Amino Acid Residues that Flank Core Peptide Epitopes and the Extracellular Domains of CD4 Modulate Differential Signaling through the T Cell Receptor

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Summary

Hen egg lysozyme 52-61-specific CD4+ T cells responded by interleukin 2 (IL-2) secretion to any peptide containing this epitope regardless of length of NH_2- and COOH-terminal composition. However, CD4- variants could only respond to peptides containing the two COOH-terminal tryptophans at positions 62 and 63. Substitutions at these positions defined patterns of reactivity that were specific for individual T cells inferring a T cell receptor (TCR)-based phenomenon. Thus, the fine specificity of major histocompatibility complex (MHC)-peptide recognition by the TCR was dramatically affected by CD4 and the COOH-terminal peptide composition. Peptides that failed to induce IL-2 secretion in the CD4- variants nevertheless induced strong tyrosine phosphorylation of $CD3\zeta$. Thus, whereas the TCR still recognized and bound to the MHC class II-peptide complex resulting in protein phosphorylation, this interaction failed to induce effective signal transduction manifested by IL-2 secretion. This provides a clear example of differential signaling mediated by peptides known to be naturally processed. In addition, the external domains of CD4, rather than its cytoplasmic tail, were critical in aiding TCR recognition of all peptides derived from a single epitope. These data suggest that the nested flanking residues, which are present on MHC class II but not class I bound peptides, are functionally relevant.

The TCR recognizes foreign antigens in the form of short peptides bound to MHC class I or II molecules (1-3). Peptides bound to MHC class I are relatively length restricted and typically only one peptide will derive from a single epitope (4-6). In contrast, MHC class II bound peptides are heterogeneous in length, and epitopes are frequently presented as large numbers of peptides nested at both the NH_2- and COOH-termini (7-10). These differences could have significant consequences for TCR recognition. Whereas only a few side chains in the center of MHC class I bound peptides would be accessible to the TCR (11-14), the extension of bound peptides at either end of the MHC class II molecule (3) raises the possibility that these flanking regions may affect, or directly interact with, the TCR. However, there is neither evidence nor precedence for this notion (15-17).

A close association between CD4 and the TCR-CD3 complex is thought to occur during antigen presentation as a result of their interaction with the same MHC class II molecule (18, 19). Early speculation that CD4 may play a role in T cell activation came from the observation that cocrosslinking CD4 with the TCR-CD3 complex resulted in a synergistic response (20, 21). More recently, supporting evidence has come from cocapping, comodulation, and fluorescence energy transfer experiments (22-26). Indeed, certain anticlonotypic antibodies have been shown to induce the comodulation of CD4 with the TCR in the absence of cross-linking by secondary antibody (22, 24). However, none of these data can rule out the possibility that this association occurs via an intermediate molecule rather than via direct binding between CD4 and the TCR. Whereas attempts to demonstrate direct physical contact have been largely unsuccessful (27), suggestive evidence has come from two recent studies using affinity chromatography of ^{125}I-labeled proteins (28), and an in vitro kinase assay (29). These difficulties may be due to the instability of this interaction (29, 30). Whereas only 5% of CD4 is associated with the TCR on a resting cell (31), up to 30% of the CD4 molecules comodulate with the TCR after specific antigen stimulation (32).

Despite these uncertainties, even an indirect association may be functionally important. Support for this notion has come from the identification of a *src* tyrosine kinase, $p56^{lck}$, which associates with the cytoplasmic tail of CD4 (33-35). Induction of CD4-TCR association with certain anticlonotypic antibodies (36) or in vitro kinase assays with $p56^{lck}$-immunoprecipitates (29) results in the phosphorylation of CD3ε and $CD3\zeta$ (also known as TCRζ). However, $p56^{lck}$ has also been

shown to perform important kinase-independent functions during T cell activation (37). Although previous studies have suggested that neither effective T cell stimulation nor CD4–TCR association can occur when CD4 cannot associate with p56^{lck} (38–41), recent studies have implied an opposing view with p56^{lck} playing a critical role in TCR-mediated signaling and T cell development in vivo in the absence of coupling to CD4 (42–44).

The physiological consequences of CD4–TCR interaction on TCR function are not clear. This question has previously been addressed using CD4 loss variants of three different H-2A^k–restricted murine T cell hybridomas specific for the immunodominant hen egg lysozome (HEL)[1] peptide 52-61 (45). Whereas all the CD4$^+$ and CD4$^-$ variants tested responded comparably to immobilized anti-TCR and anti-CD3 mAbs and naturally processed HEL, only the CD4$^+$ hybridomas responded to the synthetic tryptic peptide 46-61. These data suggested that class II–restricted T cells can only recognize a limited repertoire of peptides in the absence of CD4, and that at least some naturally processed HEL peptides can stimulate CD4$^-$ hybridomas. The recent identification of multiple nested peptides containing the core sequence 52-61, extracted from HEL-pulsed, H-2A^k positive B cells lends credence to this notion (46, 47). Furthermore, the role of CD4 and different naturally processed nested peptides in differential signaling through the TCR remains to be defined (48–52).

The initial aim of this study was to determine which among all the possible peptides containing the HEL 52-61 epitope stimulate the CD4$^-$ T cell hybridomas. These experiments led to the identification of two residues, Trp 62 and Trp 63, that are essential for TCR recognition in the absence of CD4. As a result, the following questions have been addressed. First, do peptides containing these residues have a higher affinity for either MHC class II molecules or TCR? Second, what is the sensitivity of TCR recognition to amino acid substitutions at these residues? Third, with CD4 loss variants that fail to respond in the absence of these residues, does the TCR still ligate the MHC–peptide complex? Fourth, what effect do these residues and the absence of CD4 have on signal transduction? Fifth, what regions of CD4 aid in TCR recognition of peptides that lack these residues?

Materials and Methods

Peptides. A nested set of 53 peptides consisting of every possible 12–19 mer that contains the minimal MHC binding epitope 52-61 (53, 54), was synthesized by Chiron Mimotopes (Melbourne, Australia) using pin synthesis technology. Peptides were dissolved in 10% DMSO in H$_2$O at ~100 μM. Because of variable solubility and approximate quantitation, these peptides were only used for qualitative analysis.

Crude analog peptides (see Table 2) were obtained from Chiron Mimotopes, purified to 95% by reversed-phase HPLC, and analyzed for integrity, composition, and concentration using mass

spectrometry and quantitative amino acid analysis as previously described (10).

Murine T Cell Hybridomas and Transfectants. CD4$^+$ and CD4$^-$ variants of 3A9 (P4, N49: in all cases P denotes cells which express CD4 and N denotes cells which lack CD4), A2.2B2 (P2, N22), 2B5.1 (P8, N3), and A167 (P67, N54), were isolated as previously described (45, 55) except that a different 3A9 clone, N49 rather than N30, was used as the latter, which failed to respond to HEL, was subsequently found to be unable to regain response to HEL after transfection with wild-type CD4. Variants from 4G4.1 (P5, N4) and 1C5.1 (P16, N1) (both generous gifts from Luciano Adorini, Roche Milano Ricerche, Milan, Italy) were isolated for this study. Briefly, hybridomas were double labeled with GK1.5 biotin (anti-CD4) followed by streptavidin–PE (Caltag Laboratories, San Francisco, CA) and anti-CD3–FITC (GIBCO BRL, Gaithersburg, MD). CD4$^+$ and CD4$^-$ cells with equivalent TCR–CD3 expression were sorted at one cell per well (Epics 750 series with Autoclone attachment; Coulter, Hialeah, FL). After 2 wk, approximately 24 clones were tested for CD4 and equivalence of TCR, CD2, LFA-1, CD45, CD5, LFA-1, and CD28 expression by flow cytometry (FACScan®, Becton Dickinson, San Jose, CA). At least six of these were then tested for their equivalence of sensitivity (i.e., between CD4$^+$ and CD4$^-$ clones) to immobilized anti-TCR (H57.157), all of which gave comparable results (45).

A construct that produces CD4 molecules that lack an intact cytoplasmic domain (CD4.ΔCY) was generated by exchanging T for C at bp 1,312, thereby producing a stop codon (Vignali, D. A. A., unpublished results). This leads to a cytoplasmic tail of only five amino acids (Arg Cys Arg His Asu; 56). This short tail is required to retain CD4 in the membrane. The full-length wild-type mouse CD4 cDNA (CD4.WT) and CD4.ΔCY were subcloned into pHβApr-1neo (human β-actin promoter; 57), and transfected into the 3A9 and A2.2B2 CD4 loss variants as previously described (45). The CD4.ΔCY transfectants were shown to lack a functional cytoplasmic tail by the inability of anti-CD4 mAb to coimmunoprecipitate p56^{lck} (Vignali, D. A. A., unpublished results).

Antigen Presentation Assays. Assays were performed essentially as described elsewhere (45, 55). Briefly, T cell variants (5 × 10^4, 100 μl) were cultured with 2.5 × 10^4 (100 μl) LK35.2 (murine B cell lymphoma, H2-A^{kd}) in flat-bottomed 96-well microtiter plates with synthetic peptides or HEL (Sigma Chemical Co., St. Louis, MO) at the concentrations indicated. Supernatants (50 μl) were removed after 24 h for estimation of IL-2 secretion by culturing with the IL-2–dependent T cell line CTLL-2 (5 × 10^4; 50 μl). Proliferation was determined by using a methyl thiasol-tetrazolium (MTT) assay according to the manufacturer's instructions (Promega, Madison, WI). IL-2 concentration was determined by using murine rIL-2 (Genzyme Corp., Cambridge, MA) as a standard. EC$_{50}$ values were determined by titrating peptides 10-fold from 10 μM to 100 pM, calculating the number of IL-2 U for each dilution from a rIL-2 standard.

H-2A^k–peptide Affinity Assay. Affinity of the HPLC-purified analogs was determined by competition with biotinylated HEL 46-61. H-2A^k (50 nM in 100 μl) was purified as previously described (47) and was incubated with 100 nM HEL 46-61 biotin and competitor peptide from 10 μM to 30 nM at room temperature for 48 h in McIlvaine citrate/phosphate buffer, pH5 (58), in 96-well plates that had been preblocked for 24 h with blocking buffer (3% BSA, 0.1% Tween 20, 0.02% NaN$_3$ in PBS, pH 7.2). 20 μl 1 M Na$_2$HPO$_4$ and 30 μl 10% BSA were added to the reactions and transferred to plates that had been previously precoated with anti-H-2A^k (10.2.16 at 10 μg/ml) for 24 h, washed, and then incubated

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[1] *Abbreviations used in this paper:* HEL, hen egg lysozome; IP, inositol phosphate; PLC, phospholipase C.

with blocking buffer for 2 h. After 90 min, plates were washed and probed with streptavidin–alkaline phosphatase (1:1,000 in blocking buffer; Bio-Rad, Hercules, CA). K_i values are defined as the concentration of inhibitor required to give 50% inhibition of 46-61 biotin.

Inositol Phosphate Assay. LK35.2 cells were pulsed with the appropriate peptides at 3 μM final overnight, and washed once in PBS before use. Hybridomas were labeled at 10^7/ml with 40 μCi/ml [^{3}H] myo-inositol (23.45 Ci/mmol; NEN DuPont, Wilmington, DE) at 37°C for 3 h. Cells were washed three times with inositol-deficient RPMI 1640/10% FCS containing 10 mM LiCl. An aliquot (5 × 10^5 cells) was taken for liquid scintillation counting (15 ml Aquasol; NEN DuPont) to determine the total [^{3}H] myo-inositol incorporation. An equal number (5 × 10^6 cells/sample) of LK35.2 and T cell hybridomas were mixed and spun at low speed for 1 min in a microcentrifuge to promote cell contact and incubated at 37°C for 10 min.

Medium was removed and cells lysed with 1 ml ice-cold 10% TCA, vortexed, and left at 4°C for 30 min. Samples were spun for 10 min in a microcentrifuge, TCA extracted five times with 1 ml H_2O-saturated ethyl ether, neutralized with 40 μl of 1:100 ammonium hydroxide, and diluted to 4 ml with H_2O. Samples were loaded onto a 1-ml AG1-X8 anion exchange column (100–200 mesh, formate form; Bio-Rad) and washed with 50 ml of 60-mM sodium formate/5-mM sodium borate. Inositol phosphate (IP) was eluted with 15 ml of 1 M ammonium formate/0.1 M formic acid. Aliquots of each eluate (2 ml plus 16 ml Aquasol) were counted in a liquid scintillation counter.

Tyrosine Phosphorylation Analysis. Peptide-pulsed LK35.2 cells (5 × 10^6 cells/sample) were prepared as for the IP assay, mixed with T cell hybridomas (10^7 cells/sample), spun at low speed for 1 min in a microcentrifuge, and incubated at 37°C for 5 min. Medium was removed and the cell pellet lysed with 500 μl lysis buffer containing 1% NP-40 (Fluka, Ronkonkoma, NY), 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5% glycerol, enzyme inhibitors (2 mM Pefablock [Centerchem, Stamford, CT], 25 μM aprotinin, 25 μM leupeptin), and phosphatase inhibitors (2 mM sodium orthovanadate, 2 mM sodium fluoride, 10 mM tetrasodium pyrophosphate, 1 mM EDTA). Lysates were left at 4°C for 1 h, spun for 15 min in a microcentrifuge, and precleared twice with 50 μl of a 10% suspension of Pansorbin (Calbiochem-Novabiochem Corp., San Diego, CA). Two methods of immunoprecipitation were used. (*a*) Protein A–Sepharose beads (RepliGen, Cambridge, MA) were precoated for 2 h at 4°C with either rabbit anti-PLCγ1 antisera (8 μl/10^7 cells; Upstate Biotechnologies Inc. [UBI], Lake Placid, NY, or 20 μl/10^7 cells; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-CD3ζ antisera (3 μl/10^7 cells; No. 387, gift from Larry Samelson, National Institutes of Health, Bethesda, MD). Beads (25 μl) were added to lysate and rocked at 4°C, overnight. (*b*) Antibody was added to lysates and rocked at 4°C, overnight. Protein A–Sepharose beads were then added and the samples rocked for an additional 2 h. Samples were washed twice with lysis buffer and once in modified lysis buffer with 0.1% NP-40 and without glycerol. Eluted proteins were resolved on either an 8%, for PLCγ1, or a 12%, for CD3ζ, SDS-PAGE gel, and transferred onto nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH).

Blots were blocked with 5% BSA (American Bioanalytical, Natick, MA) in TBS-T (0.2% Tween 20, 10 mM Tris-HCl, pH 8, 150 mM NaCl) at 4°C overnight. Tyrosine phosphorylation was detected with a biotinylated mAb 4G10 (0.1 μg/ml; UBI), followed by a 1:40,000 dilution of streptavidin–horseradish peroxidase (HRP) preformed complexes (Amersham, Arlington Heights, IL). Blots

were developed using enhanced chemiluminescence (Amersham). To detect the original protein, blots were stripped in 100 mM 2-ME (Bio-Rad), 2% SDS, 62.5 mM Tris-HCl, pH, 6.7, for 30 min at 50°C, washed three times and blocked with 5% nonfat dry milk in TBS-T at 4°C, overnight. Blots were probed with either mixed anti-PLCγ1 mAbs (0.5 μg/ml; UBI) or rabbit anti-CD3ζ antisera (1:200), followed by either sheep anti–mouse-HRP (1:40,000; Amersham) for PLCγ1 blot, or protein A-HRP (1:20,000; Amersham) for CD3ζ blot. Blots were developed as above.

Results

Some TCRs Can Only Recognize a Limited Number of Naturally Processed Peptides in the Absence of CD4

Loss of CD4 Dramatically Affects the Response of Some but Not All T Cells to Peptides. A panel of CD4$^+$ and CD4$^-$ murine T cell hybridomas was used to study the role of CD4 in modulating the ability of the TCR to recognize peptides containing NH$_2$- and COOH-terminal extensions of HEL 52-61. Of the hybridomas listed in Table 1, all except for the 1C5.1 variants, which were produced for this study, have been previously described (45, 47). A nested set of 53 peptides consisting of every possible 12–19 mer that contains the minimal MHC binding epitope, HEL 52-61 (53, 54) was made (Fig. 1). It should be noted that these were produced using pin-synthesis technology which facilitates the production of a large number of peptides. However, since they are crude and quantification is approximate, they were used solely for qualitative functional assessments.

All the CD4$^+$ hybridomas responded strongly to all the peptides regardless of length or composition, with the exception of the shortest peptides, which stimulated weaker responses by some of the clones (Fig. 1, *stippled bars*). In contrast, the ability of the CD4$^-$ hybridomas to recognize these peptides fell into three distinct phenotypes (Fig. 1, *solid bars*; Table 1). In phenotype A, exhibited by A2.2B2, 3A9 and 4G4.1, the only peptides that could stimulate a strong response were those that extended at least to Trp 63 (Trp/W) (e.g., peptides 52-63). In addition, this response fell off sharply with successive COOH-terminal extensions. In phenotype B, exhibited by A167, a single Trp at position 62 was sufficient, whereas in phenotype C, exhibited by 1C5.1 and 2B5.1, loss of CD4 had no effect on peptide recognition. Increase in peptide length at the NH$_2$-terminus had little effect.

These observations do not merely define the difference between CD4-dependent and -independent hybridomas as all the CD4 loss variants, regardless of phenotype, responded strongly to naturally processed HEL (Vignali, D., unpublished results). Why then are some but not all T cell hybridomas sensitive to the loss of CD4? No obvious correlation between CD4 requirement and the sensitivity of the hybridomas to HEL or TCR-VαVβ usage was seen (Table 1). Although 4G4.1 was 10 times more sensitive than 2B5.1, it was the former rather than the latter that was affected by the loss of CD4. Furthermore, these hybridomas were not dependent on costimulatory signals as fixed B cells were efficient APCs, and the presence or absence of CD28 had no effect (data not

Table 1. *Characteristics of the HEL 52-61–specific Murine T Cell Hybridomas*

Phenotype	Hybridoma	Restriction element	TCR VαVβ Usage		HEL EC$_{50}$ (μM)	Residue dependence
A	3A9	H-2A^k	Vα3	Vβ8.2	10^{-1}	Trp 62 and Trp 63
	A2.2B2	H-2A^k	Vα11	Vβ5.2	10^{-1}	Trp 62 and Trp 63
	4G4.1	H-2A^k	Vα4	Vβ8.3	3 × 10^{-2}	Trp 62 and Trp 63
B	A167	H-2Aα^{k}β^b	Vα12	Vβ8.3	10^{-1}	Trp 62
C	1C5.1	H-2A^k	Vα2	Vβ6	10^{-1}	None
	2B5.1	H-2A^k	Vα1	Vβ8.2	3 × 10^{-1}	None

The phenotype of the hybridomas was determined by the requirement of the CD4$^-$ variants for Trp 62 and Trp 63 (residue dependence) derived, in part, from Fig. 1. TCR VαVβ usage was determined either by flow cytometry and dot blot analysis using specific Vα and Vβ probes (performed by Geff Cole, Sherri Surman and David Woodland, St. Jude Children's Research Hospital, Memphis, TN) or from the literature (45, 59, 90). HEL EC$_{50}$ refers to the concentration of HEL required to give 50% stimulation. Data represent a rounded average of three to six experiments.

shown). Thus, the way in which a given TCR interacts with the peptide–MHC complex probably governs its requirement for CD4. Perhaps of greater interest are those hybridomas that respond to some but not all peptides in the absence of CD4 (phenotype A), and it is these hybridomas that are the focus of this study. In summary, these CD4 loss variants respond to HEL and the minimal epitope 52-63, but not, for example, to 52-61 or 52-62. In contrast, the CD4$^+$ hybridomas responded to all of these peptides.

COOH-terminal Peptide Residues Differentially Affect TCR Interaction. Two questions arise from the results above. First, could these data be explained on the basis of peptide affinity for H-2A^k or TCR, and second, how tolerant is the TCR to amino acid substitutions at Trp 62 and Trp 63? These questions were addressed using a second panel of HPLC-purified peptides, all of which began at the NH$_2$-terminus at position 48 (Table 2). This residue is the most predominant NH$_2$-terminus present in naturally processed peptides (46,
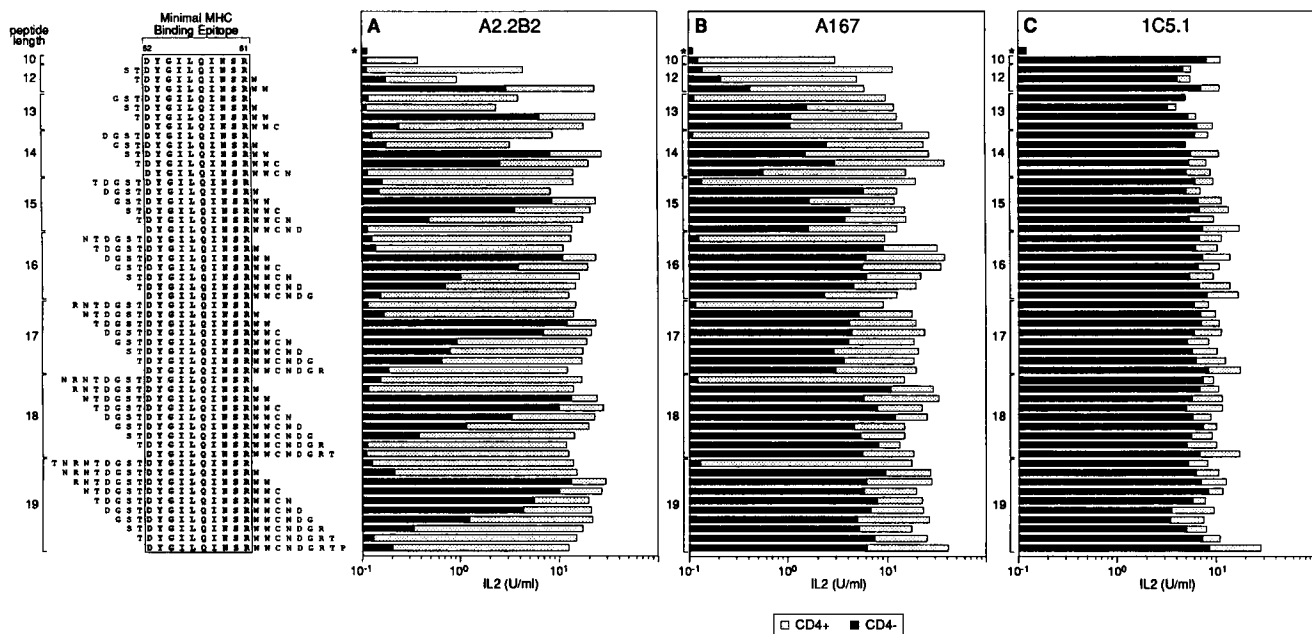

Figure 1. The loss of CD4 has contrasting effects on responsiveness to HEL 52-61 nested peptides. Peptides were synthesized and antigen presentation assays performed as described in Materials and Methods. Sequences of peptides are denoted on the left with the boxed sequence indicating the minimal H-2A^k–binding epitope (53, 54). A representative of each phenotype (*A*, *B*, or *C*) is shown. Other members of each phenotype have similar profiles and are described in Table 1. Peptide concentrations were either 10 μM (*A2.2B2* and *1C5.1*) or 1 μM (*A167*). (∗) DMSO negative control. Data are representative of four experiments. Single letter abbreviations for the amino acid residues are: *A*, Ala; *C*, Cys; *D*, Asp; *F*, Phe; *G*, Gly; *I*, Ile; *L*, Leu; *N*, Asn; *P*, Pro; *Q*, Gln; *R*, Arg; *S*, Ser; *T*, Thr; *W*, Trp; and *Y*, Tyr.

Analog Peptide	Peptide Sequence	K_i against HEL 46-61 biotin (100 nM)
48-61	DGST**DYGILQINSR**	449±89
48-61W	DGST**DYGILQINSR**W	414±130
48-61WW	DGST**DYGILQINSR**WW	550±142
48-61WWC	DGST**DYGILQINSR**WWC	454±53
48-61WWCN	DGST**DYGILQINSR**WWCN	509±48
48-61WWA	DGST**DYGILQINSR**WWA	1026±213
48-61WWAA	DGST**DYGILQINSR**WWAA	967±257
48-61A	DGST**DYGILQINSR**A	478±128
48-61AA	DGST**DYGILQINSR**AA	666±138
48-61WA	DGST**DYGILQINSR**WA	651±55
48-61AW	DGST**DYGILQINSR**AW	651±57
48-61F	DGST**DYGILQINSR**F	671±107
48-61FF	DGST**DYGILQINSR**FF	801±155
48-61WF	DGST**DYGILQINSR**WF	570±134
48-61FW	DGST**DYGILQINSR**FW	511±164
48-61FA	DGST**DYGILQINSR**FA	784±209
48-61AF	DGST**DYGILQINSR**AF	578±153
48-61QQ	DGST**DYGILQINSR**QQ	475±121
48-61-βnA-βnA	DGST**DYGILQINSR**βnAβnA	771±279

Affinity of the HPLC-purified analogs was determined by competition with biotinylated HEL 46-61. K_i values are defined as the concentration of inhibitor required to give 50% inhibition of 46-61 biotin. K_i values of controls were: HEL 46-61, 345 ± 91; RNase 43-56 (lower affinity for H-2Aᵏ), 3,535 ± 795; HEL 1-18, >10⁴; HEL 105-120, >10⁴ (the last two peptides bind to H-2Eᵏ but not to H-2Aᵏ). See legend to Fig. 1 for single amino acid code. Data represent the mean and standard error of three experiments each performed in duplicate. βnA, β-naphthylalanine.

47). One set of peptides consisted of COOH-terminal extensions of the natural sequence from position 61 to 65, with two additional peptides possessing alanine (Ala/A) substitutions at positions 64 and 65. A second set of consisted of Ala, phenylalanine (Phe/F), glutamine (Glu/Q), and β-naphthylalanine (β-nA) substitutions of Trp 62 and Trp 63. These substitutions were chosen in an attempt to delineate the two functional interactions afforded by tryptophan, namely bulky hydrophobicity and hydrogen bonding capacity. A particularly interesting substitution was β-naphthylalanine, which is a large, bulky, planar hydrophobic amino acid like tryptophan, but which lacks the N-H group required for the formation of hydrogen bonds.

The affinity of these peptides for H-2Aᵏ was determined using a competition peptide binding assay (Table 2). Using this method, no significant difference in peptide affinity could be found between the peptides, except for 48-61WWA and 48-61WWAA, which had slightly lower affinities. Most notably, the affinity of 48-61 and 48-61WW (48-63) was identical. Thus, peptide affinity for MHC class II molecules does not explain the differences observed.

In functional assays, the original observation was faithfully reproduced using these highly purified peptides (Fig. 2 A). Whereas the CD4⁺ clones isolated from the three phenotype A hybridomas, A2.2B2, 3A9, and 4G4.1, responded to all the COOH-terminal extended peptides (48-61 to 48-61WWCN), albeit to varying degrees, the CD4 loss variants only responded to COOH-terminal extensions that contained Trp 63. In previous experiments using the nested set of peptides, the response of CD4⁻ hyridomas fell off dramatically with the subsequent COOH-terminal addition of Cys 64 and Asn 65 (Fig. 1). However, using purified peptides and alanine analogs at these residues (48-61WWA and 48-61WWAA), this observation could not be satisfactorily duplicated. Indeed, with all three hybridomas, the response of both the CD4⁺ and CD4⁻ hybridomas declined proportionally. Thus, the previous observations were more likely attributable to differences in quantitation due to poor solubility of the crude peptides.

Each of the three phenotype A CD4 loss variants responded differently to the tryptophan-substituted analog peptides (Fig. 2 B). The 3A9 CD4 loss variant failed to respond to any of the analogs except for a moderate response to 48-61WA and a marginal response to 48-61WF, inferring a strict requirement for both tryptophans, particularly Trp 62. On the other hand, the A2.2B2 CD4 loss variant responded to 48-61WF and 48-61FW but to none of the other analog peptides, suggesting that a single tryptophan in either position plus a hydrophobic amino acid is required for stimulation. In contrast, the 4G4.1 CD4 loss variant was more tolerant to COOH-terminal variation, responding to 6 of the 10 analogs, although with some preference for Trp 63. It is interesting to note that the response by the CD4⁺ 4G4.1 hybridoma to 48-61AW was 100 times more sensitive than its response to 48-61WW. Thus, single amino acid substitutions in flanking regions outside the minimal MHC binding epitope can have a dramatic effect on T cell responsiveness. Furthermore, the clearly disparate recognition patterns of the three CD4 loss variants infers a TCR- rather than MHC-based phenomenon.

Differential Signaling: TCR Ligation in the Absence of IL-2 Secretion

A Role for the Extracellular Domains of CD4. Two intriguing questions are posed by these data. First, does the TCR still bind to class II molecules containing peptides that fail to induce IL-2 secretion by the CD4⁻ hybridomas? Second, is the ability of CD4 to compensate for the defect in IL-2 secretion linked to its intrinsic association with p56ᴸᶜᵏ? To address these questions, a variety of signal transduction events were analyzed in response to 46-61, 46-61WW, and 46-61FF
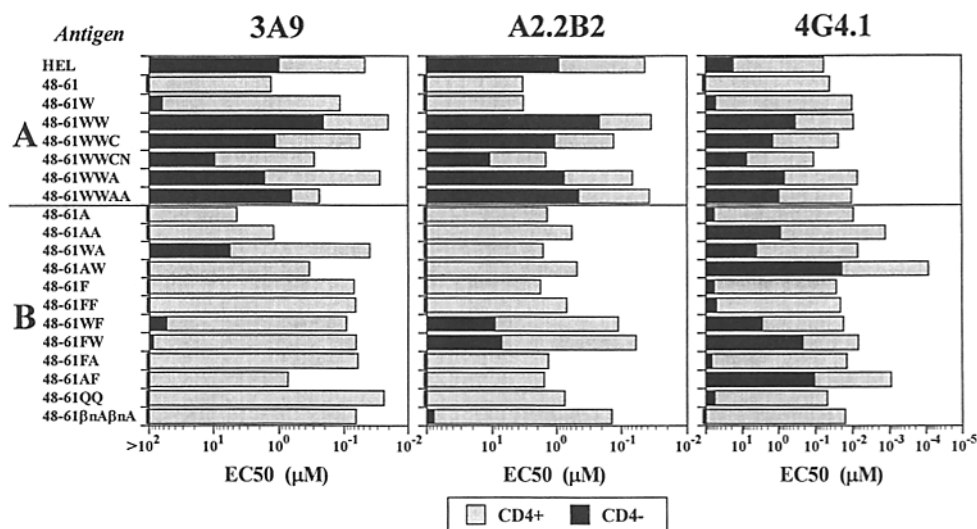
Figure 2. Differential ability of CD4 loss variants to respond to analog peptides. Peptides used in the antigen presentation assay are detailed in Table 2, and were prepared as described in Materials and Methods. (A) Includes COOH-terminal extensions of the natural sequence and two analogs containing alanine substitutions at Cys 64 and Asn65. (B) Includes peptide analogs containing substitutions of Typ 62 and Trp 63. Peptides were titrated from 10^1 to 10^{-4} μM and the data presented as EC_{50}, which is the concentration of peptide that gives 50% stimulation. Data are presented as the mean of two experiments performed in duplicate.

(peptides slightly longer at the NH$_2$-terminus were used in order to improve peptide solubility). The CD4$^+$ 3A9 T cell hybridoma was chosen for further study as it responds comparably to 48-61WW and 48-61FF (within $\frac{1}{2}$log$_{10}$; Fig. 2).

Transfection of the 3A9 CD4 loss variant (CD4$^-$) with either wild-type CD4 (CD4$^+$) or a cytoplasmic-tailless CD4 (CD4.ΔCY) restored its ability to respond to both 46-61 and 46-61FF (Fig. 3 A). Identical results were obtained with transfectants of the A2.2B2 CD4 loss variant (data not shown). Multiple clones of the CD4.ΔCY transfectants were shown to consistently respond to all peptides and failed to coimmunoprecipitate p56^{lck} with CD4 (data not shown).

Ligation of the TCR–CD3 complex initiates an array of second messenger events in addition to the secretion of IL-2. One of the best studied is the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC) to generate the intracellular second messengers, inositol 1,4,5-trisphosphate (IP$_3$), which mobilizes intracellular calcium stores, and 1,2-diacylglycerol, which activates protein kinase C (59). The generation of total IP by the CD4$^+$ and CD4$^-$ hybridomas correlated with IL-2 release, although 46-61 stimulated weaker IP production (Fig. 3 B). Surprisingly, the IP profile produced by the CD4.ΔCY transfectant was more akin to that observed with the CD4 loss variant, despite the induction of strong IL-2 secretion by all peptides. This is consistent with previous findings which demonstrated that IP production is not a prerequisite for IL-2 secretion (60).

Two of the most characterized and predominant proteins that are tyrosine phosphorylated after TCR engagement include the PLCγ1 isoform and the CD3ζ chain (also known as TCRζ) (61, 62). The latter is an integral part of the TCR–CD3 complex and has been shown, in isolation, to be sufficient for T cell activation (63–65). As expected, strong phosphorylation of both proteins was observed when the CD4$^+$ transfectant was stimulated with any peptide, whereas none was observed in the absence of peptide (Fig. 3 C). The two CD3ζ bands observed could represent phosphorylation

at either four or all six of the available tyrosine residues, with the upper band probably the hyperphosphorylated pp22 (66).

The pattern of tyrosine phosphorylation did not correlate with IL-2 secretion in either the CD4 loss variant or the CD4.ΔCY transfectant. With the former, strong phosphorylation of CD3ζ was observed with all peptides even though only 46-61WW induced detectable IL-2 secretion. Only the lower CD3ζ species was induced by all the peptides, whereas the detection of the hyperphosphorylated CD3ζ and PLCγ1 phosphorylation more closely mimicked IL-2 secretion. Thus, it is possible that the two phosphorylated forms of CD3ζ play different roles. Most interestingly, significant CD3ζ phosphorylation in the CD4 loss variant occurred after stimulation with either 46-61 or 46-61FF, providing strong evidence for TCR engagement despite the complete absence of IL-2 secretion.

The CD4.ΔCY transfectant displayed an identical pattern of phosphorylation and IP release as the CD4 loss variant (Fig. 3, B and C), but possessed an IL-2 secretion profile more akin to the CD4.WT transfectant (Fig. 3 A). These data clearly suggest that the cytoplasmic tail of CD4 needs to be present for PLCγ1 phosphorylation to occur when T cells are stimulated with 46-61 or 46-61FF. This is presumably mediated by p56^{lck}, although formal proof of this has not been presented. On the other hand, the role of p56^{lck} after stimulation with 46-61WW cannot be fully determined. Although it is clear that PLCγ1 phosphorylation can occur in the absence of the CD4 cytoplasmic tail, these data cannot rule out the involvement of p56^{lck} as several studies have shown that it does not have to be associated with CD4 to exert its effect (42, 43).

The important implications of these data are twofold. First, strong CD3ζ phosphorylation occurs in the absence of IL-2 secretion. This provides evidence of differential signaling induced by two peptides that are derived from a single epitope and that are known to be processed by APCs (47). Second, the extracellular domains of CD4, and not its cytoplasmic
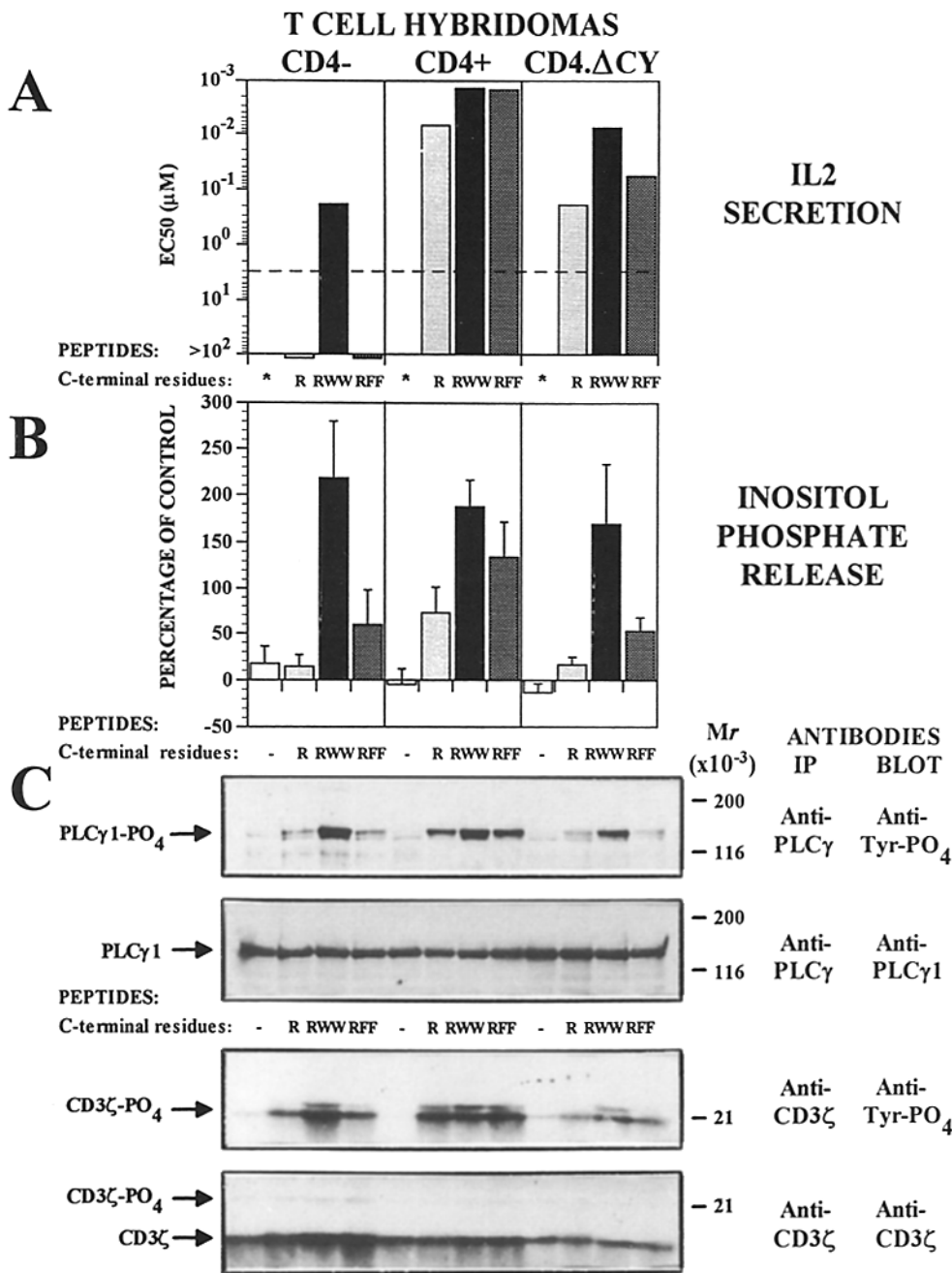
Figure 3. Analysis of signal transduction events in 3A9 transfectants. The ability of 3A9 CD4⁻, CD4⁺, and CD4.ΔCY transfectants to respond to HEL 46-61, 46-61WW, and 46-61FF was analyzed. Identical results were obtained if performed with peptides commencing at the NH₂-terminus with residue 48. (*A*) IL-2 secretion was determined as described in Materials and Methods and data expressed as EC₅₀. Peptides used are denoted as follows: (*) not determined; (−) no peptide added; (R) 46-61 (R is used because Arg 61 is the COOH-terminal residue); (*RWW*) 46-61WW; (*RFF*) 46-61FF. The horizontal dashed line marks the 3-μM peptide concentration used in the experiments shown in *B* and *C*. Any bar substantially above this line represents maximal T cell activation at this concentration. Data are representative of three experiments. (*B*) Total IP release was determined as described in Materials and Methods. Data represent the mean and standard error of two experiments and are expressed as the percentage increase over ³H-inositol–labeled T cells alone. (*C*) The antibodies used for immunoprecipitation and Western blot analysis are indicated on the right. The methodology is described in Materials and Methods. After detection of phosphorylated proteins, the same blots were stripped and redeveloped to detect total protein. The minor band under PLCγ1 is the constitutively phosphorylated PLCγ2, which is coimmunoprecipitated because of the mild crossreactivity of the antisera used. The two CD3ζ bands are likely to represent different levels of tyrosine phosphorylation. The lower CD3ζ phosphorylation seen in the CD4.ΔCY transfectant is probably due to the lower level of total CD3ζ precipitated from these cells. Data are representative of three experiments. (*PO₄*) Suffix used to indicate the tyrosine phosphorylated species; (*Tyr-PO₄*) antiphosphotyrosine mAb.

tail, are responsible for the restoration of IL-2 secretion in response to 46-61 and 46-61FF.

Discordant Dose–Response of CD3ζ Phosphorylation and IL-2 Secretion. Although there appears to be no correlation between CD3ζ phosphorylation and IL-2 secretion in the CD4 loss variant, it is possible that these events occur at much lower peptide concentrations than those required for IL-2 secretion. Thus, 46-61WW might induce stronger CD3ζ phosphorylation at much lower concentrations than 46-61FF. Peptide titration experiments with the 3A9 CD4 transfectant

revealed a good correlation of CD3ζ and PLCγ1 phosphorylation, with IL-2 secretion in response to either 46-61WW or 46-61FF (Fig. 4). However, whereas both peptides induced almost identical CD3ζ phosphorylation profiles in the CD4 loss variants, a complete absence of IL-2 secretion and little PLCγ1 phosphorylation was observed after stimulation with 46-61FF. This experiment clearly defines two separable signaling events after TCR ligation, best illustrated by the discordance in CD3ζ phosphorylation and IL-2 secretion induced by 46-61FF in the CD4 loss variant.
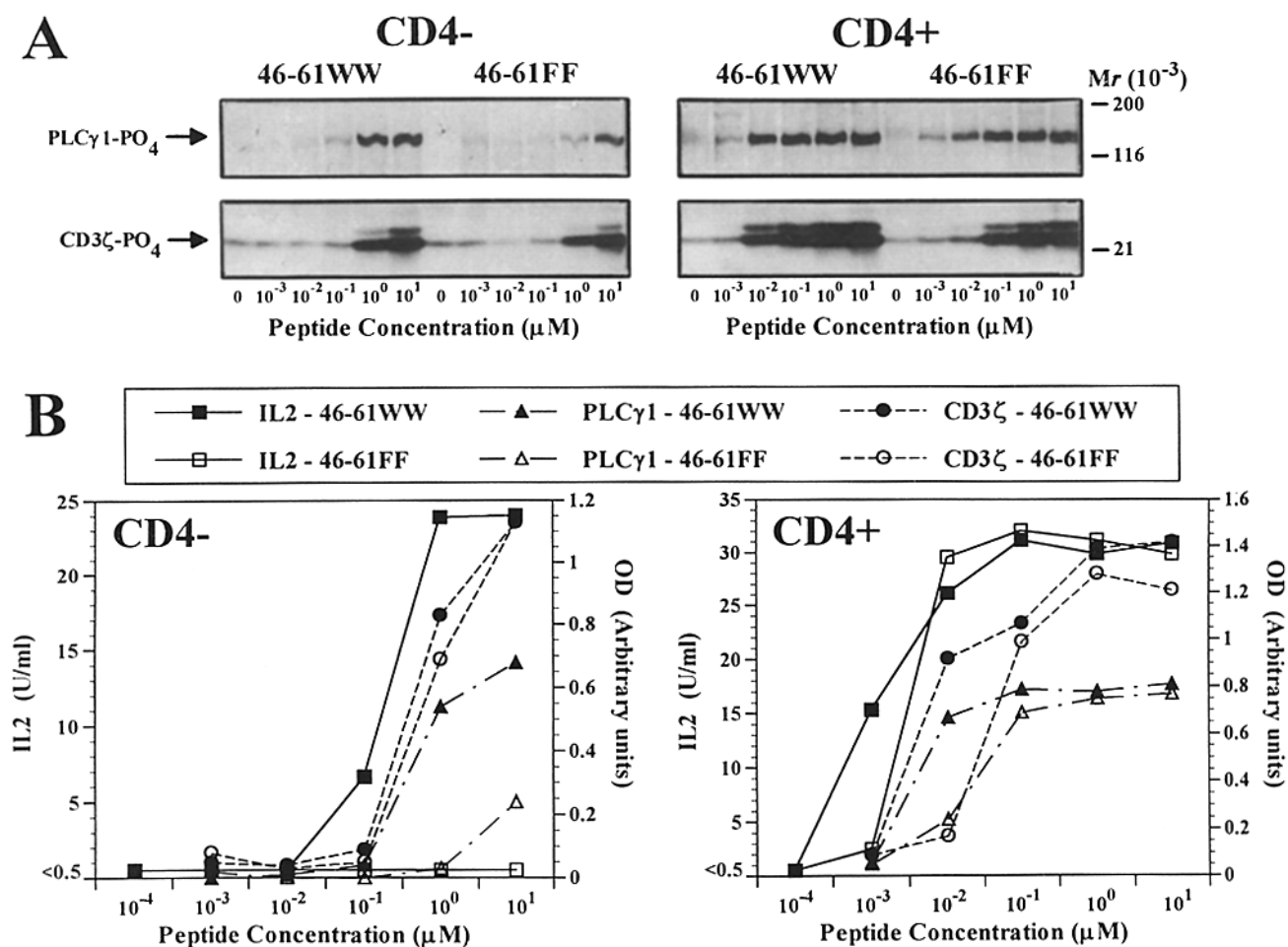
Figure 4. Evidence for TCR ligation in the absence of IL-2 secretion. (*A*) The tyrosine phosphorylation of PLCγ1 and CD3ζ was determined after stimulation of the 3A9 loss variant (CD4⁻) and its CD4⁺ transfectant with LK cells pulsed with the concentrations of 46-61WW and 46-61FF indicated. Data are representative of two experiments. (*B*) Graphs represent an overlay of the data from (*A*), represented as OD, determined using a densitometric scanner (PDI DNA35 scanner, Sparc station IPC & Quantity One V2.0 analysis software; Huntington Station, NY), with IL-2 secretion induced with the same peptide concentrations. It is important to note that the protocol for peptide stimulation used with these two protocols is, of necessity, quite different, and thus only a relative, rather than direct, comparison can be made.

Discussion

Three intriguing questions are raised by the data presented here. First, how is TCR recognition affected by Trp 62 and Trp 63? Second, what effect does this have on TCR function? Third, what is the role of CD4 in this process?

How Is TCR Recognition Affected by Trp 62 and Trp 63? In the absence of CD4, Trp 62 and Trp 63 had a dramatic effect on the responsiveness of four of the six T cell hybridomas tested. Conversely, these differences were minimal in the presence of CD4. Thus, these residues may have a direct effect on the ability of the TCR to appropriately recognize the MHC–peptide complex. Whether this involves an alteration in the conformation of the MHC–peptide complex or direct binding to the TCR remains to be determined.

One caveat to the former possibility is the fact that the Trp residues have no effect on peptide affinity for MHC class II molecules, which one might have predicted if such an interaction were taking place. Whereas some data suggest that peptide residues may affect MHC class I structure (14), it

is not yet known whether the same is true of class II molecules. Perhaps the best evidence in support of this idea is the observation that different peptides affect the mobility of MHC class II molecules in SDS-PAGE because of the generation of either "floppy" or "compact" molecules (67, 68). However, recently it has been shown that HEL 46-61 and 46-63 are both equally efficient at generating compact molecules (69). Another possibility is that the two Trp residues interact across the dimer junction proposed from the MHC class II crystal structure, thereby stabilizing the two dimers (3). However, it seems more likely that these two Trp residues are within the groove of the MHC class II molecule. Furthermore, it has not escaped our attention that the proposed placement of CD4 near the end of the peptide binding groove that contains the COOH-terminus of the peptide may be relevant.

Does the TCR directly bind to the two COOH-terminal Trp residues? Although current theoretical (15, 16) and experimental (17) observations suggest that the peptide is bound by the CDR3 loops located in the center of the TCR, no

data have yet been presented that demonstrate TCR binding to flanking peptide residues. However, the pattern of reactivity of the three hybridoma to the HEL 48-61 peptide analogs was quite distinctive, supporting the idea that these residues directly interact with the TCR. Indeed, recent studies (70) have suggested that the final configuration of TCR–MHC interaction is substantially affected by single amino acid substitutions in the bound peptide. Whichever possibility turns out to be correct, its resolution is likely to be important in our understanding of this interaction.

What Effect Does Trp62/Trp 63 Have on TCR Function? A plausible explanation for these data is based on the affinity requirements for T cell activation (71, 72). In the presence of CD4, the collective avidity of the TCR binding to contact residues in the minimal peptide epitope and CD4 binding to MHC class II molecules, is sufficient for the cell to remain above the threshold required for activation. However, in the absence of CD4, the Trp 62/63 residues become the limiting factor and their presence is a prerequisite for effective T cell activation. Although the data are consistent with this scenario, several observations are less easily explained solely on the basis of affinity requirements, and they justify further investigation. First, one might predict that T cells that possessed the highest affinity TCR would not be dependent on CD4 for recognition of HEL 48-61, whereas the converse would be true for those with low affinity TCR. Surprisingly, the opposite is true as the two most sensitive T cell hybridomas are very sensitive to CD4 loss. Second, although the CD4⁻ 3A9 T cell hybridoma failed to respond to most of the analog peptides, the CD4⁺ counterpart responded strongly to most as several analog peptides possessed EC_{50} values similar to that for 48-61WW (within ½log₁₀: e.g., 48-61FF and 48-61QQ). Whereas the resolution of these anomalies will extend our understanding of these delicate interactions, it will not affect the main conclusions of this study.

Several studies (48–52) have suggested that the TCR complex may possess the capacity for differential signaling. Perhaps only HEL 48-61WW can, in the absence of CD4, induce the "conformational change" required for complete signal transduction. Indeed, the demonstration of strong CD3ζ chain phosphorylation in the CD4 loss variants stimulated with 48-62 despite no IP or IL-2 production, demonstrates that a partial or inappropriate signal is delivered. An analogous situation also seems to occur in anergic Th1 cells which fail to produce IL-2 upon antigen stimulation but which still induce strong CD3ζ phosphorylation (52). Furthermore, these events may also underlie the function of analog peptides that act as TCR antagonists (73).

It might be tempting to conclude from these data that phosphorylation of CD3ζ is not sufficient to induce T cell activation. However, it has been shown clearly that the cytoplasmic domains of the CD3ζ chain alone can induce activation (63–65), and that its absence abrogates T cell function (74, 75). Although the importance of CD3ζ chain phosphorylation has been brought into question (76), these events strongly correlate with T cell activation (74, 77). In total, these findings suggest that the ability of CD3ζ to activate T cells is regulated when it forms part of the TCR–CD3 complex but not

when it is present on its own. Furthermore, they question the speculation that the multiple signal transduction pathways which emanate from the TCR–CD3 complex merely represent redundancy (62). Alternatively, these multiple pathways may provide the TCR with the unique capacity to respond differently depending on the ligand presented. Whatever the mechanism, it is clear that single amino acid substitutions in the flanking regions of core epitopes can have a dramatic effect on TCR recognition in the absence of CD4. This observation alone could have significant consequences for our understanding of TCR recognition and may provide an explanation for why especially long peptides are required to stimulate high affinity T cell responses (78).

What Is the Role of CD4 in this Process? It is interesting to question why the TCR needs CD4 in order to respond to peptides lacking the Trp residues. Although CD4 was initially suggested to play an adhesion role (79, 80), many have argued that this requirement cannot adequately explain its function (39, 81). Indeed, murine CD4 was recently shown to have an affinity for MHC class II molecules even lower than that of the TCR ($K_a \leq 10^4 \text{ M}^{-1}$; 82). However, it is still possible that this low affinity interaction is sufficient to compensate for any increased affinity afforded by the TCR binding directly to Trp 62 and Trp 63. It has also been suggested that signal transduction via CD4 is important for effective T cell activation and selection (39, 41, 83). However, the response of loss variants transfected with CD4 cytoplasmic tailless mutants is comparable with the response of wild-type CD4 transfectants. Taken together, these data raise the possibility that the physical interaction between CD4 and the TCR improves the quality of signaling via the TCR, and that this involves the external CD4 domains. Indeed, both physical and functional interaction of the D3/D4 domains of CD4 and the TCR–CD3 complex has recently been shown (Vignali, D. A. A., R. Mittler, and J. L. Strominger, unpublished data).

Conclusions. The data presented here clearly demonstrate that the amino acids which flank the core MHC class II binding epitope of HEL 52-61 have a profound effect on TCR recognition and that this deficiency can be compensated for by the extracellular domains of CD4, thereby defining a novel role for this molecule. Furthermore, TCR ligation and CD3ζ phosphorylation proceed normally despite a complete lack of IL-2 production, and minimal IP production and PLCγ1 phosphorylation. In fact, the latter can only be induced by CD4 or the appropriate peptide-flanking residues. Although there have been several reports in which single amino acid substitutions resulted in differential signaling through the TCR (49, 50, 73), this is, to our knowledge, the first observation involving peptides known to be naturally processed by APCs (46, 47). Indeed, of the 16 peptides that contain the core sequence 52-61 and that were recently identified from HEL-pulsed, H-2Aᵏ positive B cells, 10 contain Trp 62 and Trp 63 (47).

Several studies have demonstrated that flanking residues are critical for T cell recognition of some, but not all, clones (84–86). However, these observations are probably due to differences in TCR-VαVβ usage, whereas in the present study,

individual TCRs are studied in the presence or absence of CD4. Thus, CD4 may play an important role in reducing the TCRs "intolerance" to peptide NH_2- and COOH-terminal variability.

Considerable differences exist between the structures of CD4 and CD8 (87–89), and the characteristics of MHC class I and II bound peptides. In light of the data presented here, it is tempting to speculate that the present CD4 structure may have evolved to ensure that the TCR could recognize all naturally processed peptides bound by MHC class II molecules.

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