at 37 °C in 96-well flat-bottom culture plates. After co-culture, supernatants were harvested for the IL-2 production assay as previously reported (Fleury et al., 1991). The results of this assay are presented in such a way: on the X axis, cell names are given, while on the Y axis, the IL-2 ratio is displayed. The IL-2 ratio D^{d}DR4/D^d is calculated in such a way: the amount of IL-2 produced for a given type of cells when co-cultured with APCs expressing both the cognated Ag D^d and the class II molecules DR4 is compared with IL-2 produced for the same cells when co-cultured with APCs expressing D^d alone.

Co-receptor Assay: 7.5 X 10^4 3DT 52.5.8 cells expressing CD4 molecules (either WT, CD4 mutant, WT with mutant, or h-CD4 with m-CD4) were cocultured with 2 x 10^4 DAP-3 cells expressing human class II molecule DR1 in 200 µl complete medium for 20 hr at 37 °C in 96-well flat-bottom culture plates, in the presence of recombinant SEB (Toxin Technology) of 2-fold serial dilution from 10^5 to 10^2 pg/ml. Titration of the SEB

concentration was carried out with 3DT52.5.8 and 3DT52.5.8 transfected with the human CD4 WT. After an overnight co-culture, the supernatant was harvested for IL-2 measurement. The responses of the T cell hybridomas expressing different CD4 mutants are displayed as such: on the X-axis, the log concentrations of bacterial superantigen SEB are given; on the Y-axis, the IL-2 units produced by each mutant upon SEB stimulation are displayed (as shown in Figure 1C).

Anti-TCR stimulation : The TCR-specific antibodies F23.1 (recognizes all the Vβ8s) were used to stimulate the cells. A wide range of concentrations (3, 1, 0.3, 0.1, 0.03, 0.01, 0.003 μg/ml) of the purified mAb were coated at 4 °C in 96-well flat-bottom plates overnight. To these wells, 7.5 X 10^4 3DT 52.5.8 cells expressing different mutated CD4 molecules were added in 200 μl of complete RPMI medium and incubated for 20 hr at 37 °C. The supernatant was harvested for the IL-2 production assay later.

IL-2 Assay: The production of IL-2 was assayed by the ability of the coculture supernatant to support the proliferation of an IL-2-dependent cell line CTLL.2 using the hexaminidase colorimetric assay as previously described (Lamarre 1989). Briefly, 50 μl of the co-culture supernatant was taken and diluted in 2 fold serial dilution in complete RPMI with 2% FCS. The IL-2 values were calculated by comparing with a calibration curve performed with recombinant IL-2 (Cetus Corporation, Emeryville, CA).

EC 50 Calculation: EC 50 is defined as the concentration of a given stimulus (antigen, superantigen or anti-TCR antibody) required to obtain a half-maximal response for a cell line. For the TCR stimulation assay using monoclonal antibody F23.1 which is specific for the Vβ8.1 chain, the results were plotted as log concentrations of F23.1 on the X axis against the IL-2 values on the Y axis. Curve-fitting was done with Sigma Plot program.

against the IL-2 values on the Y axis. Curve-fitting was done with Sigma Plot program. The EC50 value was determined from the curve for each CD4 mutant, by reading out the X axis value such that the Y value equals to the half of the peak IL-2 value on the curve. The EC 50 values of all the CD4 mutants were compared to that of the wild type CD4 expressing cells, which gives the EC50 ratio (as shown in Table I). The EC50 ratio of the TCR stimulation is an index for comparing the ability of different mutants to produce IL-2

Results and Discussion

Although previous studies have favored the model that CD4 exerts its functional role as a dimer or oligomer when interacting with its physiological ligand, the mechanism and the interface of CD4 self-association is largely unclear. In order to investigate if CD4 oligomerization is critical for its interaction with class II MHC and its function in T cell activation, and to possibly define the interface of oligomerization, we have made the following approaches: (I) by co-expressing both the human and mouse CD4 WT molecules into the 3DT52.5.8 T hybridoma cell, we investigate if the presence of these molecules will enhance the T cell response or not. (II) co-expressing in the 3DT52.5.8 cells either the human or mouse CD4 WT, together with a panel of mutated human CD4 molecules. These mutant molecules involving residues from the face encompassing the CDR1, CDR3 regions of D1 and the FG loop of D2 and have been previously shown as being unable to interact with class II. We determine if the presence of the WT CD4 rescues the function of the mutants or not. (III) co-expressing either the human or mouse CD4 WT with the mutated human CD4 molecules containing residues mutated from the face of the CDR2 region in D1. We thus could be in a position to determine if the loss of class II interaction with these mutants could be rescued.

CD4 interacts with class II MHC molecules, either as an accessory molecule or as a co-receptor. The molecules involved in these two functions of CD4 are different from each other. In the accessory molecule model, CD4 can bind to a class II molecule independent of the TCR/MHC interaction: while in the co-receptor model both CD4 and the TCR bind to a same class II molecule. Due to the distinction in the two modes of CD4/class II interaction, it leads to the question if CD4 would adopt a different fashion of interaction on T cell surface. With regard to the CD4 oligomerization issue, we wonder if

CD4 oligomerizes in both function or only in the co-receptor but not the accessory molecule or vice versa. The 3DT52.5.8 cellular system provides readout for both the co-ligand (accessory molecule) and the co-receptor functions of CD4. We have co-expressed in the 3DT52.5.8 cells either both the human and mouse CD4 WT, or one of the CD4 WT with a panel of different CD4 mutant molecules from both faces of CD4 in the D1D2 fragment. Results of this study suggest strongly structural requirements for the oligomerization to occur.

L3T4 Interferes with the Function of Human CD4 to Enhance the T Cell Response

Previous studies have shown that expression of human or mouse CD4 in 3DT52.5.8 cells can enhance the T cell response to both its cognate alloantigen H-2D^d and to the bacterial superantigen SEB, when human class II molecules are present on APCs. Due to the extensive conservation between human and mouse CD4, their ability to mount an interspecies interaction with different class II alleles and isotypes (Fleury et al., 1996) and the highly conservation of the proposed CD4 binding sites on class II (König et al., 1992; Cammarota et al., 1992; König et al., 1995), it leads to the suspicion that the same structural components of human and mouse CD4 may be involved in CD4/class II interaction. Moreover, if the oligomerization event of CD4 is critical for its interaction with class II, human and mouse CD4 might use homologous structural components to form a heterodimer (h-CD4 dimerizes with m-CD4). We here would like to study if co-expression of these two molecules on T cell surface will enhance or not the binding to class II , thus arguing whether such a mechanism exists.

Both the human and mouse CD4 (L3T4) molecules were expressed in 3DT52.5.8 cells. Independent clones were generated from each transfection. The expression levels of

both molecules were monitored by staining the cells with OKT4 (anti-h-CD4) and GK1.5 (anti-L3T4) antibodies. Cytofluorescence results show that both h-CD4 and m-CD4 were expressed at high levels (Figure 1A). These double transfectant clones were screened for their ability to produce IL-2 upon stimulation with anti-TCR antibodies. Only cells expressing high levels of both h-CD4 and m-CD4 and producing IL-2 levels comparable to that of the parental cells (shown as the EC50 ratio close to 1 in Table 1) were subjected to further functional assays.

In the functional co-ligand and co-receptor assays T cell responses are dependent on the expression of the CD4 molecule. In the co-ligand assay 3DT cell expressing either h-CD4 or m-CD4 can enhance the T cell response, when co-cultured with APCs expressing either both class II molecule DR4 and the allo-antigen H-2D^d, compared to APCs expressing D^d alone. In fact, CD4$^+$ cells showed an enhanced T cell response, about 20-30 fold of IL-2 production when co-cultured with APCs expressing D^{d}DR4 as compared to those with D^d only (IL-2 ratio) (Figure 1B). The CD4$^-$ parental cells 3DT52.5.8 do not enhance this response (the IL-2 ratio is about 3-5 fold). However, when cells expressing both h-CD4 and m-CD4 molecules were studied in the co-ligand assay, they responded to the antigen in a magnitude similar to that of the CD4$^-$ cells, showing no enhancement in the IL-2 production, as their IL-2 ratios are 4-5 fold (Figure 1B). Moreover, introducing h-CD4 or m-CD4 into 3DT52.5.8 cells can also enhance the T cell response to the bacterial superantigen SEB, when APCs express human class II molecules HLA-DR1 (Fleury et al., 1996). This is shown by that the CD4$^+$ cells require at less 10 fold less SEB for the half maximal response, compared to the CD4$^-$ 3DT52.5.8 cells. When cells expressing both human and mouse CD4 were stimulated with SEB, a response similar to CD4$^-$ cells was again demonstrated (Figure 1C). Co-expression of h-CD4 and m-CD4 lead to a total abrogation of the responses in both assays. These results are

surprising since we had previously shown that human or mouse CD4 alone can enhance both the T cell responses.

The interference of the h-CD4 function by the co-expressed m-CD4 was noticed here. This could not be attributed to decreased expression levels since both h-CD4 and m-CD4 in double transfectant cells are comparably expressed to that in single transfectants. Moreover, TCR expression levels could not be attributed to the inability of double transfectants to produce IL-2 either, since the EC 50 ratios of the anti-TCR stimulation for the double transfectants range from 1-1.2. Thus the coordinate expression of both human and mouse CD4 could lead to several scenarios. First, there is oligomerization of CD4 from the same species (intra-species) but not between species (inter-species). Second, both the intra-species and inter-species oligomerization do occur. Third, the co-presence of CD4 from two different species on the cell surface will lead to a interference between both molecules, possibly in their formation of an oligomer and thus result in a loss of function. For the first two scenarios the enhancement of T cell response could still be observed. However, our results are concordant with the third model. We believe that CD4 oligomerization is essential for its functional interaction with class II, albeit as an accessory molecule or as a co-receptor. Mouse CD4, although it shares 60% homology with h-CD4, may form the oligomer by using different residues which at this stage are still unknown. Though there is a chance that some CD4 might form intra-species oligomers, these oligomer structure may not be stable enough to interact with class II. Alternatively, this interference could be due to sequestration of $p56^{lck}$ from h-CD4 by m-CD4, although this is not supported by our results (using other double transfections presented next). This issue thus requires further studies involving double transfectants expressing both WT and truncated CD4 molecules.

Based on this, we propose that in order for CD4 to stably interact with class II MHC molecules, they need to be able to form an oligomer.

Human And Mouse CD4 Wild-Type Cannot Rescue The Function When Co-Expressed With Mutated CD4 Molecules In The CDR1, CDR3 Regions Of D1, And In The FG Loop Of D2.

Previous studies have shown that mutants S19Y, Q89L and 165[SR] lost the interaction with class II MHC molecules, in both the co-ligand and the co-receptor assays (Fleury et al., 1991, 1996). These mutated residues are located on the same face of CD4. All these mutants had either lost or reduced binding to certain antibody epitopes, however, all of them retain the epitope for the CD4-specific mAb OKT4 which binds in the D3 domain of CD4. Here h-CD4 WT molecule was co-expressed with CD4 mutants in the 3DT52.5.8 cell. Both CD4 WT and mutant molecules were expressed to good levels. Mutant S19Y had reduced binding for mAb B66.6.1, mutant Q89L had reduced binding for OKT4D, while mutant 164[SR] had lost the MT151 epitope. Introduction of the h-CD4 WT molecule rescued these affected epitopes (staining results shown in Table 1).

The double transfectants expressing both the CD4 WT and mutants were studied in the two assays. In the D^d co-ligand assay, the single mutants showed responses comparable to $CD4^-$ cells, and their IL-2 ratios are 2-5 fold (Figure 2A). However, double transfectants expressing both WT and mutant CD4 responded to the alloantigen D^d in a magnitude similar to that of the $CD4^+$ and single mutants. In fact, the IL-2 ratios for the double mutants range from 1 to 8, comparable to that of the $CD4^+$ 3DT cells. Therefore it is clear that the presence of the CD4 WT could not compensate for the loss of interaction with class II for these mutants. Furthermore, when the CDR3 region mutant Q89L was

co-expressed with m-CD4, the interaction with class II would not be rescued either, as shown by only 2.3 fold IL-2 ratio.

In the co-receptor SEB assay the $CD4^+$ cells require at least 10 fold less SEB for the half maximal response, compared to $CD4^-$ 3DT52.5.8 cells. The single mutants S19Y, Q89L and 165[SR] showed a reduced response to SEB, similar to that of the 3DT52.5.8 cell. When the h-CD4 WT was co-expressed with these mutants, however, the T cell responses were not enhanced (Figure 2B). In contrast, the double transfectants still require at least 10 fold more SEB for the half maximal response, and IL-2 production reaches only 20% of that of WT $CD4^+$ cells.

These studies clearly demonstrated that when human or mouse CD4 WT are co-expressed with mutants which are deficient for class II interaction, no enhancement of the T cell responses in the co-ligand and the co-receptor assays can be observed. The exact nature of such events is not clear; however, several hypotheses can be proposed.

First, these mutations could disrupt a structure which is essential for the oligomer formation, either by directly abrogating the interface or by modifying the conformation of CD4; thus oligomers will only be formed between the WT, but not between WT and mutants, nor between mutants. Second, the oligomer interface is still intact, however, due to the loss of function of these mutants, they will affect the oligomer structure to interact with class II. Importantly it has recently been demonstrated that peptide containing residue Q89 can bind to the β2 region of class II MHC molecule with high affinity (Zhang et al., 1996), thus the first model is not favored but our own results are further confirmed (Fleury et al., 1991). Alternatively, it might suggest that these mutated CD4 molecules can still form an oligomer with the WT molecules. Due to the different avidities for class II of

these two sets of CD4 (WT vs mutant), the oligomer formed between the WT and the mutants will no longer form a stable complex with class II. This phenomenon has been reported by Sakihama, that co-expression of CD4 WT with mutant F43L results loss of function for the CD4 (Sakihama et al., 1995).

Results from these experiments suggest, for CD4 to interact with class II MHC and fulfill its function in T cell activation, it has to meet the following two requirements simultaneously. First, CD4 must be able to bind to class II; in addition, CD4 should be able to self-associate with each other to form a dimer or oligomer. If only one requirement is met. such as the case of the double transfectants expressing CD4 WT and CD4 MT, no functional interaction between CD4 and class II will occur. Furthermore, the CD4 mutant can act as dominant negative mutant to inhibit the function of the WT CD4.

Human And Murine CD4 Wild-Type Can Rescue The Function When Coexpressed With Mutants K46A And E77A

It has been shown that residues form the CDR2 region of CD4 do not play a major role in interaction with class II MHC, although point mutation mutants K46A and E77A, and double mutants K46A/P47N can not enhance the T cell response by binding to class II in both the co-ligand and co-receptor assay (Huang et al., 1996; Fleury et al., 1991). When human or mouse CD4 was co-expressed with mutants K46A and E77A into the 3DT52.5.8 cells, some very interesting phenomena were observed.

Human or mouse CD4 molecule were expressed at good levels with mutants K46A and E77A in the 3DT52.5.8 cells (Table 1). Upon generation of double-transfectants, they were studied in both assays. In the co-ligand D^d assay, it has been shown that mutants

K46A and E77A can no longer enhance the T cell response (Huang et al., 1996). When either h-CD4 or m-CD4 was co-expressed with these mutants, a significantly enhanced T cell response was observed, showing an IL-2 ratio comparable to that of the WT $CD4^+$ cells. For cells co-expressing h-CD4 and mutants, the IL-2 ratio ranges from 12 to 24 fold; while for cells co-expressing m-CD4 and mutants, it ranges from 11 to 18 (Figure 3A).

Previous studies have shown that murine CD4 molecules can also enhance the T cell response to the bacterial superantigen SEB, however, to a lesser extent compared to h-CD4 molecule, when human class II molecules were expressed on the APCs (Fleury et al., 1996). When the double transfectants expressing both h-CD4 and mutants were stimulated with SEB presented by human class II^+ APCs, they show a similar response to the h-$CD4^+$ cell, which produced 3 to 5 times more IL-2 and requires 10 fold less SEB for the half maximal response, when compared to that of the $CD4^-$ cells and the mutants (Figure 3B). When the double transfectants expressing both m-CD4 L3T4 and mutants (K46A and E77A) were stimulated with SEB, still presented by human class II^+ APCs, the responses are significantly enhanced too as compared to the $CD4^-$ cells and the mutants (Figure 3B).

This result clearly demonstrated that h-CD4 and m-CD4 can enhance the T cell responses when co-expressed with the two mutants. This is different form the previous results showing that the function of the CD4 mutants from the opposite face of the CDR2 region cannot be rescued by CD4 WT. Two possibilities can account for such a result. First, residues from the CDR2 region or on the same face of the CDR2 are involved in the class II interacting sites. Such a notion is not supported by a recent study using aromatically modified peptides which showed that residues from this region do not play a major role in binding to class II (Zhang et al., 1996). Furthermore, if they are involved in

the binding sites for class II, no rescue of function should be observed as those mutants presented the previous section. Alternatively, these residues might be not in the key contact sites for class II (Fleury et al., 1991; Huang et al., 1996), but rather on the oligomer interface. The CDR2 region is divergent among species, thus involvement of this region in binding to the highly conserved non-polymorphic region on class II across species is unlikely. On the other hand, this region is unusually prominent, suggesting a functional interaction with other molecules in addition to gp120 binding. Therefore mutations at these residues may abrogate the oligomer formation between the mutants, and between CD4 WT and mutants. However, there is still oligomers formed among the WT molecules which will in turn enable the functional interaction with class II and T cell activation.

Here by using the 3DT cellular system we have investigated the oligomerization event of CD4 in interaction with class II. Our results with the double transfectants expressing both h-CD4 and m-CD4 suggest that such a mechanism does exist in both function of CD4 and it is critical for T cell activation. Moreover, study with a panel of mutants from both faces of CD4 suggests that the oligomer interface is located in the face of the CDR2 region. The final confirmation of this result awaits further crystallographic studies.

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Table 1. Summary of Cells

	Expression Level (b)				EC50 Ratio (mt/WT) ©
Cells (a)	OKT4	GK1.5	B66.6.1	MT151	F23.1
CD4 WT	56		37	29	1
L3T4		42			0.8
CD4 L3T4 #1	40	26			1
CD4 L3T4 #2	26	22			1.2
S19Y CD4 #1	32			19	1.6
S19Y CD4 #2	39			11	1.2
Q89L CD4	71		65		1.3
Q89L L3T4 #1	53	85			1.1
Q89L L3T4 #2	49	84			1.4
165[SR] CD4	76			60	1.3
K46A CD4					1
K46A L3T4 #1	47	23			0.7
K46A L3T4 #2	45	33			0.8
E77A CD4 #1					1.2
E77A CD4 #2					1.1
E77A L3T4 #1	52	27			0.7
E77A L3T4 #2	48	27			0.7

(a) Names of cells.
(b) Expression levels for OKT4, GK1.5, B66.6.1, MT151 are defined as the mean fluorescence value of a given cell line.
(c) The EC50 ratio (mutant/WT) for F23.1 is defined in Materials and methods.

Figure Legends:

Figure 1A. FACS analysis for 3DT52.5.8 cells transfected with both human and mouse CD4. The upper panel shows staining with both GAM.FITC and GAM.PE without the primary antibodies. The lower panel is staining with OKT4.FITC (anti-human CD4) and GK1.5.PE (anti-mouse CD4).

Figure 1B. Quantitative analysis of the T cell response in the co-ligand assay. 3DT52.5.8 cells expressing both human and mouse CD4 were co-cultured with DAP-3 cells expressing both the alloantigen D^d and the class II MHC molecule DR4. Results were presented as such: the Y axis is expressed as the IL-2 ratio (D^d DR4/D^d), as described in the materials and methods section. On the X axis, the names of the cells are indicated.

Figure 1C. Quantitative analysis of the T cell response in the co-receptor assay. 3DT52.5.8 cells expressing both human and mouse CD4 were stimulated with the bacterial superantigen SEB presented by human class II molecules DR1 expressed on the DAP-3 cells. The SEB concentration displayed on X-axis are: 10^5, 2.5 X 10^4, 1.25 X 10^4, 6.25 X 10^3, 3.13 X 10^3, 1.56 X 10^3, 7.8 X 10^2, 3.9 X 10^2, 1.95 X 10^2, 0.98 X 10^2 pg/ml. On the Y-axis the IL-2 units are presented.

Figure 2A. Quantitative analysis of the T cell response in the co-ligand assay. 3DT52.5.8 cells expressing either human or mouse CD4 with different CD4 mutants from the CDR1, CDR3 of D1 and the FG loop of D2, were co-cultured with DAP-3 cells expressing alloantigen D^d and human class II molecule DR4. Results were presented the same way as in Figure 1B.

Figure 2B. Quantitative analysis of the T cell response in the co-receptor assay. 3DT52.5.8 cells expressing either human or mouse CD4 with CD4 mutants from the CDR1, CDR3 of D1 and the FG loop of D2, were stimulated with the bacterial superantigen SEB presented by human class II molecules DR1 expressed on the DAP-3 cells. The SEB concentration used here are the same as the one in Figure 1C.

Figure 3A. Quantitative analysis of the T cell response in the co-ligand assay. 3DT52.5.8 cells expressing either human or mouse CD4 with different CD4 mutants from the CDR2 region of D1 were co-cultured with DAP-3 cells expressing alloantigen D^d and human class II molecule DR4. Results were presented the same way as in Figure 1B.

Figure 3B. Quantitative analysis of the T cell response in the co-receptor assay. 3DT52.5.8 cells expressing either human or mouse CD4 with CD4 mutants from the CDR2 region of D1 were stimulated with the bacterial superantigen SEB presented by human class II molecules DR1 expressed on the DAP-3 cells. The SEB concentrations are the same as the one in Figure 1C.

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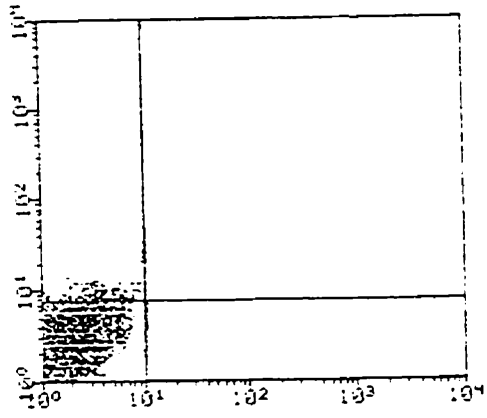

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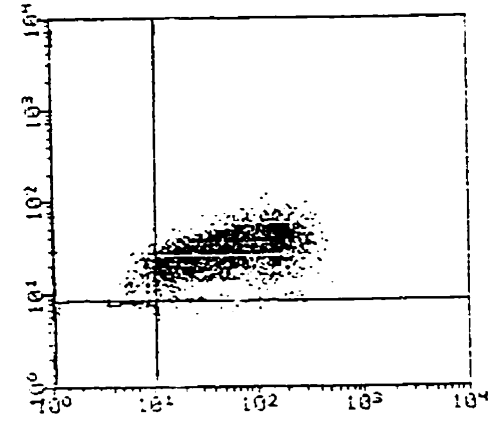


OKT4.FITC

Figure 1A

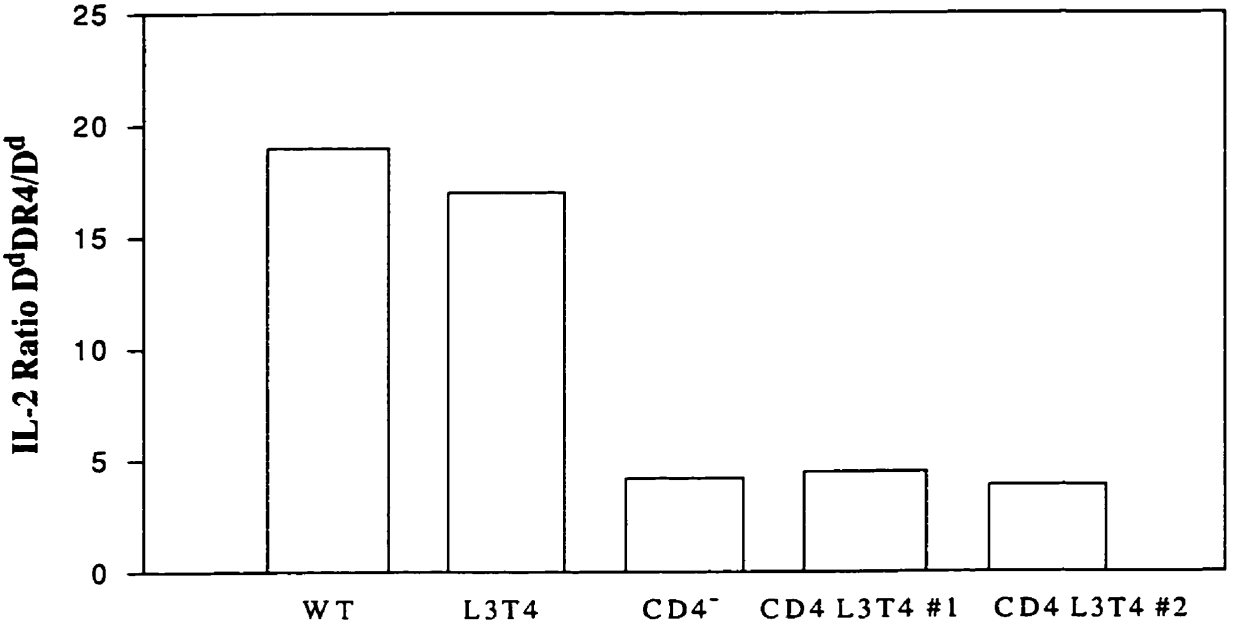


Figure 1B

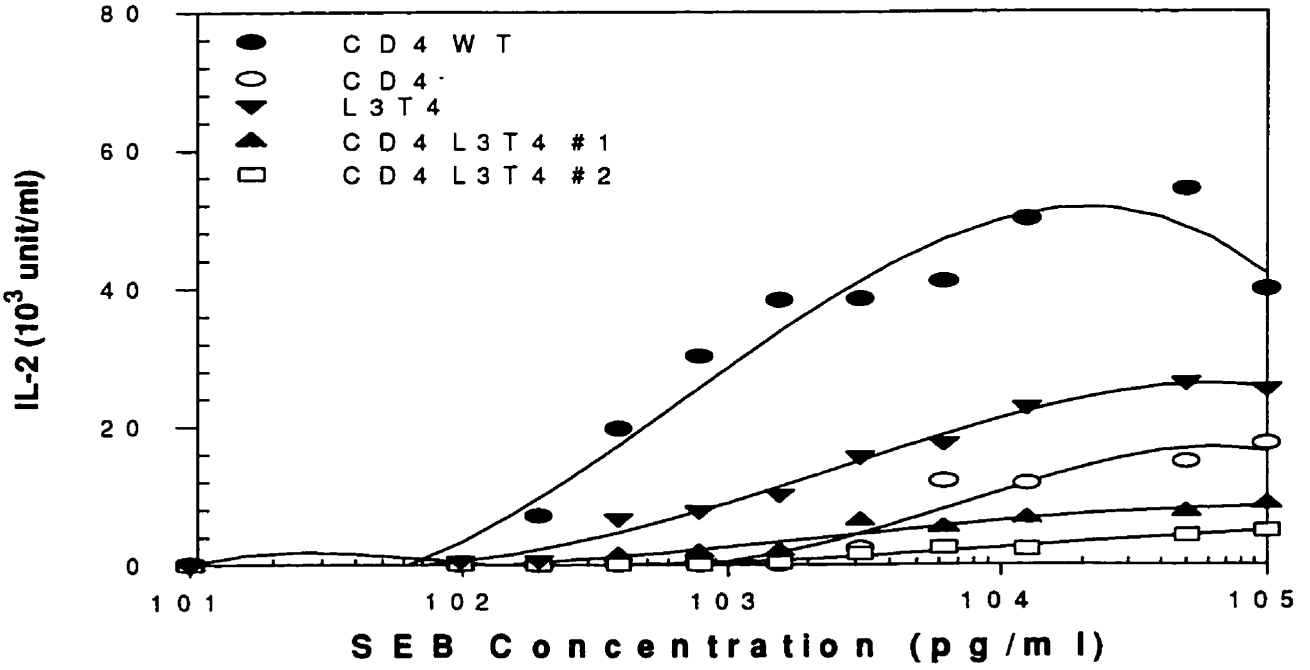
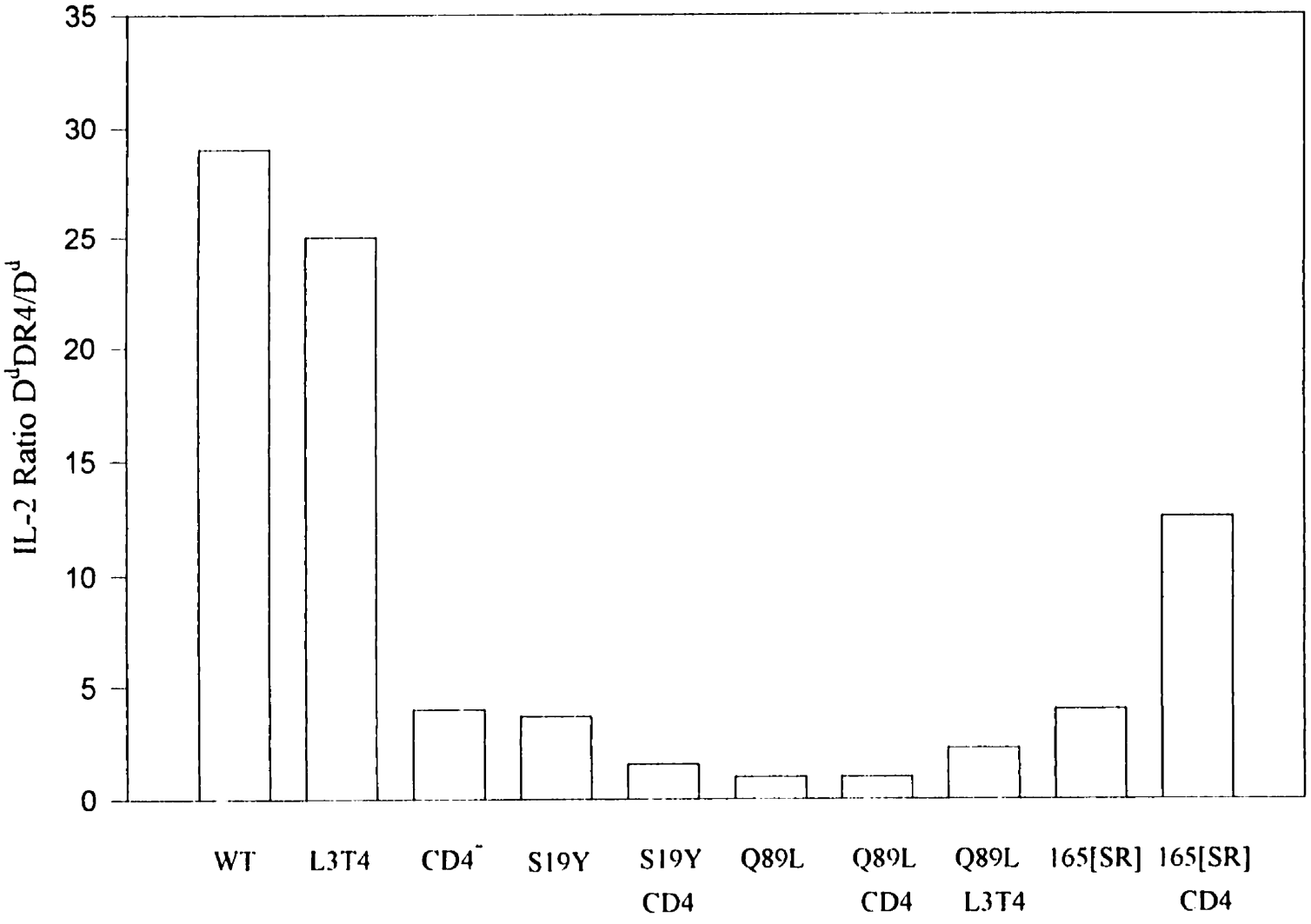


Figure 1C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30
25
20
15
10
5
0
WT
L3T4
CD4-
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S19Y CD4
Q89L
Q89L CD4
Q89L L3T4
165[SR]
165[SR] CD4

Figure 2A

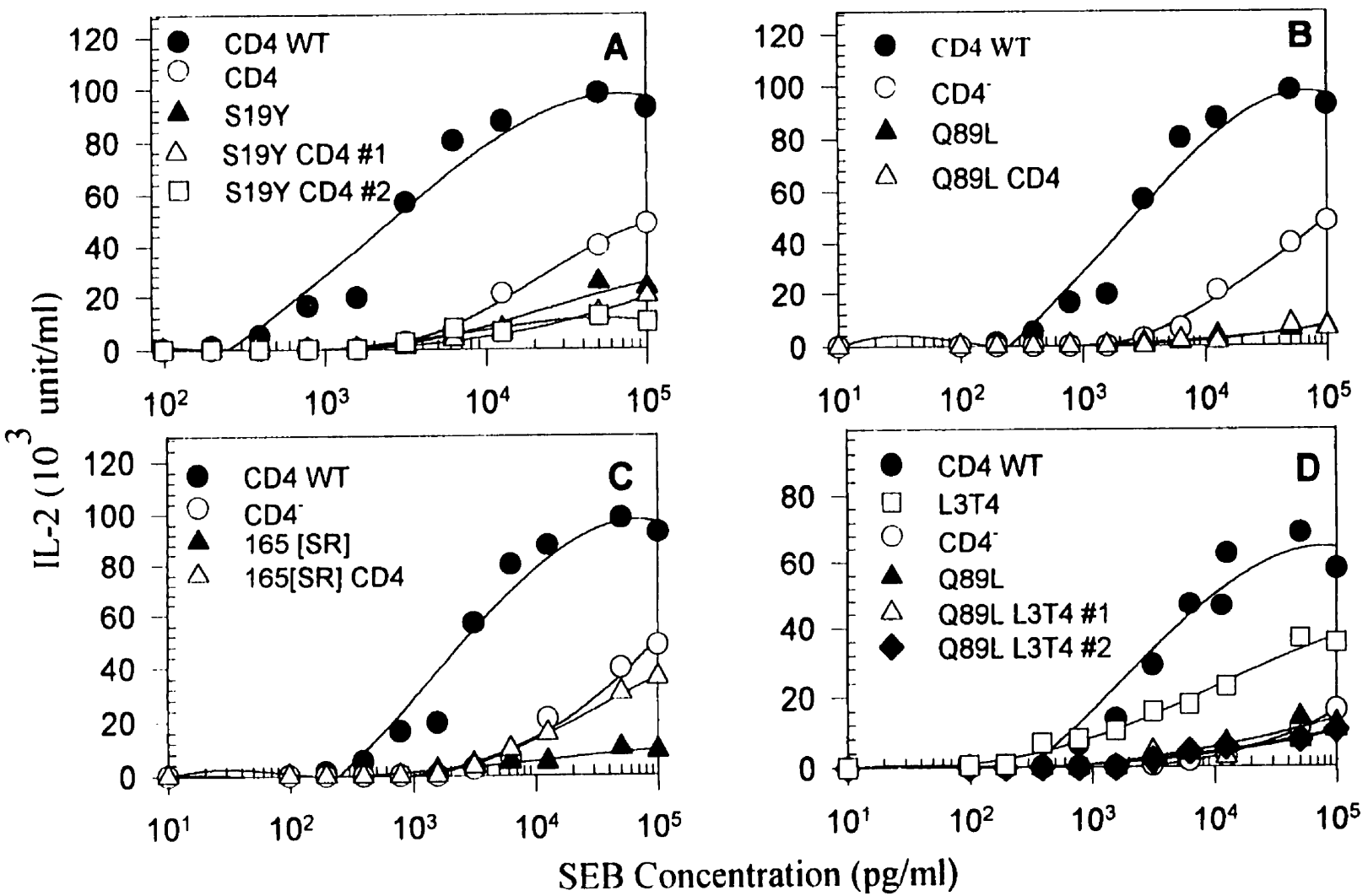
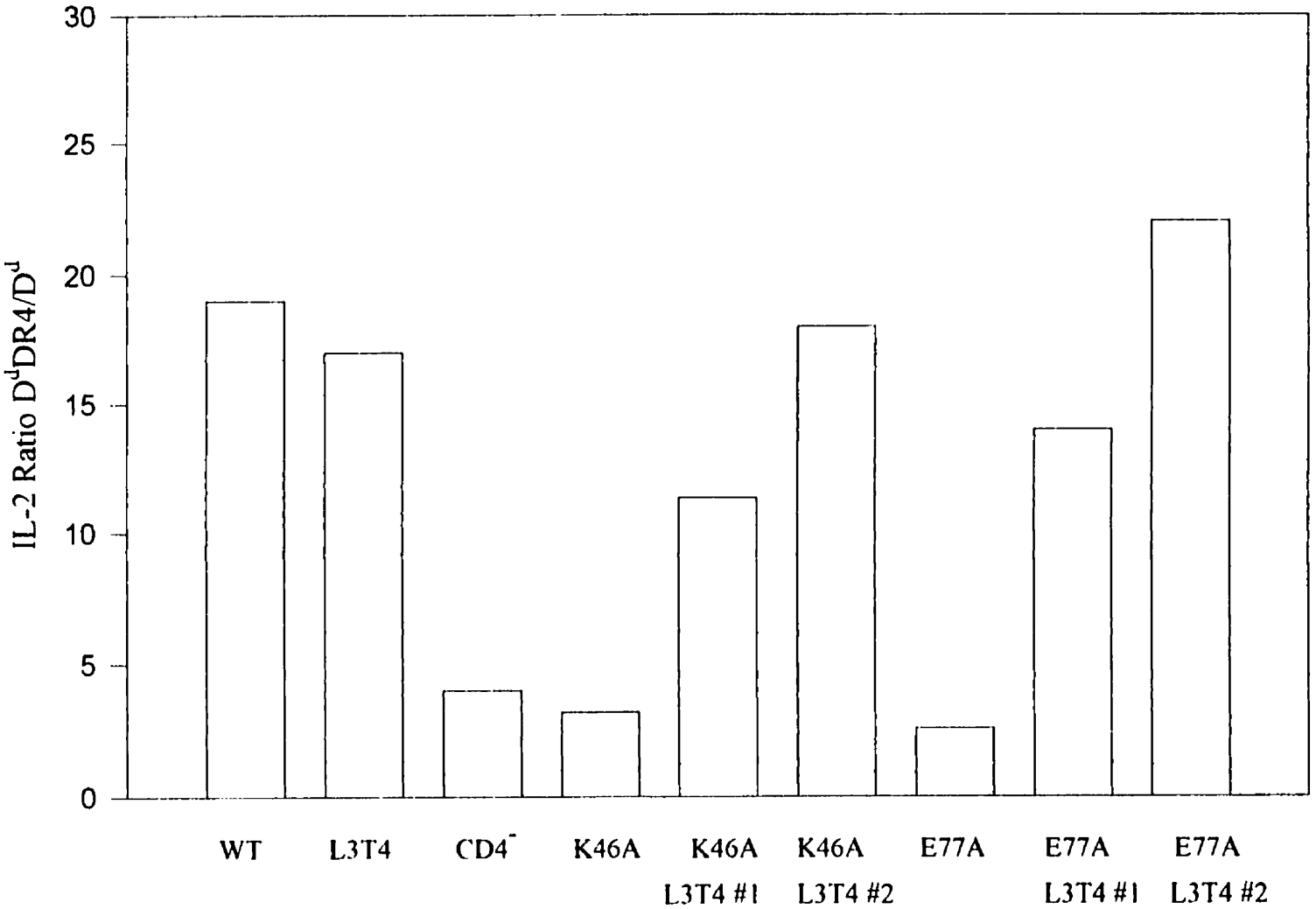

A
CD4 WT
CD4
S19Y
S19Y CD4 #1
S19Y CD4 #2
B
CD4 WT
CD4-
Q89L
Q89L CD4
C
CD4 WT
CD4-
165 [SR]
165[SR] CD4
D
CD4 WT
L3T4
CD4-
Q89L
Q89L L3T4 #1
Q89L L3T4 #2
IL-2 (10^3 unit/ml)
SEB Concentration (pg/ml)

Figure 2B


30
25
20
15
10
5
0
IL-2 Ratio D^dDR4/D^d
WT
L3T4
CD4-
K46A
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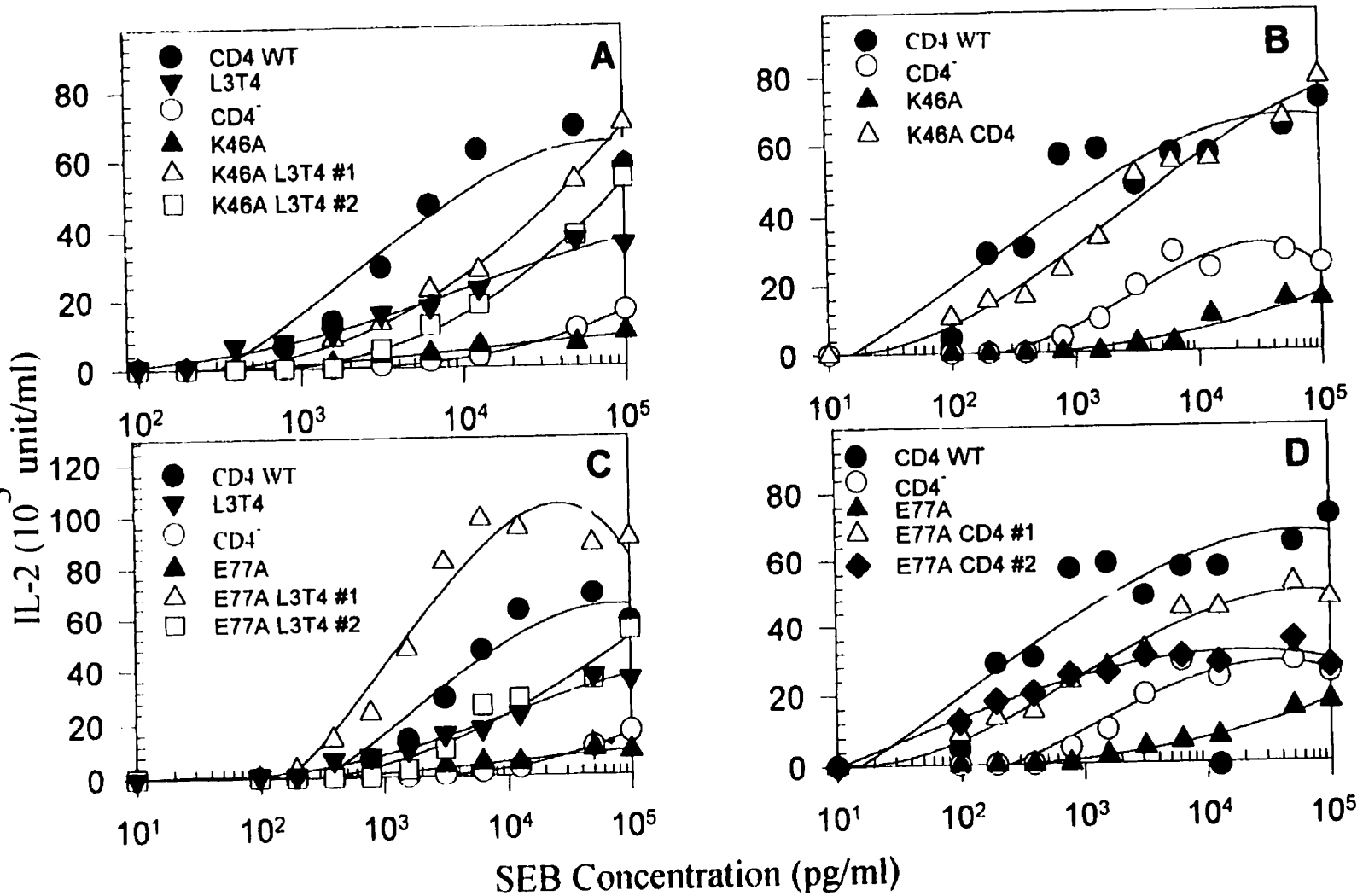
K46A L3T4 #2
E77A
E77A L3T4 #1
E77A L3T4 #2

Figure 3A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CD4 WT
L3T4
CD4-
K46A
K46A L3T4 #1
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B
CD4 WT
CD4-
K46A
K46A CD4
C
CD4 WT
L3T4
CD4-
E77A
E77A L3T4 #1
E77A L3T4 #2
D
CD4 WT
CD4-
E77A
E77A CD4 #1
E77A CD4 #2
IL-2 (10^3 unit/ml)
SEB Concentration (pg/ml)

Figure 3B

Chapter 5

General Discussion

The CD4 molecule is expressed on approximately 60% of peripheral blood T lymphocytes where the TCRs are selectively restricted to MHC class II molecules on antigen presenting cells. CD4 positive T cells are not only involved in the cellular response, but they also provide "help" to the humoral response; they thus play a central role in the immune system. Although CD4 is a phenotypic marker for these T cells, it has indeed an important role in T cell development and T cell activation by interacting with class II MHC molecules. The CD4/class II interaction is very important for the modulation of T cell activation. Our knowledge of this interaction is still limited although important contributions have been made by this lab and several other groups. The understanding of CD4/class II interaction at the molecular level depends largely on the availability of the crystal structure, on site-directed mutagenesis of each molecule and on functional studies. The crystal structures of both human D1D2 and rat D3D4 fragments of CD4 and human class II MHC molecule HLA-DR1 have been successfully solved (Ryu et al., 1990, Wang et al., 1990; Brady et al., 1993; Brown et al., 1993). Various approaches to map the contact residues on both CD4 and class II molecules for this interaction have been carried out (Fleury et al., 1991; Bowman et al., 1990; Moebius et al., 1992, 1993). Final characterization of this interaction will require the co-crystallization of these molecules.

Although previous experiments provide a great deal of information to our understanding of the CD4/class II event, however, questions still remain to be answered In an effort to further characterize the class II MHC contact sites on CD4, to study the possible dimerization/oligomerization events of CD4 upon interaction with class II molecules, and to determine the inter-species interaction of CD4/class II, we have made approaches presented in the previous chapters. Here we would like to focus our discussion on the functional significance of all these sites of CD4 mapped in contact with class II, in regard with the possible dimerization/oligomerization events of CD4 and class II, even the TCR.

1. Structural and Functional Analysis of the Interaction between the CD4 Molecule and the Class II MHC Molecule: Mapping the Contact Sites on CD4

1.1 General Models of The Functional Roles of CD4 in T Cell Activation: Structural Components for Each Model

As described in the introduction, CD4 binds to its physiological ligand-the class II MHC molecules in the non-polymorphic regions (Cammarota et al., 1992; Konig et al., 1992; 1995). Two models have been proposed for the functional roles of CD4 in T cell activation, the adhesion molecule (accessory molecule) and the co-receptor (Janeway, 1992). In the adhesion function of CD4, it is believed that the CD4/class II interaction can mediate cell-cell adhesion between T cells and APCs which in turn will increase the chance for the TCR to encounter the Ag/MHC ligand on the APC surface, thus augmenting T cell stimulation at a given Ag density. In the adhesion function of CD4, the involvement of the CD4 cytoplasmic tail-associated tyrosine kinase $p56^{lck}$ is not believed to be critical. However, it has been shown that expression of $p56^{lck}$ in the CD4-transfected CHO cells can enhance their adhesion to the class II MHC-positive B lymphocytes (Kinch et al., 1994). By analogy with other receptors associated to tyrosine kinase such as the EGF receptor it is more than likely that tyrosine phosphorylation of the receptor co-modulates its affinity for its ligand. However, in the co-receptor model of CD4, evidences suggested that there is a physical association between the TCR/CD3 complex and CD4 (both molecules share the same class II MHC ligand); and the involvement of $p56^{lck}$ is critical for transmitting the CD4-mediated signal to downstream events. Although the involvement of $p56^{lck}$ in the CD4 co-receptor function is clearly demonstrated, in some experimental systems CD4 has been observed to increase the T cell responsiveness in the absence of association with $p56^{lck}$ (Zerbib et al., 1995; Killeen & Littman, 1993). Both in vitro and in vivo Lck-independent CD4 function is hypothesized to reflect the ability of CD4 to act as adhesion molecules. Alternatively, it may suggest the involvement of CD4 in other signal transduction pathways by associating with molecules other than Lck.

1.2 Cellular Assay Systems Used to Characterize Each Function of CD4 and the CD4/Class II MHC Interaction: the Major Differences between Each Assay System and their Physiological Relevance

As described in previous chapters there are mainly three types of cellular assays used to investigate the functional impact of CD4/class II interaction, namely the adhesion assay, the co-ligand assay and the co-receptor assay. Here these assay systems, their structural components and the advantage and disadvantage of each system will be discussed from the structural/functional point of view.

Adhesion Assay:

The strict correlation of CD4 expression and MHC class II restriction of T cells led to the suggestion that CD4 might interact directly with class II. In an attempt to demonstrate this, a TCR-independent adhesion assay was developed (Doyle and Strominger 1987). It involved the CV1 fibroblastic cells expressing high levels of CD4 and class II^+ B lymphocytes. At the high level of CD4 expression the adhesion between the two cells was clearly demonstrated; however, at physiological levels of CD4, interaction with class II alone is insufficient to mediate cell-cell adhesion. Later, a similar TCR-independent adhesion assay involving CD4-transfected COS cells and class II^+ B lymphocytes has been employed by the group of Reinherz to determine the class II binding sites on CD4 (Clayton et al., 1989). The readout of this assay is the rosette formation between the two cells. Again levels of expression plays a critical role in this assay.

Co-ligand Assay:

$CD4^+$ T cells recognize Ags presented by class II molecules on APCs, and both CD4 and the TCR bind to class II molecules. Hence, the contribution of CD4/class II interaction in T cell activation is difficult to be discriminated from that of the TCR/class II interaction. With the availability of the 3DT52.5.8 cells and by transfecting CD4 in theses cells it became possible to study this issue (Greenstein et al., 1984). The advantages of the

3DT cellular system are the followings: first, in this particular system CD4 and TCR have different MHC ligands (CD4 binds to class II; while the TCR binds to class I), thus it allows to distinguish the interaction of CD4/MHC from the one of the TCR/MHC/Ag. It is believed that CD4 act as an adhesion molecule here since it is not involved in the TCR/class II/Ag interaction. Second, different from the adhesion assay mentioned above, this assay monitors the CD4/class II interaction in the context of T cell activation, in addition to cell-cell adhesion. Thus this assay is named co-ligand assay in order to be discriminated from the TCR-independent adhesion system (Fleury et al., 1991) In this assay the IL-2 production is monitored. This assay has been used by the Sekaly's group to study different mutations in CD4 and their effects in interaction with class II (Fleury et al., 1991; Fleury et al., 1996; Huang et al., 1996).

Co-receptor Assay

Other cellular system using T cell hybridomas which are specific for class II MHC-restricted Ags and the efficient T cell responses depend on the expression of CD4 and the CD4/class II interaction. In these systems CD4 is believed to act as a co-receptor for T cell activation (Bowman et al 1990; Huang et al 1996). The read-out is often an enhanced T cell response (IL-2 production) resulting from the CD4/class II interaction, as compared to the $CD4^-$ T cells. All these assays are termed co-receptor assays. The 3DT52.5.8 cells express the TCR Vβ8.1 chain and can be stimulated by the bacterial antigen SEB when presented by class II molecules, therefore the 3DT system indeed provides a co-receptor assay in addition to the co-ligand assay. It is therefore beneficial for us to use the 3DT system since in one single cellular system two functions of CD4 can be studied.

The major difference between the three assay systems is that: the CD4/class II interaction is dissected in the T cell environment or not. Although the adhesion system involving the CD4-transfected COS cells is commonly employed in studying the CD4/class II interaction, many cellular components expressed in T cells which have already been shown to have an impact on CD4/class II interaction are missing, namely the TCR, the tyrosine kinase $p56^{lck}$, CD45, and many more.

The TCR

The major difference between the adhesion assay and the functional co-ligand and co-receptor assays is the involvement of the TCR or lack thereof. Although the adhesion assay provides information on the class II/CD4 interaction, it does not represent the physiological situation in which CD4 is expressed on class II MHC restricted T cells and CD4 interaction with the TCR is important for T cell activation. It can not explain the thymic selection of single CD4 positive cells based on the MHC specificity of the TCR. Moreover, it has been shown at least for the other co-receptor CD8 that stimulation via the TCR can result in a significantly enhanced CD8/class I interaction and mediate cell-cell adhesion (O'Rourke et al., 1990). Finally, co-clustering of CD4 and TCR can be detected at the cell contact site of MHC/Ag, when recognition of Ag occurs (Kupfer et al., 1987) It is believed that the interaction of CD4 with other molecules such as the TCR will modulate the CD4/class II interaction. However, all these events have no representation in the COS cell adhesion system.

$p56^{lck}$

It was initially proposed that the adhesion function of CD4 is not dependent on its cytoplasmic tail-associated $p56^{lck}$. However, in a later report it was clearly shown that expression of $p56^{lck}$ in CD4-transfected CHO cells can enhance the adhesion of CHO cells to class II+ B lymphocytes; while the expression of a constitutively activated Lck molecule (the Lck F505) prevents cell-cell adhesion (Kinch et al., 1994). The mechanism for such a phenomenon is still not well characterized; however, the effect of Lck in redistribution of CD4 and other cytoskeletal proteins at the cell surface is evident.

In the co-ligand function of CD4, it was initially suggested that the CD4-associated $p56^{lck}$ is not involved. However, our data do suggest a role for CD4/Lck association in the co-ligand function of CD4. By expressing mutants in 3DT52.5.8 cells two CD4 which can no longer associate with Lck (either the truncated CD4 or the CD4 mutant which bears alanine substitutions at the Lck-association residue cysteines 420 and

422 on the cytoplasmic tail of CD4), we have demonstrated that these mutants can no longer result in an enhanced IL-2 production in the co-ligand assay. (Figure 1, Figure 2) As expected, the importance of the CD4/Lck association in the co-receptor assay was demonstrated as well.

CD45

It has been shown that the tyrosine phosphatase CD45 can associate with a number of T cell surface molecules, including CD4 (Mittler et al., 1991 JI; Dianzini et al., 1990). This association of CD45 with molecules important in TCR signaling may be important for the generation of optimal signal transduction. A recent study demonstrated that there is a preferential association of CD4 and the low molecular weight CD45 isoform after co-capping with anti-CD4 antibody (Dianzini et al., 1992; Leitenberg et al., 1996). The association of CD45 with CD4 is of particular interest since CD4-associated Lck has been shown as a potential substrate for the CD45 phosphatase activity. However, in a recent report (Leitenberg et al., 1996) it has been demonstrated the association of CD4 with low molecular weight isoform of CD45 is independent of the cytoplasmic domain of CD45 which contains the phosphatase activity, but on the external domain of CD45. The structural basis and functional importance of this phosphatase-independent association are not well understood at this stage. Nevertheless, the association of CD4 with CD45 which may involved in other signaling pathway other than Lck will modulate its function. We do not know if human CD4 introduced in the murine 3DT52.5.8 cells will result an efficient association with CD45. Further experiments are required for a better understanding of the mechanism.

Taken together, this leads us to believe that the adhesion system, although it provides information of CD4/class II interaction, it lacks the modulation of this interaction by various molecules in T cells, thus its physiological reflection is limited. On the other hand, the co-receptor functional assays have the advantage of representing the physiological situation in T cells where the CD4 and the TCR interact with the same

Dd STIMULATION

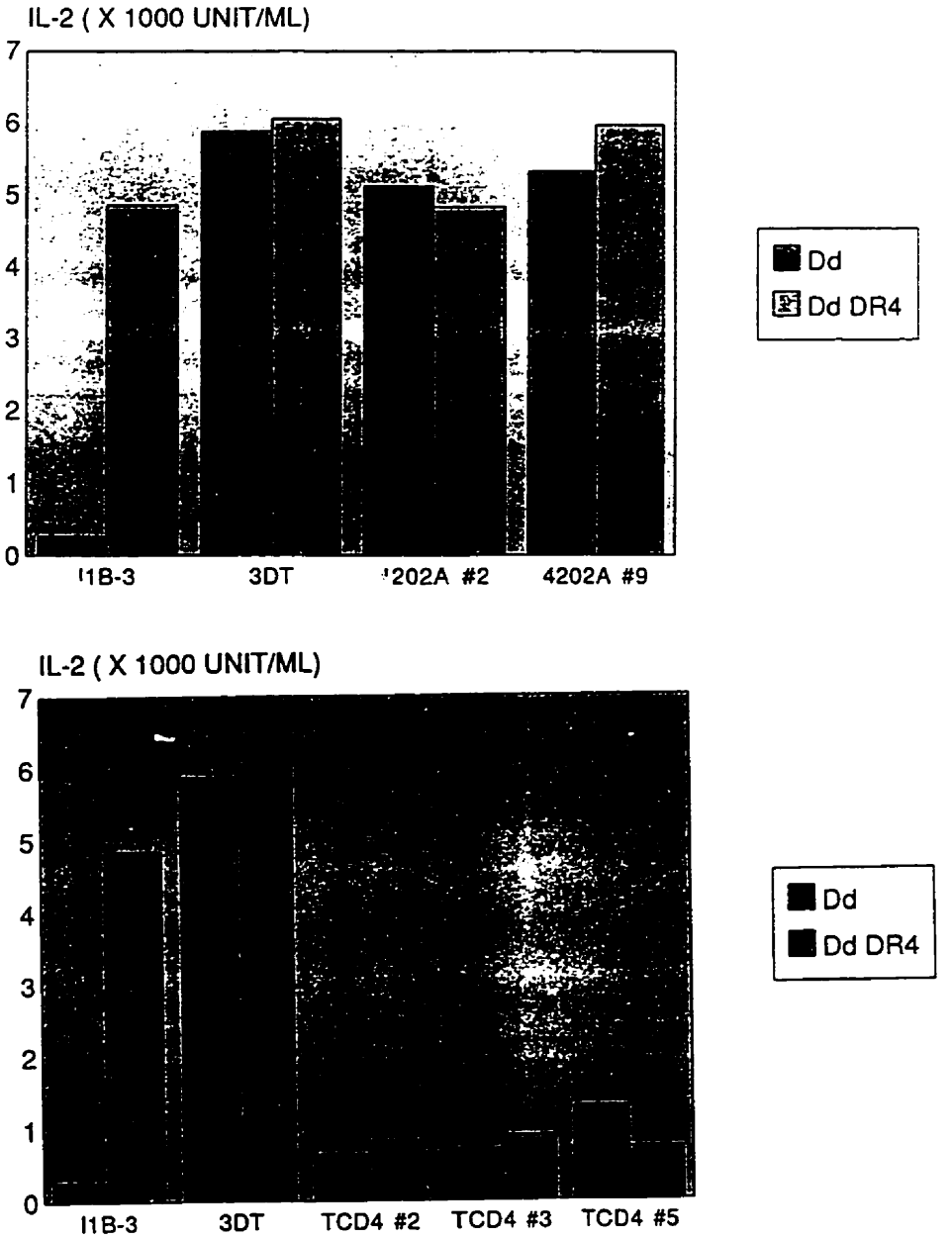

Figure 1

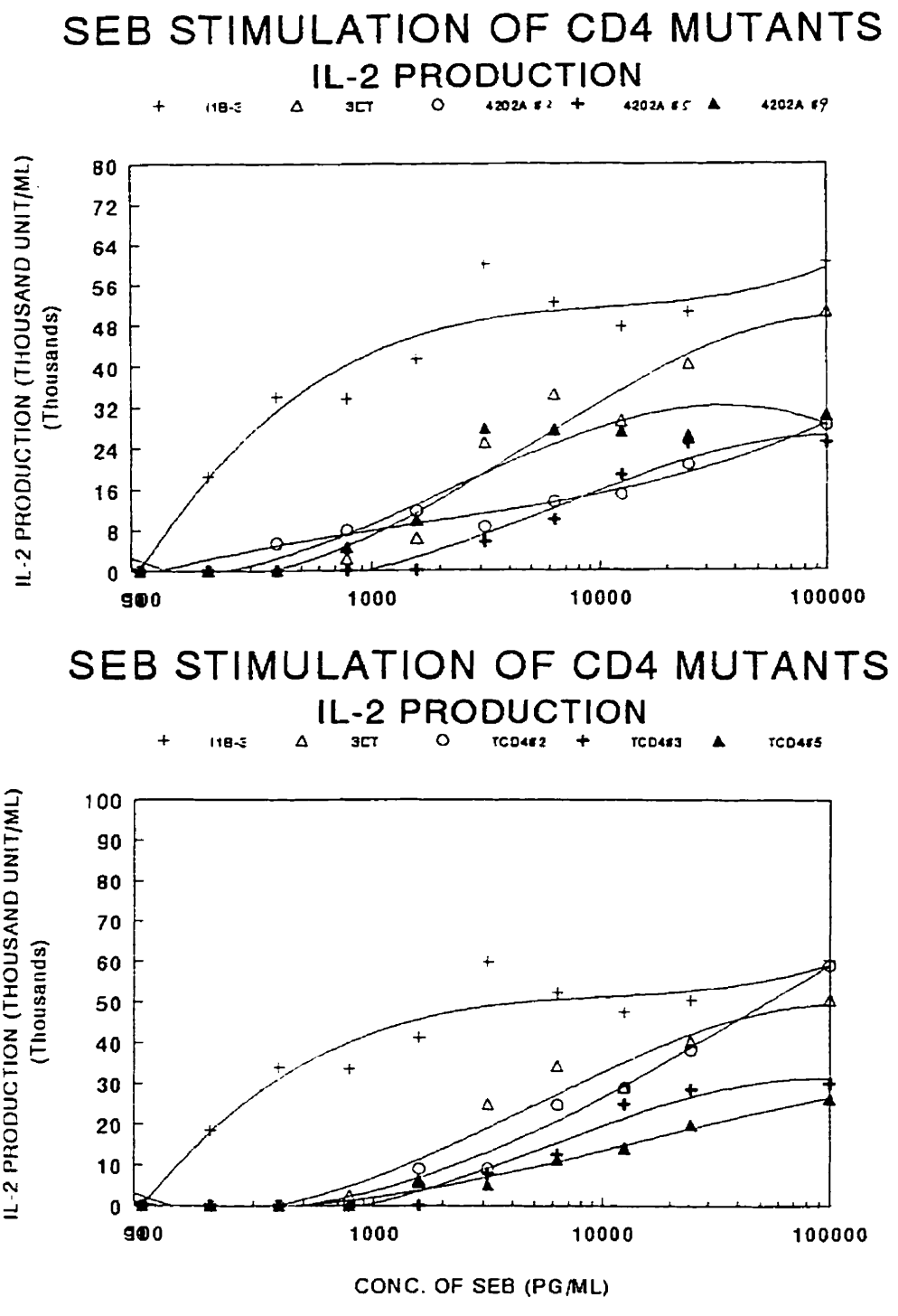
SEB STIMULATION OF CD4 MUTANTS
IL-2 PRODUCTION
+ 118-3 △ 3CT ○ 4202A #2 + 4202A #5 ▲ 4202A #9
IL-2 PRODUCTION (THOUSAND UNIT/ML)
(Thousands)
80
72
64
56
48
40
32
24
16
8
0
900
1000
10000
100000
SEB STIMULATION OF CD4 MUTANTS
IL-2 PRODUCTION
+ 118-3 △ 3CT ○ TCD4#2 + TCD4#3 ▲ TCD4#5
IL-2 PRODUCTION (THOUSAND UNIT/ML)
(Thousands)
100
90
80
70
60
50
40
30
20
10
0
900
1000
10000
100000
CONC. OF SEB (PG/ML)

Figure 2

ligand, however there are limitations: interpretation of results is often complicated by the involvement of the three molecules on the interacting cell surfaces: CD4, the TCR and class II molecules. Hence CD4 residues which when mutated have an effect in T cells response might be the true contact residues for the class II MHC molecule or conformational residues for CD4. They could be involved in the docking site for the TCR, or even involved in a function of CD4 itself, such as forming a dimer/oligomer interface. The co-ligand assay which mimics the adhesion function of CD4 in the T cell activation can simplify the situation in the co-receptor assay, but do not over-simplify the situation compared to the adhesion assay. However, if CD4 plays a co-ligand role under physiological condition is yet unknown. Furthermore, it has been shown in thymic selection that both CD8 and the TCR have to bind to a single class I MHC molecule in order to be selected, though such a role of CD4 has not been demonstrated. In fact, in transgenic mice expressing the truncated CD4, over-expression of this molecule can rescue the selection of $CD4^+$ T cell (Killeen et al., 1993). This suggests that CD4 can act independent of $p56^{lck}$, presumably, either as an adhesion molecule or via another signal transduction pathway.

There are enough evidences supporting both the adhesion and the co-receptor models of the functional role of CD4, however, it is now widely accepted that the primary physiological function of CD4 is to bind and recognize the same MHC molecule as the TCR, rather than just to increase the intra-cellular avidity by mediating adhesion. By doing so the co-receptor CD4 can maintain the antigen specificity of the TCRs, as well as augment the T cell response. This effect of co-receptor is most prominent for recognition of antigens at limiting conditions, either that antigens have low to modest affinity to the TCR, or the concentrations of antigens are limited.

1.3 The Class II MHC Molecule Interaction on CD4 Involved Multiple Sites of CD4

Role of Residues from the Face encompassing the CDR1, the CDR3 regions of D1 and the FG loop of D2 of CD4 in Interaction with Class II:

Using the above discussed assay systems, the class II MHC interaction site(s) on CD4 have been mapped through crystal structure informed site-directed mutagenesis. Residues located on both faces of CD4 have been studies: one face encompasses the CDR1 and the CDR3 Regions in D1 Domain and the FG Loop in D2 Domain of CD4; the opposite face contains mainly residues from the CDR2 region of D2.

The picture emerging from these mutational studies of the CD4-MHC interaction is still rather confusing. Though results from the 3DT co-ligand assay on the face encompassing the CDR1, the CDR3 regions of D1 and the FG loop of D2 (Fleury et al., 1991) were mostly confirmed by subsequent studies using the adhesion assay (Moebius et al., 1992; 1993), major differences exists in the implication of residues from the CDR2 face. In the adhesion assay, residues from the CDR2 region are implicated; however, in the co-ligand study, some puzzling effects were found. Single substitution mutants from the CDR2 region, T45P and G47R had an effect in the co-ligand assay. Since a deletion mutant in the CDR2 region (deletion 43-49) can still interact with different class II isotypes and alleles inter-species and intra-species (Fleury et al., 1991; 1996), and based on the face the in miniature swine CD4 polymorphism have been identified in the CDR2 region, the face of CD4 containing mostly the CDR2 region was not regarded as an essential determinant for class II interaction.

In an effort to further define the molecular interaction on CD4 with class II, we have focused our attention on the role of the exposed residues located in the D1 and D2 domains of CD4, especially those located on the face composed of the CDR1, the CDR3 regions of D1 and the FG loop of D2, as well as those on the opposite face of the CDR2 region. Furthermore, the structure and function of the region between the D2 and D3 domain, the so-called hinge region has been also characterized.

Results from this investigation (presented in Chapter 2) confirmed and further implicated residues from the face of the CDR1, CDR3 regions of D1 and the FG loop of

D2 in interaction with class II. Residues from the CDR2 region seemed to be implicated as well (The role of the CDR2 region will be discussed in next section). This multiple sites mapped on CD4 for class II binding is not a surprise, however, this indicates that more residues or regions in class II should be involved. Recent studies have suggested this by showing that more residues outside the highly conserved β2 segment (residues 137-143) as well as from the α2 region are implicated in CD4/class II interaction (Fleury et al., 1995; Konig et al., 1995). In fact similar results were obtained showing multiple sites on CD8 are involved in interaction with MHC class I molecule (Sun et al., 1995). Although one report suggested that this face could be involved in dimerization of CD4 via the CDR3 region, further investigation using aromatically modified peptide analogs did not support it. On the contrary, it demonstrated that CDR3 peptides can bind to the β2 domain of class II (Zhang et al., 1996). The implication of multiple sites in this face of CD4 in class II interaction strongly suggest that the major class II contact site lies on this face. However, data from this study and identification of the a2 sites in class II suggest that this face of CD4 might not be the only class II interaction site. Finally, a much larger surface of CD4 is implicated in the co-receptor function compared to the co-ligand one. This suggest a different mode of interaction between CD4/TCR/class II might be employed.

Role of Residues from the Face Containing the CDR2 Region of D1 Domain in the Function of CD4

The CDR2 region in D1 of CD4 is the most divergent region, compared to that of the CDR1 and CDR3 regions of D1 and the FG loop of D2. Sequence alignment of the CDR1, CDR2, CDR3 regions of D1 and the FG loop of D2 between human and mouse CD4 allowed us to determine the conservation across species. The CDR1 and CDR3 regions show 54% and 67% homology between h-CD4 and m-CD4, respectively. The FG loop shows 39% homology, however, the CDR2 shows only 38%. Among the 21 residues in the CDR2 only 8 residues are conserved. Moreover, Gustafsson et al (1993) reported that there is an extensive allelic polymorphism in the CDR2 region of the miniature swine

CD4. In fact, three amino acids located within this region are polymorphic, including F43, the critical residue for the HIV gp120 binding.

As discussed in the previous section, the role of the CDR2 region in class II interaction is mostly controversial. Results from the adhesion assay and the co-receptor assay system By155.16 all showed that the CDR2 region is implicated in interaction with class II (Bowman et al., 1990; Moebius et al., 1992; 1993); however, results from the 3DT functional co-ligand and co-receptor assay are not strongly in favor of it (Fleury et al., 1991; Fleury et al 1996).

The CDR2 region was not suggested as a class II contact site form the co-ligand assay results, as a deletion mutant including residues 43-49 was still able to bind to different class II molecules (Fleury et al., 1991; 1996). However, when this mutant was studied in the co-receptor assay, it acted differently by failing to enhance the class II-dependent T cell response. Moreover, single alanine substitutions at conserved residues 46 (still in the CDR2 region) and 77 (on the same face of the CDR2) can not interact with class II in both assays (Huang et al., 1996). From this study, it seems that residues from this region have indeed a role in the interaction with class II. This region might be either as a second contact site (the minor site) for class II, as both $\alpha 2$ and $\beta 2$ regions of class II are involved in binding to CD4 (Konig et al.,1992; Cammarota et al., 1992; Konig et al., 1995); or they may be involved in a function of itself, such as self-association via this domain of CD4.

Role of the Hinge region of CD4

Although the NH2-terminal two domains of human CD4 and the COOH-terminal two domains of rat CD4 have been successfully crystallized (Ryu et al., 1990; Wang et al., 1990; Brady et al., 1993), the crystal of the soluble CD4 molecule containing only the extracellular D1-D4 domains has not been obtained with high resolution (Kwong et al., 1990). Despite the lack of sufficient information on the full length CD4 molecule from crystallographic studies, they still shed some light on the extracellular portion of CD4

structure. For all the crystals obtained containing the four-domain human CD4 (without the transmembrane and the cytoplasmic portions), their limited diffractions have suggested that an internal flexibility might exist in CD4. This internal flexibility of CD4 is in accordance with the proteolytic studies which have demonstrated that the junction between D2 and D3 domains are particularly sensitive to the cleavage of enzymes (Ibegbu et al., 1989; Healey et al., 1990; Richardson et al., 1988). Taken together, it indicates that the junction between D2 and D3 domains of CD4 might act as the hinge region, since it is consistent with the structural features of the hinge region, such as that in Igs and CD8. However, up to now no functional characterization of this region has ever been carried out.

In the work presented in chapter 2, the functional importance of the so-called "hinge region" was explored for the first time. We assessed the effects of mutations in this region on the structural conformation of CD4, and on the co-ligand and co-receptor functions in T cell activation. Our results revealed the importance of this region in maintaining the overall structure of CD4, particularly the conserved residues 175-177 in this region, as shown by the loss or affected antibody epitopes throughout D1 to D4 domains. Furthermore, mutations also affected the CD4/class II MHC interaction drastically, in the co-ligand and co-receptor assays.

Taken together, these data suggest that the region between D2 and D3 domains play a very important role in maintaining a structure that is critical for CD4 function, just like the hinge region. The crystallographic information of this region is still lacking, however, a direct role in interaction with class II is not suggested. As provided by electro-micrographic studies that when residues 180 or 180-181 were deleted, the CD4 molecule turns into a straightened rod-like structure instead of a bent structure. Thus the abrogation of CD4/class II interaction in these hinge region mutants might be attributed to the loss of inter-domain flexibility in CD4. By giving flexibility to the molecule, the hinge region enables CD4 to adapt a conformation such that CD4 interaction with other surface molecules and even self-association of CD4 become possible.

The existing of such a hinge region in CD4 has further significance. With such a flexibility, CD4 can bend instead of being just a rigid rod-like molecule. Therefore it will not be surprising to find out that there are more than one CD4 contact site on class II MHV molecule. It is also possible that such a region plays an important role in interaction with other cell surface molecules in addition to class II and the TCR, such as CD45. Furthermore, as recent studies have shown that for HIV infection, a co-receptor on the cell surface is required. Chemokine receptors have been identified as such a co-receptor, which are the 7-transmembrane G protein-coupled receptors. The extracellular domain of the chemokine receptors are about 100 amino acids. As the binding site for gp120 is located in the D1 domain, it is not hard to imagine that a bent structure of CD4 is required for the simultaneous binding of CD4 and the chemokine receptors to the envelop protein of HIV and for HIV infection.

3. Dimerization or Oligomerization of CD4 is Required for CD4 to Fulfill Its Functional Roles in the T Cell Activation

3.1 Structural and function basis for CD4 dimerization to occur and crystallographic evidences for CD4 dimerization

The CD4 molecule is expressed as a monomer on the T cell surface, unlike other members of the Ig gene superfamily, such as Igs and CD8 which can be easily immunoprecipitated as dimers from the cell surface. However, most cell surface receptors involved in signaling are either dimer in nature or induced to dimerize upon ligand binding. Receptor dimerization have an important role in signal amplification and transduction to downstream events. Furthermore, crystallographic and functional studies have at times brought in evidences that CD4 may dimerize or oligomerize upon interaction with its ligand and this dimerization/oligomerization event is critical for the functional role of CD4 in T cell activation (Kwong et al., 1990; Sakihama et al., 1995a and 1995b; Konig et al.,

1995; 1996). In order to trigger the tyrosine kinase $p56^{lck}$ oligomerization of CD4 is required.

The first evidence of CD4 oligomerization was obtained in 1990 from crystallographic analysis of the soluble CD4 molecule (contains 4 extracellular domains but no transmembrane and cytoplasmic tail) (Kwong et al., 1990). This finding did not get too much attention until the human class II molecule HLA-DR1 was also crystallized as a superdimer (dimer of the αβ heterodimer) (Brown et al., 1993). Moreover, the recently-solved crystal structure of the Vα chain of the myelin basic protein peptide-specific TCR is also a dimer. Thus based on all these crystallographic information, and the fact that many cell surface receptors involved in signal transduction are either dimers in nature or are induced to dimerize, we hypothesized that CD4 may dimerize upon binding to class II MHC molecules on the APCs and this dimer formation is critical for the function of CD4 during T cell activation.

The availability of D1D2 and D3D4 fragments of CD4 enables to search for a possible dimer interface in CD4, based on analogy of crystal structures of CD4 to that of Igs and CD8. Compared to Ig, the loops connecting the CC' and FG strands (correspond to the CDR3 region of Ig) in D1 of CD4 are shortened by several residues. Importantly, in Ig and CD8 the CC' and FG loops help to make up the interface of a dimer of two Ig-like domains. Therefore, it suggests that dimerization through these two loops are unlikely In contrast, the C'C'' loop (homologous to the CDR2 region of Ig) in D1 of CD4 is unusually prominent and extended by several residues. This region in CD4 is important for the binding of HIV gp120. An extended C'C'' loop is also found in CD8 (Leahy et al., 1992). Although it has been speculated that this loop may be involved in a function common to CD4 and CD8 but not to Igs, such a function has not been firmly established (Leahy et al., 1995). Unlike in the case of D1, the CC' and FG loops of D3 are not shortened and are of comparable length to the homologous loops in Ig. However there are two glycosylation sites in the D3D4 fragment. One glycosylation site lies on the F strand of D3- Asn 270 which is conserved in CD4 from all species sequenced so far except from

dog. Another glycosylation site is located in the AB loop of D4 which is observed in CD4 sequence from human, mouse, dog, monkey and cat, but not in rat CD4. In rat CD4, an additional glycosylation site is found to lie close to the membrane (position 367, after G strand), which is spatially similar to the site in the AB loop of CD4 from other species. The presence of the conserved N-linked glycosylation on the F strand of D3 and AB loop of D4 would spatially interfere with dimer formation or other interactions of CD4 through this face of D3. Therefore, the CC' and FG loops are unlikely to mediate dimerization of CD4 as in Igs and CD8. However, the face of CD4 free of any carbohydrates could be involved in interaction with either self or other proteins, i. e., MHC class II and TCR. (Figure 3).

3.2 Dimerization or Oligomerization Events of the CD4 Molecule is Critical for Its Functional Roles in T cell Activation: co-ligand and co-receptor

The work presented in Chapter 4 was designed to address the following questions: Does CD4 dimerizes? If so, then where?

We have already demonstrated that the structural basis of CD4/class II interaction is conserved, at least between human and mouse. If dimerization is essential for this interaction and it does occur among CD4 from the same species, then whether dimers will be formed or not between h-CD4 and m-CD4 depends on the conservation of the structure involved in such a function. This hypothesis is tested by expressing two sets of CD4 in the 3DT52.5.8 cells: human and mouse CD4 WT. Results from these double transfectants showed that although they express good levels of both CD4 and the TCR molecules and are capable of producing IL-2 upon anti-TCR stimulation, they did not respond to cognate Ags with an enhanced IL-2 response. This effect has been demonstrated in both assays. Thus an interference of human CD4 function by murine CD4 was observed, even though both molecules are capable of binding to class II molecules. We interpret our results as followings: h-CD4 and m-CD4, although they share 60% of homology and both are capable of mounting an inter-species interaction with class II

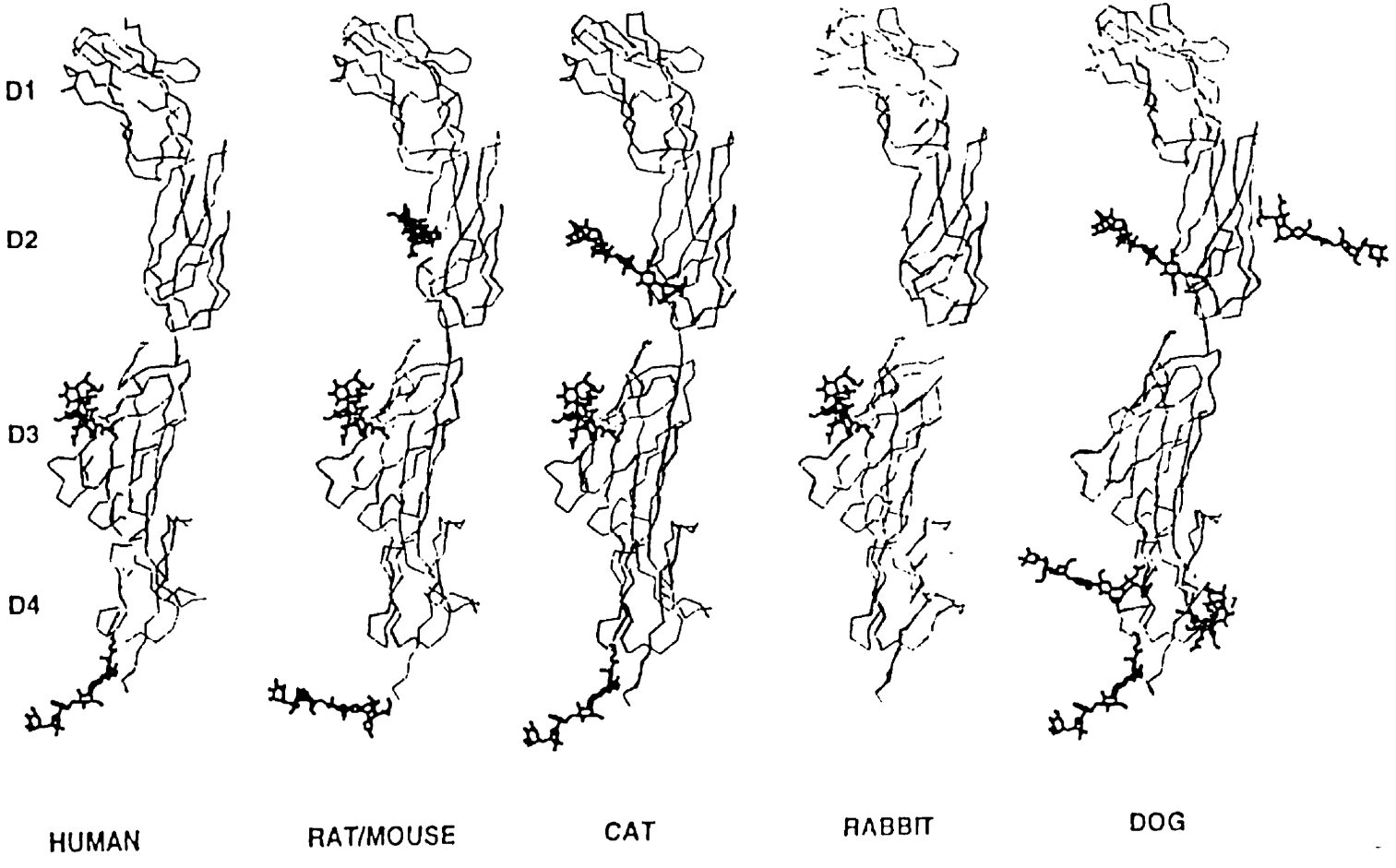

Figure 3.Glycosylation Sites on Different CD4 are Conserved (From Brady 1996).

molecules, may still use different residues from the same structural domains to form a dimer/oligomer. As both molecules are expressed on the cell surface of the double tranfectants, they might do so by inhibiting each other from forming stable oligomer complexes. Alternatively, these inhibitory effects may be due to sequestration of $p56^{lck}$ from h-CD4 by m-CD4, but this is not supported by our further studies using other double mutants. Thus our results clearly showed that oligomerization of CD4 does occur; and for CD4 to fulfill the two functions in T cell activation, CD4 must be able to interact with class II as well as be able to dimerize.

3.3 The CD4 Molecule Forming the Dimer/Oligomer Complex Mostly Probably through the Face Encompassing the CDR2 Region of D1 Domain

Although initial evidences of CD4 dimerization came from crystallization, these studies failed to pinpoint the interface of dimerization. Furthermore, this interface is largely unknown and quite intriguing, since the structural components of CD4 whose counterparts are involved in forming dimers in Igs and CD8 are differently formed. Moreover, a recent functional study has suggested that CD4 oligomerizes via the D3D4 domain, on the face opposite to the OKT4 binding sites (Sakihama et al., 1995). However, the refined crystal structures of D3D4 fragment of rat CD4 failed to show that (Lange et al., 1994).

In this study the dimer interface was determined. Our strategy was co-expressing CD4 WT with mutants from both faces of CD4 which affect the CD4/class II interaction. Results presented in Chapter 4 revealed some very interesting phenomenon. Co-expression of h-CD4 with those class II MHC interaction-deficient CD4 mutants from the face encompassing the CDR1 (S19Y), CDR3(Q89L) regions of D1 and the FG loop of D2 (165[SR]) did not rescue the function of mutants. This can be explained as such: both WT and mutant CD4 can still form oligomers; however, as one set of CD4 is not capable of bind class II, it will in turn render this oligomer incompetent of a stable interaction with class II, thus no functional enhancement by CD4 can be observed. alternatively, these

mutated residues could be involved in dimer formation directly, although such a notion is not supported (Zhang et al., 1996).

When h-CD4 or m-CD4 molecules were co-transfected with mutants on the face of CD4 encompassing the CDR2 region of D1, results different from the above mentioned double transfectants were obtained. Again, two possibilities could account for such an observation. First, residues from this face of CD4 (the CDR2 face) could be the class II contact site rather than be involved in dimer formation. However, our results (Fleury et al., 1991; 1996) and others' using aromatically modified peptides (Zhang et al., 1996) did not support the notion that the CDR2 region is a class II interaction site, at least not for the β2 region in class II. Alternatively, these residues might not be the key contact sites for class II (Huang et al., 1996; Fleury et al., 1991), but rather could be involved in a function of self-association.

The CDR2 region, as mentioned before, is rather divergent, thus binding to the conserved non-polymorphic α2 and β2 regions of class II seems unlikely. However, several residues located in the CDR2 region are still conserved through evolution, such as L44 and K46. Moreover, from the crystal structure the most distinct part on CD4 compared to Igs and CD8 lies in this region, indicating a function unique to CD4. The CDR2 region is also unusually prominent, suggesting a role in interaction with other cell surface molecules in addition to gp120. Taken together, our results suggest a possible role of the face on CD4 encompassing the CDR2 region in formation of oligomer of CD4, upon interaction with class II.

3.4 The Oligomerization Events of CD4 in the CD4/Class II/TCR Interaction, Evidences and Importance In T cell Activation

As mentioned before, crystallographic data for the recombinant soluble CD4 suggesting that CD4 oligomerizes in different crystals obtained. Moreover, the recurrent self-association of the four-domain CD4 molecules in different crystals have suggested

that CD4, like Igs and CD8, might exert its functional role as a dimer. However, such an association has not been revealed for the D1D2 fragment or the D3D4 fragment The reason for the lack of dimer formation in those crystals is not known, however, it may well suggest that the hinge region, by giving flexibility to the CD4 molecule, may be important for dimer formation. Alternatively, it may point to the role of the transmembrane and cytoplasmic regions of CD4 in this dimerization, as these regions provide membrane anchor and are involved in intracellular signaling. As it has been shown that ligand-induced dimerization of cell surface receptor requires membrane attachment or clustering to facilitate receptor dimerization or aggregation (Davis A et al., 1994)). It is also possible that dimerization of CD4 occurs following interaction with other cell surface molecules, such as class II MHC and the TCR.

Recent functional studies have shed light on this oligomerization issue by bringing in functional evidences suggesting that oligomerization of CD4 is critical for CD4/class II interaction and for T cell activation. Using a TCR-independent adhesion assay, Sakihama et al (1995) have suggested that oligomerization is required for the CD4/class II interaction. Moreover, using different CD4 co-receptor assays as readout, Konig et al., have demonstrated that both $\alpha 2$ and $\beta 2$ regions of class II are involved in interaction with CD4. Importantly, both regions are located opposite to each other, thus binding to a single CD4 simultaneously seems impossible. Here by co-expressing two different sets of CD4 molecules (WT vs WT; Wt vs mutants) into the 3DT52.5.8 cells and studied them in the co-ligand and co-receptor assays, we have clearly demonstrated that oligomerization of CD4 is critical for the function roles of CD4 in T cell activation: co-ligand and co-receptor.

Previous experiments have shown that CD4 and class II molecules would co-cluster at the T cell-APC contact sites (Kupfer et al., 1987). If CD4 oligomerization occurs (there are enough evidences for that), the interaction with class II can bring class II molecules close together thus the oligomerization of class II will likely occur. The increased local concentrations of both CD4 and class II may be a driving force for the

dimerization of both molecules. This in turn will as well result in oligomerization of the TCR. This oligomerization event will augment the activation signal and result in a much stronger T cell response.

Although functional evidences for the oligomerization of class II MHC and the TCR are still lacking; however, crystallographic data have been obtained which showed human class II molecule HLA-DR1 (Brown et al., 1993), and the Vα chain of the MBP peptide-specific TCR are dimers in crystal. There are more and more evidences suggesting that cell surface molecules involved in receptor/ligand interaction are either dimer in nature or induced to dimerize upon binding to ligand. This dimerization event has a role in generating and amplifying intracellular signal.

4. Model of CD4/class II MHC Interaction

The model we would like to propose based on our studies is following: CD4 interact with class II molecules, mainly through the face encompassing the CDR1, CDR3 regions of D1 and the FG loop of D2; while the face encompassing the CDR2 region of D2 is involved as part in oligomerization of CD4 upon interaction with its physiological ligand class II MHC. The oligomerization is critical for both functions of CD4 in T cell activation, namely, the co-ligand and the co-receptor function. (Figure 4).

5. Future Prospective

5.1. Complementation experiments

As regions throughout both faces on CD4 and class II molecules are implicated in their interaction, it leads us to wonder the exact nature of interaction. In other word, we would like to explore the corresponding interaction sites on both (for example, β2 region of class II interact with the face encompassing the CDR1, CDR3 and the FG loop of CD4- we call it Face A, not the opposite face of CD4).

Charged residues implicated in CD4/class II interaction will be swapped in such a way: residue A from CD4 will be replaced residue B on class II, and vice versa. If a

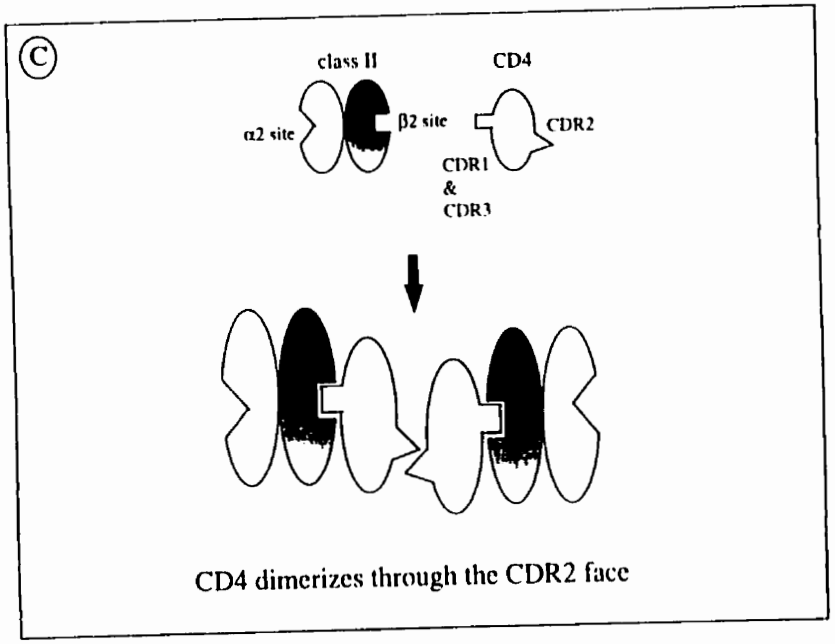

Figure 4. Model

functional interaction is rescued, suggesting a direct interaction between these two residues. This will provide us further information on the structural relation between two molecules. Nevertheless, the full understanding of this CD4/class II interaction relies on the co-crystallization of these two molecules.

5.2. Generating CD4 transgenic mice for CD4 mutants

Up to now the co-receptor function of CD8 has been demonstrated only in CD8 transgenic mice, such a role of CD4 in T cell thymic selection has yet to be revealed. In order to investigate that CD4 does act as a co-receptor in T cell selection, and to explore the functional implication of CD4 residues implicated in binding to class II in both functions, especially those implicated differentially in both functions, transonic mice expressing CD4 mutant in the CDR2 region will be generated. As a deletion mutant Del 43-49 affects only co-receptor function of CD4, but not the co-ligand function. If selection of CD4 single positive T cells occurs in transgenic mice for CDR2 mutants, it suggests that CD4 indeed plays a role as the co-receptor in thymic selection.

5.3. Implication in HIV

The recent identification of the HIV infection co-receptor has opened a new door for HIV research. Hence understanding the interaction of CD4/HIV envelope protein with the HIV coreceptor becomes very important for the eventual controlling of the disease. As HIV Env protein binds both CD4 and the chemokine receptor, then certain structural domains on CD4 may be important for this interaction. It has recently been shown that chemokine receptors are the HIV co-receptor. They are the 7-transmembrane G-protein coupled receptor, the extracellular portions are small, compared to that of CD4. Thus the CD4 may adapt a bent-over structure by its flexible hinge region, this enables the simultaneously binding of both CD4 and chemokine receptors to Env and allow the HIV virus entry. By expressing CD4 mutants in the hinge region, the importance of this region in HIV infection will be determined.

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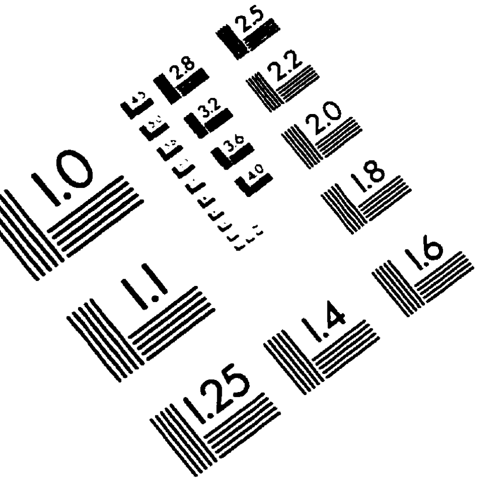
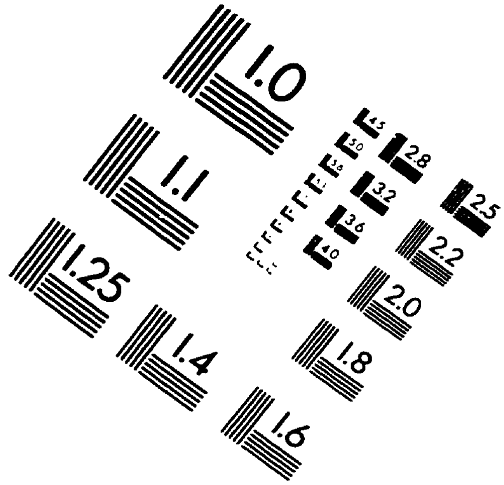
Claims for Original Research

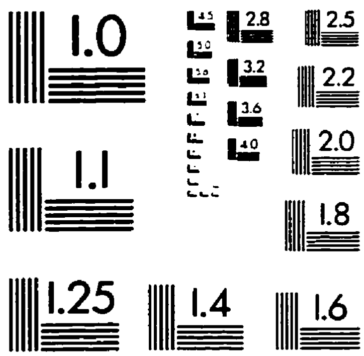
The work presented in this thesis will make original contributions in the understanding of CD4/class II interaction in T cell activation at molecular level at the following aspects.

The CD4/class II interaction was investigated for the first time in an internally controlled cellular system in which provided assays for monitoring both the co-ligand and co-receptor functions of CD4. This study addressed previous controversial results from studies involving different cellular systems, especially the role of the CDR2 region in both functions of CD4. The finding that residues from both faces of CD4 are implicated in interaction with class II MHC molecules seems to be consistent with the results that class II molecules interact with CD4 via at least two opposite regions in both $\alpha 2$ and $\beta 2$ domains. Based on further characterization of residues from the face containing the CDR2 region of D1 domain, we suggested that the a possible dimerization or oligomerization of CD4 via this part is likely to occur. This is one of the first functional evidences supporting the model of CD4 or even class II oligomerization, following the crystallographic data. Furthermore, the region connecting the D2 and D3 domain and their possible involvement in a function such as that of a "hinge region" in Igs and CD8 has been investigated for the first time. This study brings in the first evidence that this region plays an important role in maintaining the CD4 structure and in rendering the flexibility required for the functional interaction of CD4 with different cell surface molecules. Finally, the structural basis for an inter-species CD4/class II MHC interaction between human and mouse was explored for the first time. Our results demonstrated that the structural components important for the inter-species interaction is also conserved for the cross-species interaction between CD4 and class II molecules.

IMAGE EVALUATION TEST TARGET (QA-3)







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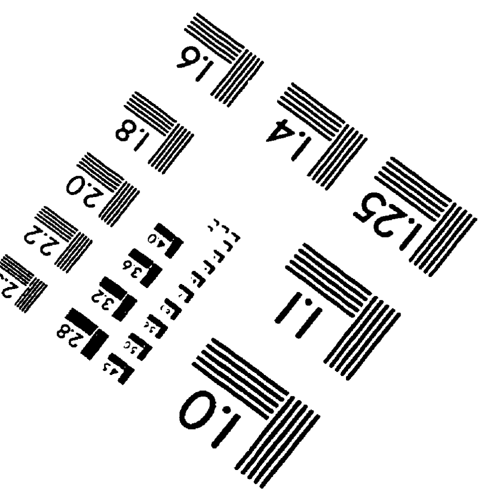

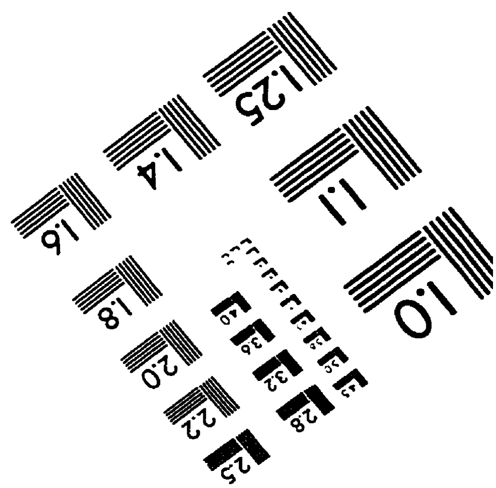

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