

Molecular Requirements for T Cell Recognition by a Major Histocompatibility Complex Class II-restricted T Cell Receptor: The Involvement of the Fourth Hypervariable Loop of the V α Domain

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Summary

The role of two central residues (K68, E69) of the fourth hypervariable loop of the V α domain (HV4 α) in antigen recognition by an MHC class II-restricted T cell receptor (TCR) has been analyzed. The TCR recognizes the NH₂-terminal peptide of myelin basic protein (Ac1-11, acetylated at NH₂ terminus) associated with the class II MHC molecule I-A^a. Lysine 68 (K68) and glutamic acid 69 (E69) of HV4 α have been mutated both individually and simultaneously to alanine (K68A, E69A). The responsiveness of transfectants bearing wild-type and mutated TCRs to Ac1-11-I-A^a complexes has been analyzed in the presence and absence of expression of the coreceptor CD4. The data demonstrate that in the absence of CD4 expression, K68 plays a central role in antigen responsiveness. In contrast, the effect of mutating E69 to alanine is less marked. CD4 coexpression can partially compensate for the loss of activity of the K68A mutant transfectants, resulting in responses that, relative to those of the wild-type transfectants, are highly sensitive to anti-CD4 antibody blockade. The observations support models of T cell activation in which both the affinity of the TCR for cognate ligand and the involvement of coreceptors determine the outcome of the T cell-antigen-presenting cell interaction.

Key words: T cell receptor • V α domain • fourth hypervariable loop • antigen recognition • CD4 coreceptor

For the vast majority (90–95%) of T cells, the T cell receptor (TCR) is composed of a heterodimer of highly diverse transmembrane α and β chains, whereas a minor population bear $\gamma\delta$ TCRs. These $\alpha\beta$ or $\gamma\delta$ heterodimers associate with the nonpolymorphic CD3 and TCR ζ chains to form the functional TCR-CD3 complex. The $\alpha\beta$ heterodimer interacts with cognate peptide-MHC (pMHC) complexes, whereas the CD3-TCR ζ chain complex is involved in signal transduction. These TCR-associated polypeptides contain tyrosine motifs (called immune receptor tyrosine-based activation motifs, or ITAMs), which are the targets of phosphorylation following TCR triggering (1). ITAM phosphorylation leads to recruitment of SH2 domain containing proteins, such as ZAP-70 and subse-

quent signaling cascades (1), and is one of the early events of T cell activation (2, 3).

The extracellular domains of the TCR resemble the Fab arms of an antibody at both the sequence and structural levels, although there are also significant differences in both complementarity determining regions (CDRs) and overall fold for both α and β chains (4–6). Structural modeling of TCR-pMHC complexes led to the suggestion that the less diverse CDRs 1 and 2 of the TCR α and β chain make contacts with the α helices of the MHC molecule, whereas the highly variable CDR3 loops interact directly with the antigenic peptide (7–9). More recent X-ray crystallographic structures of mouse and human TCR $\alpha\beta$ -pMHC class I complexes (10–13) are consistent with these proposed three-dimensional models insofar as CDR3 residues overlie, but do not always make intimate contact with, antigenic peptide, whereas CDR1 and CDR2 residues are positioned to contact primarily MHC residues with more limited peptide contacts. The X-ray structures (10–13) demonstrate a diagonal orientation of the TCR with respect to pMHC in a configuration that is most likely to be general, at least for pMHC class I complexes. Earlier struc-

¹Abbreviations used in this paper: APC, antigen-presenting cell; CD, circular dichroism; CDR, complementarity determining region; E69, glutamic acid 69; E69A, mutation of E69 to alanine; HV4 α , the fourth hypervariable loop of the V α domain; ITAM, immune receptor tyrosine-based activation motif; K68, Lysine 68; K68A, mutation of K68 to alanine; MBP, myelin basic protein; PBS, phosphate-buffered saline; pMHC, peptide-major histocompatibility complex; TCR, T cell receptor.

ture-function studies of both MHC class I and class II restricted TCRs led to the proposal of a similar orientation (14–16), but whether this configuration is generally observed in TCR $\alpha\beta$ -pMHC class II complexes awaits the elucidation of the corresponding X-ray structures.

Current models of T cell activation support a role for the affinity/avidity of the TCR–pMHC as being a key parameter in the outcome of the T cell–antigen-presenting cell (APC) interaction (17). In particular, the off-rate of the TCR–pMHC complex appears to play a central role (17, 18). The coreceptors CD4 or CD8 can also increase the avidity of the interaction and/or affect the signaling efficiency via associated proteins such as p56^{lck} (19–23). Using binding assays with living CTLs (24) and soluble molecules in surface plasmon resonance experiments (25), CD8 has been demonstrated to increase the affinity of the TCR for cognate pMHC ligand. In contrast to affinity based models, for effective signaling the need for a conformational change in the TCR post-ligand binding without a major role for avidity has also been proposed (26, 27). Furthermore, data have been presented in support of a requirement for ordered oligomerization or aggregation of TCRs (28–30), which for optimal signaling may be followed by coreceptor recruitment (31–33). Conformational and affinity/avidity models are therefore not mutually exclusive; both may be relevant in “sequential engagement” models (31, 32, 34) where there is a need for a threshold time of TCR occupancy to be reached to induce the necessary configuration of TCR–pMHC complexes of a sufficient half-life to allow coreceptor association. This concept is supported by recent data demonstrating that CD4 can enhance responses to agonist ligands but not to antagonist ligands due to the shorter half-life of the TCR antagonist–MHC complex (31). This is also consistent with the proposed role of kinetic proofreading (35, 36) in increasing the fidelity of T cell recognition.

Toward the aim of better understanding the molecular nature of T cell recognition, in the current study we have analyzed the effect of alteration of amino acids in the fourth hypervariable loop of the TCR α chain (HV4 α) on T cell responsiveness. The crystal structure of a TCR V α domain indicated that residues in HV4 α are almost coplanar with the CDR loops, which led to the suggestion that they have the potential to interact with the pMHC complex (4). More recently, the structure of a human TCR complexed with an HIV Tax peptide bound to HLA-A2 (11) shows that the highly conserved residue K68 of HV4 α contacts HLA-A2 by forming hydrogen bonds to residues T163 and E166 of this MHC class I molecule. These amino acids are conserved in MHC class I but not in class II molecules (37). However, in other structural studies describing class I-restricted TCRs, HV4 α residues do not contact pMHC ligand (10, 12, 13). The functional role of HV4 α residues in antigen responsiveness has not to date been elucidated, although recent *in vitro* binding studies with the soluble 2C (murine) TCR and pMHC have indicated a minor but significant role for HV4 α residues in contributing to the binding energy of this interaction (38).

In this study, we have analyzed the role of HV4 α residues of a TCR derived from the encephalitogenic T cell hybridoma 1934.4 (39) in antigen responsiveness. Using the X-ray crystallographic structure of the 1934.4 TCR V α domain as a guide (4), amino acid substitutions of the two central, exposed residues of HV4 α (Lysine 68 [K68], glutamic acid 69 [E69]) have been introduced. Wild-type (WT) and mutated TCRs have been expressed in T cell transfectants and IL-2 production following antigen exposure analyzed in the presence and absence of CD4 coexpression. Our results indicate that in the absence of CD4 coexpression, mutation of K68 to alanine (K68A) results in greatly reduced IL-2 production, whereas mutation of E69 to alanine (E69A) has a comparatively minor effect. Furthermore, expression of the coreceptor CD4 can compensate for the reduced responsiveness of transfectants bearing the K68A mutant TCR. The data indicate that HV4 α residues play an important role in modulating T cell responsiveness and support models for T cell activation in which both affinity of TCR for pMHC ligand and coreceptor density determine the outcome of the T cell–APC interaction (31, 32, 34).

Materials and Methods

Cell Lines, Antibodies, and Peptides. The CD4⁺, TCR $\alpha\beta$ ⁺ cell line 58 α ⁺ β ⁺ (40) was provided by Dr. Stephen Hedrick (University of California at San Diego, CA), with permission from Dr. Bernard Malissen (INSERM-CNRS, Marseille-Luminy, France). The I-A^b expressing B cell line, PL-8 (derived by fusing LPS-activated splenocytes from H-2^b mice with the M-12.C3 lymphoblast line, [41]) was used as the APC line and was provided by Dr. David Wraith (University of Bristol, UK). The hybridoma producing the anti-V β 8 mAb F23.1 (42) was a gift from Drs. John Kappler and Philippa Marrack (University of Colorado Health Science Center, Denver, CO). Hybridomas expressing the anti-I-A mAbs Y3P (HB-183), 10.2.16 (TIB-93), and the anti-CD4 mAb GK1.5 (TIB-207) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Anti-CD3 ϵ mAb 145-2C11 (CRL-1975; ATCC) was purchased from PharMingen (San Diego, CA). The horseradish peroxidase-conjugated anti-mouse/rat IgG antibodies used as secondary antibodies for immunofluorescence studies were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). The NH₂-terminal peptide Ac1-11 of rat myelin basic protein (MBP) and an analog in which lysine at position 4 is substituted by tyrosine (Ac1-11[4Y]), were synthesized at the peptide synthesis unit of the Howard Hughes Medical Institute, UT Southwestern Medical Center, Dallas, Texas.

Expression Plasmids. The α and β shuttle vectors (43) used as TCR expression vectors in this study were a kind gift of Dr. Mark Davis (Stanford University, Palo Alto, CA). The construction of the 1934.4 α and β chain expression plasmids using the 1934.4 V α and V β domain genes (isolated as in reference 44), was carried out using 1934.4 TCR-specific oligonucleotide primers and essentially the strategy of Patten and colleagues (43). Mutagenesis of HV4 α residues K68 and E69 (numbering as in reference 37; in reference 4 these residues are numbers 67 and 68, respectively) together with E69 were carried out as described by Kunkel et al. (45). E69 was substituted by alanine, and K68 and E69 were both substituted by alanine to generate the mutants

E69A and K68AE69A, respectively. The oligonucleotides used for mutagenesis were: 5' AGG TGG CTG CTT TAT TG 3' for E69A, and 5' GAG GTG GCT GCT GCA TTG TAT GT 3' for K68AE69A. Mutated bases are indicated by underlining. K68 was substituted by alanine to generate K68A using the splicing by overlap extension method (46) and the following complementary oligonucleotides (mutated bases underlined): 5' GTG GCT TCT GCA TTG TAT GT 3' and 5' ACA TAC AAT GCA GAA GCC ACC 3'. The presence of the mutations, and the absence of second site mutations, was confirmed by nucleotide sequencing using the Thermo Sequenase ³³P radiolabeled terminator cycle sequencing system (Amersham, Arlington Heights, IL). All (sub)cloning steps and PCRs were carried out using standard methods. An expression plasmid encoding murine CD4 (14) was a generous gift of Dr. Syamal Datta (Northwestern University Medical School, Chicago, IL).

Generation of Transfectants. For transfections, the 1934.4 α (mutant and WT), 1934.4 β , and CD4 expression plasmids were purified using Qiagen (Chatsworth, CA) plasmid midi-prep kits. 5 μ g of α shuttle vector and 10 μ g of β shuttle vector were used per transfection. The plasmid encoding the murine CD4 molecule (14) was used together with α and β shuttle vectors at 10 μ g per transfection. As transfection recipient, the TCR⁻, CD4⁻ cell line 58 α - β ⁻ (40) was used (1×10^7 cells per transfection). Cells were washed once with ice-cold Hepes-buffered saline, resuspended in 0.5 ml phosphate-buffered saline (PBS) and placed on ice in Bio-Rad (Hercules, CA) electroporation cuvettes (0.4 cm gap). The relevant constructs were added and mixed with the cells by gentle pipetting. Cells were kept on ice for 10 min and pulsed in a Bio-Rad Gene Pulsar at 250V/cm and 960- μ F capacitance. After pulsing, cells were immediately diluted into ice-cold non-selective medium (RPMI 1640 supplemented with 10% FCS, 5×10^{-5} M 2-ME, 100 U/ml of penicillin G, and 100 μ g/ml streptomycin) and placed on ice for 10–15 min. Electroporated cells were plated into two 96-well plates at 100 μ l/well. 2 d later, 100 μ l of 2 \times selection medium (2 μ g/ml mycophenolic acid, 400 μ g/ml xanthine) was added per well. 4 d later, 80% of the medium was replaced with fresh selective medium. Mycophenolic acid-resistant transfectants (30–50 per transfection) were obtained and screened for surface expression of the transfected TCRs by flow cytometry.

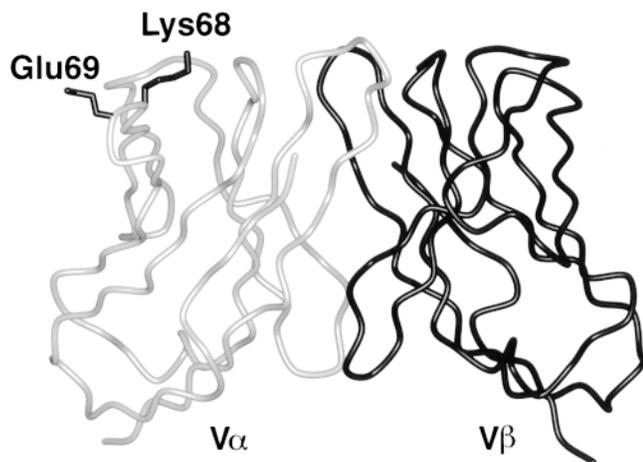


Figure 1. α -carbon trace of the structurally solved 1934.4 V α domain (grey) associated with the modeled 1934.4 V β domain (black) (4). HV4 α residues K68 and E69 are shown with their side chains in black. The figure was generated using the programs Bobsript and Raster3D (70, 71).

Analysis of Cell Surface Expression of TCR and CD4 by Flow Cytometry. For analysis of cell surface expression of TCR and CD4, transfectants (1×10^5 /well in a 200 μ l volume) were incubated with 10 μ g/ml of anti-V β 8 mAb (F23.1) or with 10 μ g/ml anti-CD4 mAb (GK1.5) at 4°C for 1 h. Cells were washed three times with PBS containing 1% BSA (Sigma Chemical Co., St. Louis, MO) and were incubated with anti-mouse IgG-FITC conjugate for TCR staining or anti-rat IgG-FITC conjugate for CD4 staining at 4°C for 1 h. Controls were incubated with secondary conjugates only. Cells were washed three times with 1% BSA containing PBS, resuspended in PBS, and analyzed in a flow cytometer (Becton Dickinson). Results were analyzed using the Cellquest program.

Stimulation by Cross-Linking Antibodies or PMA/Ionomycin. Transfectants (1×10^5 /well in 96-well plates) were stimulated either with 10 ng/ml PMA + 500 ng/ml ionomycin, plate-bound anti-V β 8 antibody, F23.1, or plate bound anti-CD3 ϵ antibody, 145-2C11. For the antibody stimulations, plates were incubated overnight with 10 μ g/ml antibody and washed twice with PBS before adding the transfectants. Controls were run in parallel without any stimulation. Culture supernatants were harvested after 24 h, and after a single freeze-thaw, 80 μ l aliquots were incubated with an IL-2-dependent cell line, CTLL-2 (TIB214; ATCC; 5×10^3 cells/well). Cells were pulsed 24 h later with 1 μ Ci of ³H/well for 16–18 h and thymidine incorporation analyzed by liquid scintillation spectroscopy (LKB Pharmacia, Uppsala, Sweden). All assays were done in triplicates and data is expressed as mean values of triplicate measurements following background subtraction (in cpm) \pm SD.

Stimulation by Cognate Peptide:MHC Class II. Transfectants (1×10^5 /well in 96-well plates) were incubated with graded doses of the peptides Ac1-11 or Ac1-11[4Y] together with PL-8 cells (1×10^5 /well) as APCs. No peptide was added to the control wells. For anti-CD4 or anti-I-A^b inhibition, graded doses of anti-CD4 (GK1.5) or anti-I-A (Y3P, 10.2.16) mAbs were used. Appropriate isotype matched antibodies were used as controls. The responses of transfectants were measured by IL-2 production that was detected by the proliferative response of CTLL-2 cells as above. All stimulation assays were done in triplicates and data expressed as percent of response to the plate-bound anti-CD3 ϵ antibody, 145-2C11.

Circular Dichroism (CD) Analyses. Far-ultraviolet CD analyses were performed as previously described (44), using an AVIV model 60DS circular dichroism spectrophotometer at 25°C and a cell of 0.2 cm path length.

Results

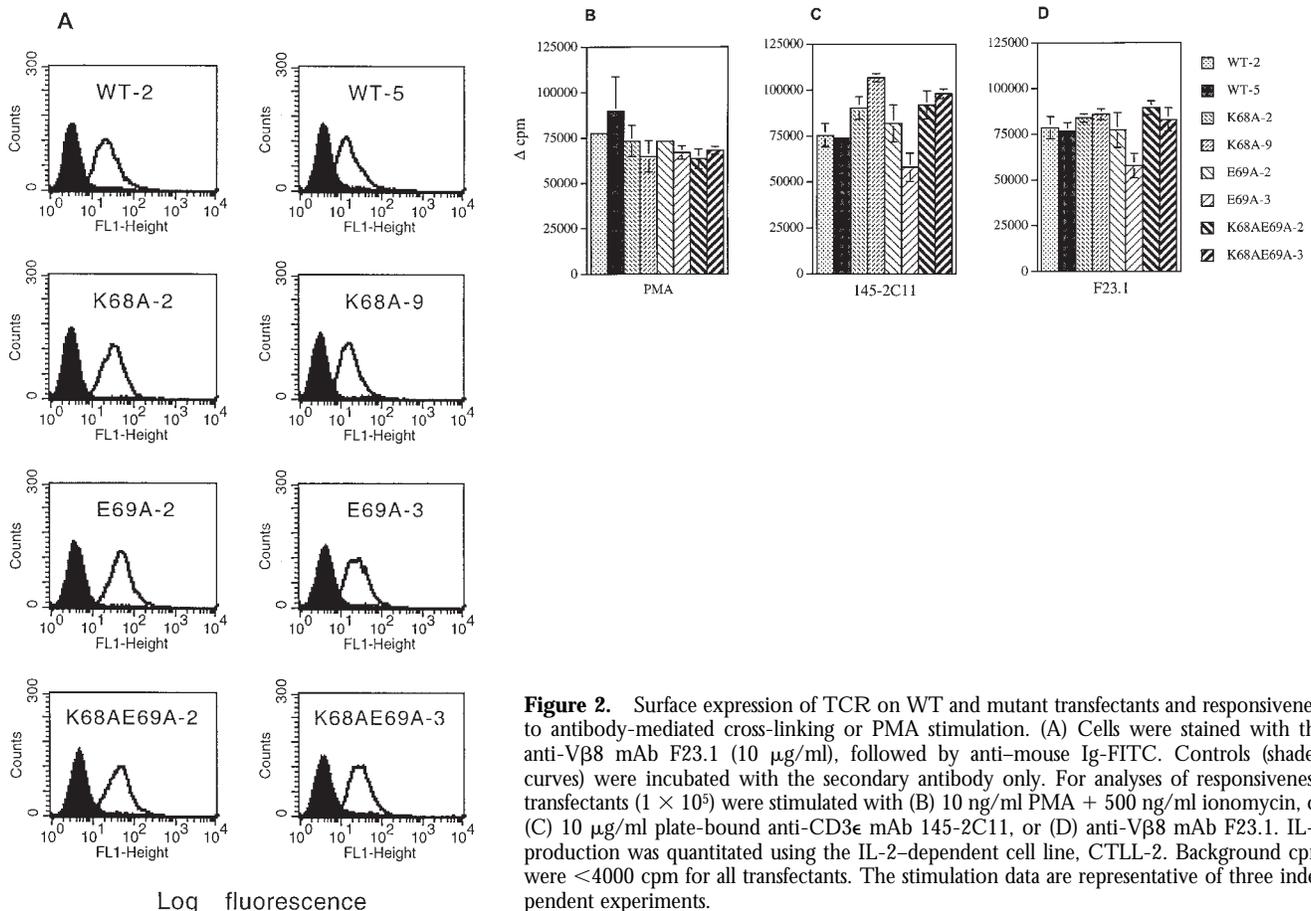
Generation of WT and Mutant TCR Transfectants. To analyze the effects of mutating HV4 α residues 68 and 69 of the 1934.4 TCR on antigen responsiveness, WT and mutated α chain genes were transfected with the WT β (V β 8.2-J β 2.3) chain into a TCR α - β ⁻ thymoma, 58 α - β ⁻ (40). This murine T cell thymoma line lacks an endogenous TCR, but has a functional CD3-TCR ζ complex that can be expressed on the cell surface with the transfected TCR α and β chains. Fig. 1 shows the location of the residues that were targeted for mutagenesis on the X-ray crystallographic structure of the 1934.4 V α domain (4).

Mycophenolic acid-resistant transfectants were analyzed for expression levels of surface TCR by flow cytometry using a V β 8-specific mAb, F23.1. Since the expression levels varied between transfectants, transfectants with comparable

levels of surface TCR were chosen for further analysis (Fig. 2). These transfectants were analyzed for responsiveness (assessed by quantitating IL-2 production) to PMA in addition to anti-V β 8 (F23.1) and anti-CD3 ϵ (145-2C11) antibody-mediated cross-linking. Responses to all three types of stimulation did not differ markedly, indicating that the signaling machinery of the transfectants was intact and that the V α mutations do not affect signaling via antibody-mediated β chain cross-linking (Fig. 2 D). Differences observed upon 145-2C11 (Fig. 2 C) stimulation probably reflect minor differences in the surface TCR levels observed in Fig. 2 A. To control for variability, responses to cognate pMHC (below) have been normalized with respect to those obtained from stimulation with 145-2C11 and expressed as percentages of 145-2C11 responses. A similar approach was taken by Patten and colleagues for the analysis of anti-cytochrome c-I-E^k responses by transfectants expressing the WT 2B4 TCR and its mutated derivatives (43).

Responsiveness of WT and Mutant Transfectants to Cognate Antigen. The 1934.4 TCR recognizes the NH₂-terminal 11-mer (or nonamer) of myelin basic protein (MBP) associated with I-A^u and the acetylation of the NH₂ terminus of this peptide (abbreviated to Ac1-11) is essential for T cell recognition (39). Position 4 analogs (position 4 substituted by alanine and tyrosine, designated Ac1-11(4A) and Ac1-

11(4Y), respectively) of this peptide bind with higher affinity to I-A^u (47, 48), resulting in shifts of dose response curves of 1934.4 hybridoma cells (47). We tested the ability of the WT versus mutant transfectants to recognize the Ac1-11 peptide presented in the context of the MHC class II molecule I-A^u. When Ac1-11 was used for stimulation (Fig. 3 A), the WT transfectants respond, albeit poorly, whereas there is an almost undetectable response observed for the three types of mutant transfectants (K68A, E69A, and K68AE69A). This poor responsiveness is most likely due to the lack of CD4 expression by the transfectants, which in other systems is known to decrease antigen responsiveness (31, 49). Therefore, to make quantitative comparisons between the WT and mutant transfectants in the absence of CD4, we tested their responses to the higher affinity peptide Ac1-11(4Y). Fig. 3 B shows that the response of the WT transfectants to Ac1-11(4Y) is significantly better compared with their response to Ac1-11 (Fig. 3 A), and this is reminiscent of the data of others for the parent 1934.4 hybridoma (47, 48, 50). The mutant transfectants also show higher and detectable levels of responses to the higher affinity peptide. Replacement of glutamic acid at position 69 with alanine (E69A) results in a slight, but significant, reduction in response as compared with WT; however, the double mutants (K68AE69A) and the



K68A mutants show substantial decreases in their responses relative to WT transfectants.

The data indicate that lysine at position 68 of HV4 α plays a crucial role in the TCR-pMHC interaction, most likely by either directly contacting Ac1-11-I-A^u or indirectly by affecting the conformation of neighboring CDRs in the V α domain. Importantly, several observations indicate that the mutations analyzed in this study do not have significant effects on the conformation of the TCR V α domain. First, the WT and mutant transfectants express similar levels of surface TCR, and aberrant folding of the TCR α chain due to mutation would be expected to affect this. Second, CD spectroscopic analysis of the WT and mutant V α domains, expressed as recombinant proteins in *Escherichia coli*, indicate that the V α domains are folded into structures of high β -sheet content (data not shown). Unfortunately, the lack of an anti-V α 4.2 antibody that is conformationally dependent precludes analyses using such a reagent.

CD4 Coexpression Enhances the Responsiveness of the Transfectants. Since the differences in response to peptide were most striking between the WT and the K68A mutant, transfectants expressing these TCRs were analyzed further. The T cell coreceptor, CD4, has been shown to enhance the capability of thymocytes and mature T cells to recognize antigen (reviewed in references 19 and 51). To analyze whether CD4 can compensate for the effect of the K68A mutation, cotransfections of the 1934.4 WT or K68A mu-

tant α chain together with WT β chain and CD4 expression constructs using the 58 α - β ⁻ thymoma cell line as recipient were carried out. The surface TCR and CD4 levels are comparable between the WT and K68A transfectants (Fig. 4 A), although the TCR levels for the transfectant K68A/CD4-32 are slightly lower. These CD4⁺ transfectants show similar responses to 145-2C11 and F23.1 stimulation (Fig. 4, B and C). In addition to shifting the dose response curve of the WT transfectants, cotransfection of CD4 almost completely (K68A/CD4-12 mutant) or partially (K68A/CD4-32 mutant) restores the K68A mutant responses to cognate pMHC to the levels seen with the WT transfectant, WT/CD4-43 (Fig. 5). The WT transfectant WT/CD4-33 is, however, still more responsive than the two mutant transfectants and this is particularly so for the Ac1-11 peptide (Fig. 5 A). This significant gain of function for the K68A mutants suggests that either the increased avidity and/or enhancing the efficiency of TCR signaling by CD4 coexpression can compensate, in part at least, for the suboptimal nature of the K68A TCR-pMHC interaction. In addition, the enhancing effect of CD4 is much greater for the K68A mutants than for the WT transfectants (Figs. 3 and 5).

To demonstrate that the increased IL-2 production by the K68A mutants is due to CD4 coexpression, the sensitivity of the WT and K68A transfectant responses following Ac1-11(4Y)-I-A^u exposure to blockade by the anti-

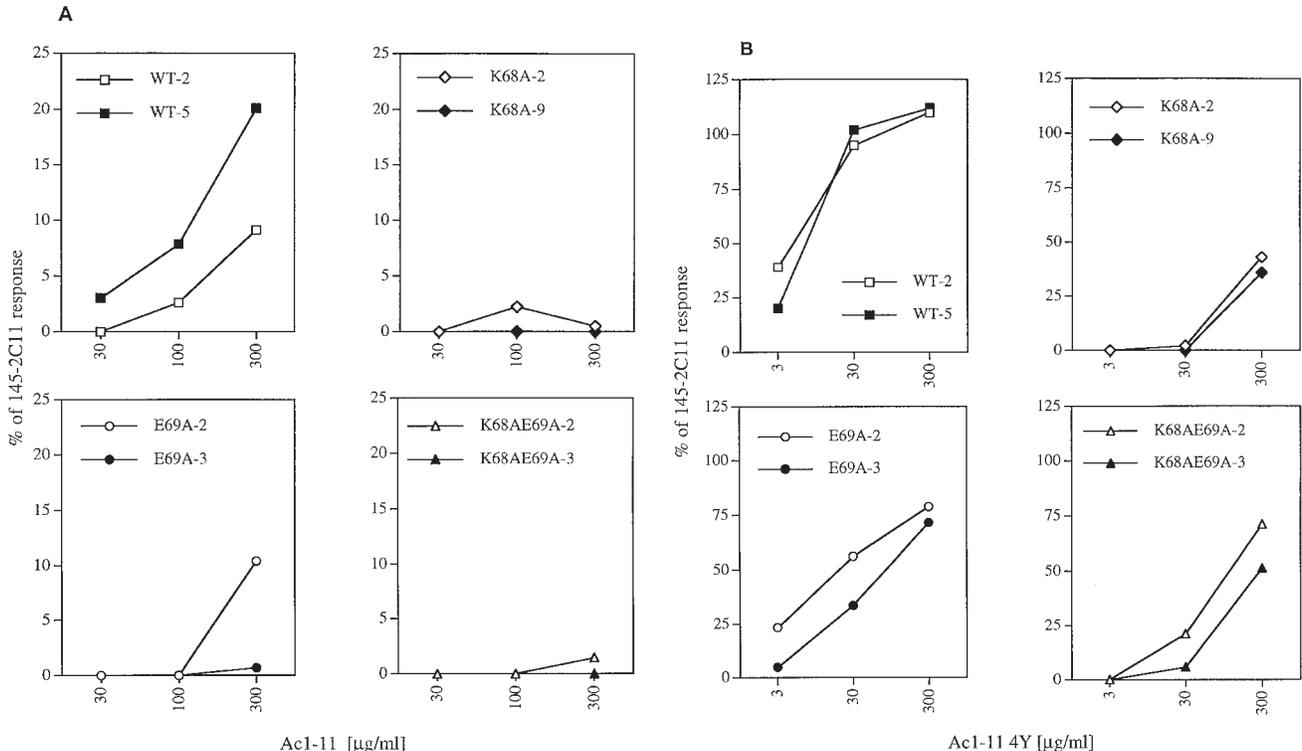


Figure 3. Responses of transfectants to MBP peptides. 10^5 PL-8 cells/well were cocultured with 10^5 transfectants/well and the concentrations of (A) Ac1-11, or (B) Ac1-11[4Y] peptides as shown. Culture supernatants were harvested at 24 h and analyzed for IL-2 activity as in Fig. 2. Data is expressed as percent of 145-2C11 response shown in Fig. 2. SDs were <15%. Background cpm were <4000 cpm for all transfectants. The data shown are from one representative experiment of a total of three separate experiments.

CD4 mAb GK1.5 was investigated. K68A transfectants are significantly more sensitive to the effects of GK1.5 than the WT transfectants (Fig. 6 A). Importantly, this difference is observed for K68A/CD4-12 relative to WT/CD4-43, and these two transfectants show essentially the same dose response to Ac1-11(4Y)-I-A^u. The effects of GK1.5 are consistent with the observation that WT transfectants without CD4 coexpression are responsive to antigen, in contrast to the K68A transfectants (Fig. 3). Finally, the sensitivity of the WT and K68A transfectants to blockade by anti-I-A antibodies was also investigated, because this was expected to reveal differences in affinities of the corresponding TCR-pMHC complexes. Two anti-class II mAbs (10-2.16 and Y3P) that recognize I-A^u were used, and for both anti-MHC class II antibodies the K68A transfectants are more sensitive to inhibition than the WT transfectants (Fig. 6 B; data shown only for 10.2.16). As with the GK1.5 inhi-

tion, this difference in sensitivity to anti-MHC class II inhibition is seen when the WT/CD4-43 and K68A/CD4-12 transfectants are compared. Taken together with the similarity of the dose responses to cognate antigen in the absence of antibodies for these two transfectants (Figs. 5 and 6), this indicates that the affinity of the K68A TCR for cognate antigen is lower than that of the WT TCR.

Discussion

To date, there are no high-resolution structural data available for a TCR-pMHC class II complex, but functional studies (14, 15) of class II-restricted TCRs indicate that the orientation of the TCR is similar to the diagonal configuration observed in the X-ray structures of several class I-restricted TCRs (10-13). The crystal structures indicate that HV4 α residues sometimes (11), but not always

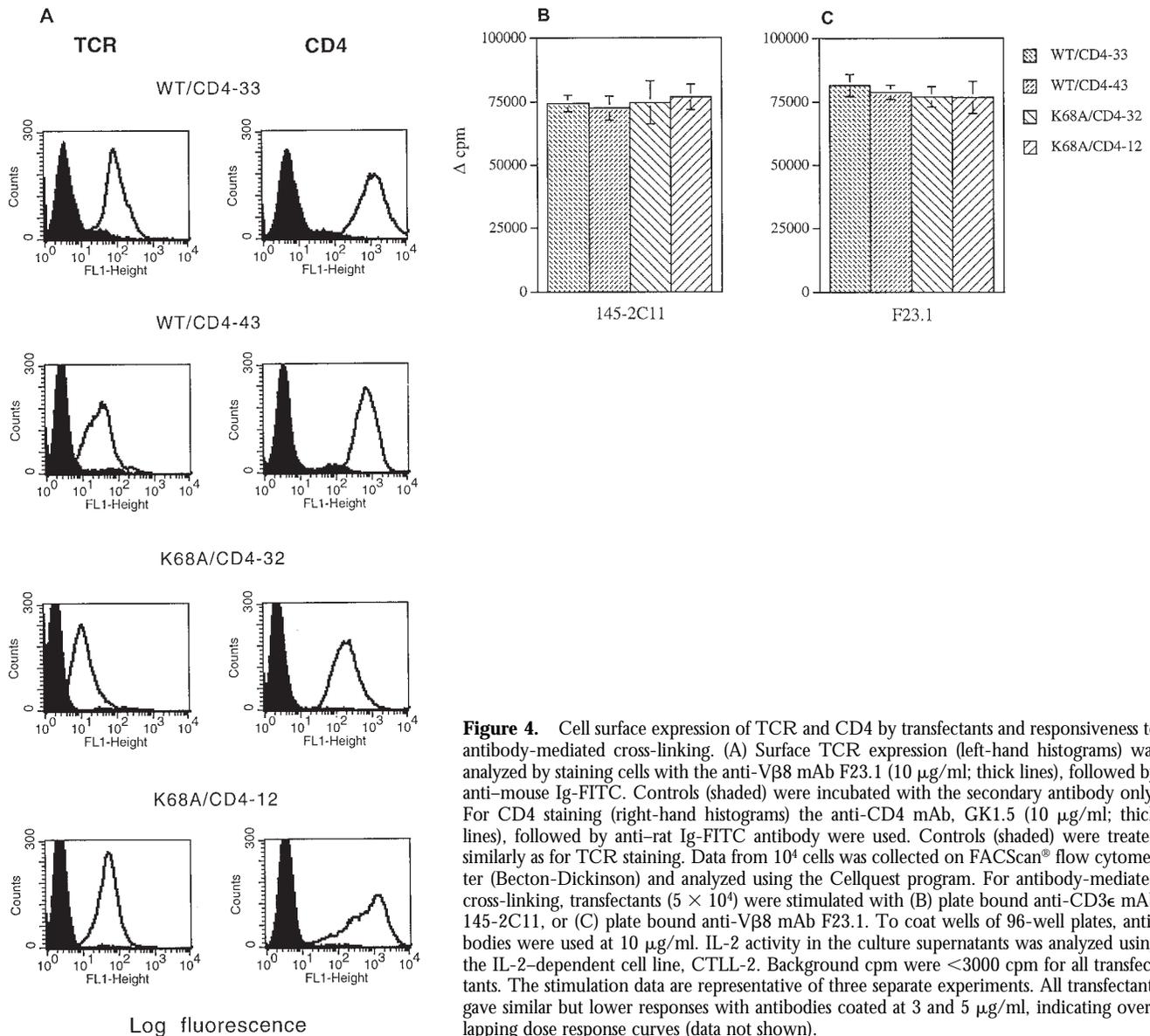


Figure 4. Cell surface expression of TCR and CD4 by transfectants and responsiveness to antibody-mediated cross-linking. (A) Surface TCR expression (left-hand histograms) was analyzed by staining cells with the anti-V β 8 mAb F23.1 (10 μ g/ml; thick lines), followed by anti-mouse Ig-FITC. Controls (shaded) were incubated with the secondary antibody only. For CD4 staining (right-hand histograms) the anti-CD4 mAb, GK1.5 (10 μ g/ml; thick lines), followed by anti-rat Ig-FITC antibody were used. Controls (shaded) were treated similarly as for TCR staining. Data from 10^4 cells was collected on FACScan[®] flow cytometer (Becton-Dickinson) and analyzed using the Cellquest program. For antibody-mediated cross-linking, transfectants (5×10^4) were stimulated with (B) plate bound anti-CD3 ϵ mAb 145-2C11, or (C) plate bound anti-V β 8 mAb F23.1. To coat wells of 96-well plates, antibodies were used at 10 μ g/ml. IL-2 activity in the culture supernatants was analyzed using the IL-2-dependent cell line, CTLL-2. Background cpm were <3000 cpm for all transfectants. The stimulation data are representative of three separate experiments. All transfectants gave similar but lower responses with antibodies coated at 3 and 5 μ g/ml, indicating overlapping dose response curves (data not shown).

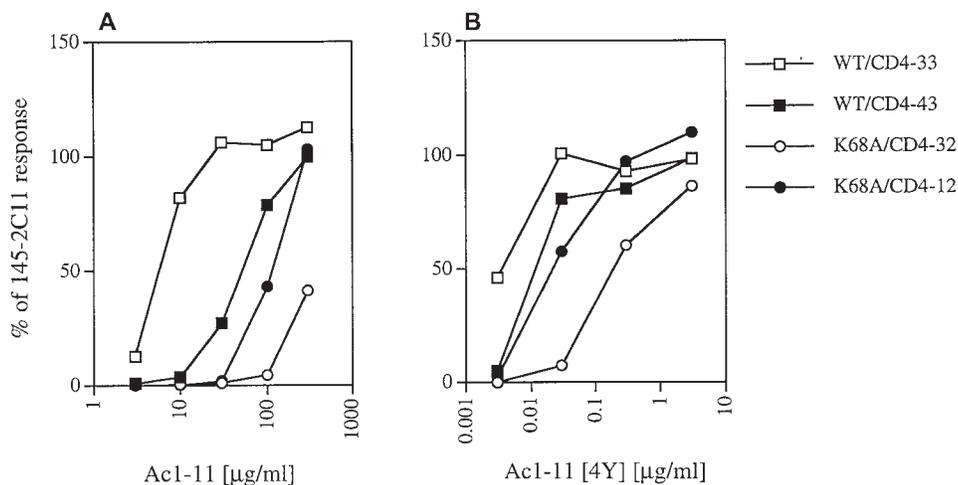


Figure 5. Responses of transfectants to Ac1-11 and the higher affinity analog Ac1-11[4Y]. 5×10^4 PL-8 APCs/well were cocultured with 5×10^4 transfectants/well and the concentrations of (A) Ac1-11, or (B) Ac1-11[4Y] peptides as shown. Culture supernatants were harvested after 24 h and analyzed for IL-2 activity as in Fig. 2. Data is expressed as percent of 145-2C11 response shown in Fig. 4. Background cpm were <3000 cpm for all transfectants. SDs were $<12\%$, and the data are representative of three independent experiments. Simultaneous analysis of CD4⁺ and CD4⁻ transfectants led to similar results to those shown in Figs. 3 and 5 (data not shown).

(10, 12, 13), make contact with cognate ligand. In addition, binding studies using a recombinant class I-restricted TCR indicate a minor contribution for these residues to the binding energy of the interaction (38). However, the functional relevance of HV4 α residues in T cell activation have not, to our knowledge, been investigated for either class I- or class II-restricted TCRs, and the current study addresses this issue for an autoreactive TCR that recognizes MBP Ac1-11 bound to I-A^u. In contrast to amino acids located in HV4 α , the functional significance of HV4 β residues have been more extensively analyzed, and this TCR region is

known to play a role in contacting several bacterial and endogenous superantigens (52–54). In addition, recent modeling/X-ray structural studies for the interaction of the murine 2C TCR with allo-ligand have indicated that the HV4 β residue R69 might contribute toward the binding energy through electrostatic effects (55).

Our findings identify a role for HV4 α residues of the 1934.4 TCR in pMHC recognition. This is consistent with the X-ray crystallographic structure of the 1934.4 V α domain (V α 4.2), in which HV4 α forms part of a relatively flat continuous surface with CDR1, 2, and 3, leading to the

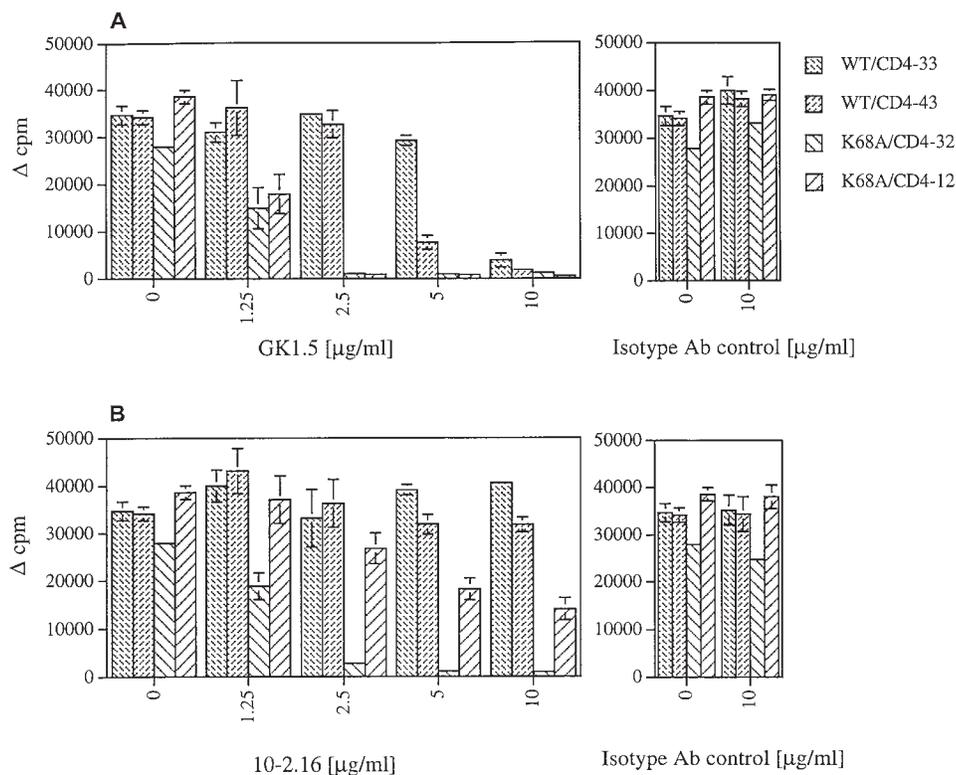


Figure 6. Effect of anti-CD4 and anti-MHC class II antibodies on the responses of the transfectants to peptide. Transfectants (5×10^4 /well) were cocultured with PL-8 APCs (5×10^4 /well) and $0.3 \mu\text{g/ml}$ of Ac1-11[4Y] peptide in the absence or presence of the concentrations of (A) the anti-CD4 antibody, GK1.5, or (B) the anti-MHC class II antibody, 10-2.16, as shown. Supernatants were harvested after 24 h and analyzed for IL-2 as in Fig. 2. Appropriate isotype matched control antibodies (rat IgG2b for GK1.5, and mouse IgG2b for 10-2.16) were used at $10 \mu\text{g/ml}$. Background cpm were <1500 cpm for all transfectants. Data are representative of two independent experiments.

earlier suggestion that it might be involved in interactions with cognate pMHC ligand (4). In the current study, the role of the two central exposed residues (K68, E69) of this loop in pMHC recognition have been investigated by expressing 1934.4-derived TCRs with mutations to alanine at these positions in T cell transfectants. K68 is highly conserved in murine and human $V\alpha$ sequences, whereas the variability at position 69 is higher (37). Antigen responsiveness of the TCR transfectants has been analyzed in the presence and absence of CD4 coexpression and compared with that of 1934.4 WT transfectants. These studies have shown that K68 plays an important role in antigen responsiveness, as assessed by quantitating IL-2 secretion, whereas E69 has a lesser role. In addition, mutation of both K68 and E69 to alanine within the same TCR results in responsiveness to Ac1-11(4Y)-I-A^u complexes that is intermediate between that of the transfectants expressing K68A and E69A TCRs. However, transfectants expressing this double mutant show an almost undetectable response similar to that of the K68A mutants when presented with the lower affinity peptide Ac1-11 in the context of I-A^u. Thus, the effect of the double mutation appears to be analogous to that of the K68A mutation, reiterating the importance of K68 in the interaction.

The role of K68 in pMHC responsiveness of the 1934.4 TCR could be through several possible mechanisms that are not mutually exclusive. First, K68 may contact either peptide or I-A^u (or both) in the pMHC complex either directly or via ordered water, which has been shown to play a role in stabilizing antibody-antigen interactions (56, 57). Electrostatic interactions, which can occur over distances up to 20 Å (58), may also occur between K68 and cognate pMHC. Consistent with this possibility and assuming that the I-A^u structure is similar to that of the recent I-A^k/I-A^d structures (59, 60), several acidic I-A β^u residues (E84, E85; numbering as in reference 37) would contact/be in proximity to HV4 α if the orientation of 1934.4 TCR binding resembles that in TCR-pMHC class I complexes (10-13). Alternatively, the effects of mutation of K68 may be indirect through destabilization of other CDR loops of $V\alpha 4.2$ that are involved in pMHC binding. However, we favor a more direct effect of the mutation, since the X-ray crystallographic structure of $V\alpha 4.2$ (4) indicates that K68 is exposed and does not make stabilizing H-bonds with CDR residues. Furthermore, it is unlikely that the mutations analyzed in this study have resulted in gross structural perturbations since the CD spectra of the corresponding recombinant $V\alpha$ domains indicate that they are correctly folded, and the WT and mutant transfectants express similar levels of surface TCR.

Regardless of the mechanism by which the effect of mutation of K68 occurs, the almost total abrogation of responsiveness of the CD4⁻ mutant transfectants to Ac1-11-I-A^u complexes is unexpected since K68 would be predicted to be at the periphery of the interacting surfaces of TCR and pMHC. However, the absence of CD4 together with the low affinity of this peptide for I-A^u most likely contribute toward the unresponsiveness, and in this context CD4⁻

WT transfectants also respond relatively poorly to this ligand. In contrast, when the density of cognate pMHC is increased by using Ac1-11(4Y) which has a ~1000-fold higher affinity for I-A^u (61), responsiveness of the K68A and K68AE69A transfectants is observed albeit at considerably lower levels than that of the WT transfectants. This supports the concept that the K68A mutant TCR binds to cognate antigen (Ac1-11-I-A^u complexes) with an affinity/avidity which, in the absence of CD4, falls below the threshold needed for effective signaling (IL-2 production in the current study). Consistent with this, coexpression of CD4 partially compensates for the defective signaling of transfectants expressing K68A. The enhancing effect of CD4 could be through one or more mechanisms. First, CD4 may increase the affinity/avidity of the TCR for pMHC by stabilizing the trimeric complex via CD4-MHC interactions (19-21, 33), or by increasing the affinity of the TCR-pMHC interaction in an analogous way to that demonstrated for CD8 (24, 25). Second, CD4 is able to recruit the phosphotyrosine kinase p56^{lck} to the TCR-CD3 complex increasing the strength of the signal delivered postantigen recognition by the TCR (22, 23). In the current study, this could compensate for the loss in affinity of the K68A mutant. Consistent with this, several studies have shown a significant loss of CD4 effects on T cell activation in the absence of p56^{lck}-CD4 interaction (62-64). However, CD4 lacking its cytoplasmic lck-binding domain can still augment T cell reactivity (65), and in one study the extracellular domains of CD4 and not its cytoplasmic tail are responsible for restoration of IL-2 secretion (66).

Recent studies have shown that reduced CD4 availability can convert the functional and biochemical effects of agonist peptides into those characteristic of partial agonists (32) or partial agonists into antagonists (67, 68). Reciprocally, CD4 coexpression can convert partial agonists into agonists, but has no effect on antagonist activity (31). The lack of effect of CD4 on antagonists which form short-lived TCR-pMHC complexes (17) supports the hypothesis that CD4 engagement follows TCR-pMHC complex formation, and that the latter complex needs to be sufficiently long lived to allow CD4 recruitment (31, 32). By extension, in the current study, the enhancing effect of CD4 coexpression on the responsiveness of the K68A mutant suggests that the half life of the K68A TCR-pMHC interaction is long enough to allow CD4 recruitment to the ternary TCR-pMHC complex. However, without CD4 coexpression, the interaction of this TCR with Ac1-11-I-A^u complexes appears to be below the threshold needed for T cell activation. In contrast, the WT 1934.4 TCR-pMHC interaction appears to be sufficiently long lived in the absence of CD4 to result in signaling. This is also consistent with the relative resistance of this latter interaction to blockade by the anti-CD4 antibody, GK1.5. In addition, the enhancement in responsiveness by CD4 coexpression is much greater for the K68A mutant than the WT TCR. The effect of CD4 therefore appears to be maximal for suboptimal TCR-pMHC interactions that attain a threshold affinity, and this is consistent with observations using

other antigen recognition systems (31, 69). However, in these other systems, the effects of CD4 on responses to agonists/partial agonists/antagonists were analyzed, and this is in contrast to the current study where the affinity of the TCR-pMHC (agonist) interaction has been affected by mutating the TCR. Taken together, our data provide further support for sequential engagement models of T cell signaling in which both the affinity (off-rate) of the TCR-pMHC complex and coreceptor involvement affect the outcome of T cell contact with APCs bearing cognate ligands (31, 32, 34).

In summary, a single amino acid substitution of the exposed, highly conserved residue K68, which is located outside the CDRs of the TCR, appears to have a significant impact on the outcome of the interaction between a TCR and its pMHC ligand. Given the indications that the diagonal orientation observed for the binding of class I-restricted TCRs to pMHC complexes is general (10–13), it is likely that the functional role of this HV4 α amino acid that we have defined will be observed for other TCR-pMHC interactions.

We are indebted to Drs. Bertram Ober and Mark Mummert for providing the wild-type TCR transfection constructs and advice with CD analyses, respectively. We also thank Drs. Nicolai van Oers and Stephen Thompson for critical review of the manuscript and Dr. Mischa Machius for assistance with Fig. 1.

This work was supported by grants from the National Multiple Sclerosis Society (RG-2411), National Institutes of Health (AI/NS 42949) and Yellow Rose Gala. E.S.Ward is an Established Investigator of the American Heart Association (Grant 9640277N).

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Received for publication 5 August 1998 and in revised form 20 October 1998.

References

1. Wange, R.L., and L.E. Samelson. 1996. Complex complexes: signaling at the TCR. *Immunity*. 5:197–205.
2. Iwashima, M., B.A. Irving, N.S. van Oers, A.C. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science*. 263:1136–1139.
3. van Oers, N.S., W. Tao, J.D. Watts, P. Johnson, R. Aebersold, and H.S. Teh. 1993. Constitutive tyrosine phosphorylation of the T cell receptor (TCR) ζ subunit: regulation of TCR ζ -associated protein tyrosine kinase activity by TCR ζ . *Mol. Cell. Biol.* 13:5771–5780.
4. Fields, B.A., B. Ober, E.L. Malchiodi, M.I. Lebedeva, B.C. Braden, X. Ysern, J.K. Kim, X. Shao, E.S. Ward, and R.A. Mariuzza. 1995. Crystal structure of the V α domain of a T cell antigen receptor. *Science*. 270:1821–1824.
5. Bentley, G.A., G. Boulot, K. Karjalainen, and R.A. Mariuzza. 1995. Crystal structure of the beta chain of a T cell antigen receptor. *Science*. 267:1984–1987.
6. Bentley, G.A., and R.A. Mariuzza. 1996. The structure of the T cell antigen receptor. *Annu. Rev. Immunol.* 14:563–590.
7. Jorgensen, J.L., U. Esser, B.F. de St. Groth, P.A. Reay, and M.M. Davis. 1992. Mapping T-cell receptor-peptide contacts by variant peptide immunization of single-chain transgenics. *Nature*. 355:224–230.
8. Davis, M.M., and P.J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature*. 334:395–398.
9. Claverie, A., A. Prochnicka-Chaloufour, and L. Bougueleret. 1989. Implications of a Fab-like structure for the T-cell receptor. *Immunol. Today*. 10:10–13.
10. Garcia, K.C., M. Degano, R.L. Stanfield, A. Brunmark, M.R. Jackson, P.A. Peterson, L. Teyton, and I.A. Wilson. 1996. An $\alpha\beta$ T cell receptor structure at 2.5Å and its orientation in the TCR-MHC complex. *Science*. 274:209–219.
11. Garboczi, D.N., P. Ghosh, U. Utz, Q.R. Fan, W.E. Biddison, and D.C. Wiley. 1996. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature*. 384:134–141.
12. Garcia, K.C., M. Degano, L.R. Pease, M. Huang, P.A. Peterson, L. Teyton, and I.A. Wilson. 1998. Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen. *Science*. 279:1166–1172.
13. Ding, Y.H., K.J. Smith, D.N. Garboczi, U. Utz, W.E. Biddison, and D.C. Wiley. 1998. Two human T cell receptors bind in a similar diagonal mode to the HLA-A2/Tax peptide complex using different TCR amino acids. *Immunity*. 8:403–411.
14. Hong, S.C., A. Chelouche, R.H. Lin, D. Shaywitz, N.S. Braunstein, L. Glimcher, and C.A. Janeway. 1992. An MHC interaction site maps to the amino-terminal half of the T cell receptor alpha chain variable domain. *Cell*. 69:999–1009.
15. Sant'Angelo, D.B., G. Waterbury, P. Preston-Hurlburt, S.T. Yoon, R. Medzhitov, S.C. Hong, and C.A. Janeway. 1996. The specificity and orientation of a TCR to its peptide-MHC class II ligands. *Immunity*. 4:367–376.
16. Sun, R., S.E. Shepherd, S.S. Geier, C.T. Thomson, J.M. Sheil, and S.G. Nathenson. 1995. Evidence that the antigen receptors of cytotoxic T lymphocytes interact with a common recognition pattern on the H-2K^b molecule. *Immunity*. 3:573–582.
17. Lyons, D.S., S.A. Lieberman, J. Hampl, J.J. Boniface, Y.

- Chien, L.J. Berg, and M.M. Davis. 1996. A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity*. 5:53–61.
18. Alam, S.M., P.J. Travers, J.L. Wung, W. Nasholds, S. Redpath, S.C. Jameson, and N.R. Gascoigne. 1996. T-cell-receptor affinity and thymocyte positive selection. *Nature*. 381:616–620.
 19. Janeway, C.A., Jr. 1992. The T cell receptor as a multicomponent signaling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Annu. Rev. Immunol.* 10:645–674.
 20. Clayton, L.K., M. Sieh, D.A. Pious, and E.L. Reinherz. 1989. Identification of human CD4 residues affecting class II MHC versus HIV-1 gp120 binding. *Nature*. 339:548–551.
 21. Doyle, C., and J.L. Strominger. 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature*. 330:256–259.
 22. Veillette, A., M.A. Bookman, E.M. Horak, and J.B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56^{lck}. *Cell*. 55:301–308.
 23. Turner, J.M., M.H. Brodsky, B.A. Irving, S.D. Levin, R.M. Perlmutter, and D.R. Littman. 1990. Interaction of the unique N-terminal region of tyrosine kinase p56^{lck} with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell*. 60:755–765.
 24. Luescher, I., E. Vivier, A. Layer, J. Mahieu, F. Godeau, B. Malissen, and P. Romero. 1995. CD8 modulation of T-cell antigen receptor-ligand interactions on living cytotoxic T lymphocytes. *Nature*. 373:353–356.
 25. Garcia, K., C. Scott, A. Brunmark, F. Carbone, P. Peterson, I. Wilson, and L. Teyton. 1996. CD8 enhances formation of stable T-cell receptor/MHC class I complexes. *Nature*. 384:577–581.
 26. Janeway, C.A., Jr. 1995. Ligands for T cell receptors: hard times for avidity models. *Immunol. Today*. 16:223–225.
 27. Yoon, S.T., U. Dianzani, K. Bottomly, and C.A. Janeway. 1994. Both high and low avidity antibodies to the T cell receptor can have agonist or antagonist activity. *Immunity*. 1:563–569.
 28. Reich, Z., J.J. Boniface, D.S. Lyons, N. Borochoy, E.J. Wachtel, and M.M. Davis. 1997. Ligand-specific oligomerization of T-cell receptor molecules. *Nature*. 387:617–620.
 29. Symer, D.E., R.Z. Dintzis, D.J. Ciamond, and H.M. Dintzis. 1992. Inhibition or activation of human T cell receptor transfectants is controlled by defined, soluble antigen arrays. *J. Exp. Med.* 176:1421–1430.
 30. Monks, C.R., B.A. Freiberg, H. Kupfer, N. Sciaky, and A. Kupfer. 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature*. 395:82–86.
 31. Hampl, J., Y. Chien, and M.M. Davis. 1997. CD4 augments the response of a T cell to agonist but not to antagonist ligands. *Immunity*. 7:379–385.
 32. Madrenas, J., L.A. Chau, J. Smith, J.A. Bluestone, and R.N. Germain. 1997. The efficiency of CD4 recruitment to ligand-engaged TCR controls the agonist/partial agonist properties of peptide-MHC molecule ligands. *J. Exp. Med.* 185:219–229.
 33. Konig, R., X. Shen, and R.N. Germain. 1995. Involvement of both major histocompatibility complex class II α and β chains in CD4 function indicates a role for ordered oligomerization in T cell activation. *J. Exp. Med.* 182:779–787.
 34. Madrenas, J., and R.N. Germain. 1996. Variant TCR ligands: new insights into the molecular basis of antigen-dependent signal transduction and T cell activation. *Semin. Immunol.* 8:83–101.
 35. McKeithan, T. 1995. Kinetic proof reading in T-cell receptor signal transduction. *Proc. Natl. Acad. Sci. USA*. 92:5042–5046.
 36. Rabinowitz, J.D., C. Beeson, D.S. Lyons, M.M. Davis, and H.M. McConnell. 1996. Kinetic discrimination in T-cell activation. *Proc. Natl. Acad. Sci. USA*. 93:1401–1405.
 37. Kabat, E.A., T.T. Wu, H.M. Perry, K.S. Gottesman, and C. Foeller. 1991. Sequences of Proteins of Immunological Interest. Vol. 1, 5th Ed. U.S. Department of Health and Human Services, Washington, DC.
 38. Manning, T.C., C.J. Schlueter, T.C. Brodnicki, E.A. Parke, J.A. Speir, K.C. Garcia, L. Teyton, I.A. Wilson, and D.M. Kranz. 1998. Alanine scanning mutagenesis of an $\alpha\beta$ T cell receptor: mapping the energy of antigen recognition. *Immunity*. 8:413–425.
 39. Wraith, D.C., D.E. Smilek, D.J. Mitchell, L. Steinman, and H.O. McDevitt. 1989. Antigen recognition in autoimmune encephalomyelitis and the potential for peptide-mediated immunotherapy. *Cell*. 59:247–255.
 40. Letourner, F., and B. Malissen. 1989. Derivation of a T cell hybridoma variant deprived of functional T cell receptor α and β chain transcripts reveals a non-functional α mRNA of BW5147 origin. *Eur. J. Immunol.* 19:2269–2274.
 41. Wraith, D.C., D.E. Smilek, and S. Webb. 1991. MHC-binding peptides for immunotherapy of experimental autoimmune disease. *J. Autoimmun.* 5 (Suppl A):103–113.
 42. Staerz, U., H.G. Rammensee, J.D. Benedetto, and M.J. Bevan. 1985. Characterisation of a murine mAb specific for an allotypic determinant on T cell antigen receptor. *J. Immunol.* 134:3994–4000.
 43. Patten, P.A., E.P. Rock, T. Sonoda, B. Fazekas de St. Groth, J.L. Jorgenson, and M.M. Davis. 1993. Transfer of putative complementarity determining region loops of T cell receptor V domains confers toxin reactivity but not peptide/MHC specificity. *J. Immunol.* 150:2281–2294.
 44. Ward, E.S. 1992. Secretion of T cell receptor fragments from recombinant *Escherichia coli* cells. *J. Mol. Biol.* 224:885–890.
 45. Kunkel, T.A., J.D. Roberts, and R.A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367–382.
 46. Horton, R.M., H.D. Hunt, S.N. Ho, J.K. Pullen, and L.R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene*. 77:61–68.
 47. Fairchild, P.J., R. Wildgoose, E. Atherton, S. Webb, and D.C. Wraith. 1993. An autoantigenic T cell epitope forms unstable complexes with class II MHC molecules: a novel route for escape from tolerance induction. *Int. Immunol.* 5:1151–1158.
 48. Mason, K., D.D. Denney, and H. McConnell. 1995. Kinetics of the reaction of a myelin basic protein peptide with soluble I-A^d. *Biochemistry*. 34:4874–4878.
 49. Marrack, P., R. Endres, R. Shimonkevitz, A. Zlotnik, D. Dialynas, F.W. Fitch, and J. Kappler. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. II. Role of the L3T4 product. *J. Exp. Med.* 158:1077–1091.
 50. Wraith, D.C., B. Bruun, and P.J. Fairchild. 1992. Cross-reactive antigen recognition by an encephalitogenic T cell receptor. *J. Immunol.* 149:3765–3770.
 51. Rothenberg, E.V. 1994. Signaling mechanisms in thymocyte selection. *Curr. Opin. Immunol.* 6:257–265.

52. Pullen, A.M., T. Wade, P. Marrack, and J.W. Kappler. 1990. Identification of a T cell receptor β chain that interacts with the self-superantigen Mls-1. *Cell*. 61:1365–1374.
53. Choi, Y., A. Herman, D. DiGiusto, T. Wade, P. Marrack, and J. Kappler. 1990. Residues of the variable region of the T-cell-receptor β -chain that interact with *S. aureus* superantigens. *Nature*. 346:471–473.
54. Fields, B.A., E.L. Malchiodi, H. Li, X. Ysern, C.V. Stauffer, P.M. Schlievert, K. Karjalainen, and R.A. Mariuzza. 1996. Crystal structure of a T-cell receptor β -chain complexed with a superantigen. *Nature*. 384:188–192.
55. Speir, J.A., K.C. Garcia, A. Brunmark, M. Degano, P.A. Peterson, L. Teyton, and I.A. Wilson. 1998. Structural basis of 2C TCR allorecognition of H-2L^d peptide complexes. *Immunity*. 8:553–562.
56. Fischmann, T.O., G.A. Bentley, T.N. Bhat, G. Boulot, R.A. Mariuzza, S.E.V. Phillips, D. Tello, and R.J. Poljak. 1991. Crystallographic refinement of the three-dimensional structure of the FabD1.3-lysozyme complex at 2.5 Å resolution. *J. Biol. Chem.* 266:12915–12920.
57. Tulip, W.R., J.N. Varghese, W.G. Laver, R.G. Webster, and P.M. Colman. 1992. Refined crystal structure of the influenza virus N9 neuraminidase-NC41 Fab complex. *J. Mol. Biol.* 227:122–148.
58. McCoy, A.J., V. Chandana-Epa, and P.M. Colman. 1997. Electrostatic complementarity at protein/protein interfaces. *J. Mol. Biol.* 268:570–584.
59. Scott, C.A., P.A. Peterson, L. Teyton, and I.A. Wilson. 1998. Crystal structures of two I-A^d-peptide complexes reveal that high affinity can be achieved without large anchor residues. *Immunity*. 8:319–329.
60. Fremont, D.H., D. Monnaie, C.A. Nelson, W.A. Hendrickson, and E.A. Unanue. 1998. Crystal structure of I-A^k in complex with a dominant epitope of lysozyme. *Immunity*. 8:305–317.
61. Fugger, L., J. Liang, A. Gautam, J. Rothbard, and H. McDevitt. 1996. Quantitative analysis of peptides from myelin basic protein binding to the MHC class II protein, I-A^u, which confers susceptibility to experimental allergic encephalomyelitis. *Mol. Med.* 2:181–188.
62. Miceli, M.C., P. von Hoegen, and J.R. Parnes. 1990. Adhesion versus coreceptor function of CD4 and CD8: role of the cytoplasmic tail in coreceptor activity. *Proc. Natl. Acad. Sci. USA*. 88:2623–2627.
63. Collins, T.L., S. Uniyal, J. Shin, J.L. Strominger, R.S. Mittler, and S.J. Burakoff. 1992. p56^{lck} association with CD4 is required for the interaction between CD4 and the TCR/CD3 complex for optimal antigen stimulation. *J. Immunol.* 148:2159–2162.
64. Glaichenhaus, N., N. Shastri, D.R. Littman, and J.M. Turner. 1991. Requirement for association of p56^{lck} with CD4 in antigen specific signal transduction in T cells. *Cell*. 64:511–520.
65. Xu, H., and D.R. Littman. 1993. A kinase-independent function of Lck in potentiating antigen-specific T cell activation. *Cell*. 74:633–643.
66. Vignali, D.A.A., R.T. Carson, B. Chang, R.S. Mittler, and J.L. Strominger. 1996. The two membrane proximal domains of CD4 interact with the T cell receptor. *J. Exp. Med.* 183:2097–2107.
67. Mannie, M.D., J.M. Rossier, and G.A. White. 1995. Autologous rat myelin basic protein is a partial agonist that is converted into a full antagonist upon blockade of CD4. Evidence for the integration of efficacious and non-efficacious signals during T cell antigen recognition. *J. Immunol.* 154:2642–2645.
68. Vidal, K., B.L. Hsu, C.B. Williams, and P.M. Allen. 1996. Endogenous altered peptide ligands can affect peripheral T cell responses. *J. Exp. Med.* 183:1311–1321.
69. Viola, A., M. Salio, L. Tuosto, S. Linkert, O. Acuto, and A. Lanzavecchia. 1997. Quantitative contribution of CD4 and CD8 to T cell antigen receptor serial triggering. *J. Exp. Med.* 186:1775–1779.
70. Esnouf, R.M. 1997. An extensively modified version of MolScript that includes greatly enhanced coloring capabilities. *J. Mol. Graph.* 15:133–138.
71. Meritt, E.A., and M.E.P. Murphy. 1994. Raster3D Version 2.0: a program for photorealistic molecular graphics. *Acta Cryst. D*. 50:869–873.