

## The Two Membrane Proximal Domains of CD4 Interact with the T Cell Receptor

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### Summary

During T cell activation, CD4 is intimately involved in colocalizing the T cell receptor (TCR) with its specific peptide ligand bound to class II molecules of the major histocompatibility complex (MHC). Previously, the COOH-terminal residues, Trp62/63, which flank the immunodominant epitope of hen egg lysozyme (HEL 52–61), were shown to have a profound effect on TCR recognition. CD4 maintains the fidelity of this interaction when short peptides are used. To determine which portion of CD4 was responsible for this effect, a series of CD4 mutants were made and transfected into CD4 loss variants of two HEL 52–61-specific T cell hybridomas. Surprisingly, some CD4 mutants that failed to interact with MHC class II molecules (D2 domain mutant) or with p56<sup>lk</sup> (cytoplasmic-tailless mutant) restored responsiveness. Nevertheless, a significant reduction in association between cytoplasmic-tailless CD4 and the TCR, as determined by fluorescence resonance energy transfer, was observed. Thus, neither colocalization of CD4 and the TCR nor signal transduction via CD4 was solely responsible for the functional restoration of these T cell hybridomas by wild-type CD4. However, substitution of the two membrane proximal domains of murine CD4 (D3 and D4) with domains from human CD4 or intercellular adhesion molecule 1 not only abrogated its ability to restore function, but also substantially reduced its ability to associate with the TCR. Furthermore, the mouse/human CD4 chimera had a potent dominant negative effect on T cell function in the presence of equimolar concentrations of wild-type CD4. These data suggest that the D3/D4 domains of CD4 may interact directly or indirectly with the TCR–CD3 complex and influence the signal transduction processes. Given the striking structural differences between CD4 and CD8 in this region, these data define a novel and unique function for CD4.

CD4 and CD8 have been shown to be critical for both T cell development and peripheral activation (1, 2). These coreceptors assist in the localization of the antigen-specific TCR with class II or I molecules of the MHC, respectively, thereby increasing the avidity of this interaction (3–5). Furthermore, both CD4 and CD8 are capable of transducing signals into the T cell by virtue of their association with the nonreceptor tyrosine kinase, p56<sup>lk</sup> (6). Despite these functional similarities, CD4 and CD8 are structurally quite distinct (7). CD4 has four extracellular Ig-like domains in tandem, whereas CD8 contains only one Ig-like domain and a thin, proline-rich, highly O-linked glycosylated stem, and is dimerized. The most divergent portions of these molecules are membrane proximal, and thus in plane with the TCR–CD3 complex. Whether these structural differences represent evolutionary drift or, more likely, a distinguishing function for one of these molecules, has not been established.

Substantial evidence that CD4 and the TCR–CD3 complex interact both physically and functionally now exists (3–5). Although CD8, which also associates with p56<sup>lk</sup>, and a cytoplasmic-tailless CD4 were each found to enhance the function of an MHC class II-restricted T cell hybridoma, maximal stimulation only occurred with the intact CD4 molecule (8). Thus, CD4 and the TCR have to interact with the same MHC molecule, and at least one site of CD4–TCR interaction occurs intracellularly. Fluorescence resonance energy transfer (FRET)<sup>1</sup> has so far provided the best evidence for direct physical association (9–11). While both murine (m) and human (h) CD4 were found to interact with the TCR–CD3 complex, this ability was ab-

<sup>1</sup>Abbreviations used in this paper: FRET, fluorescence resonance energy transfer; h, human; HEL, hen egg lysozyme; ICAM-1, intercellular adhesion molecule 1; m, murine; tTA, tetracycline-controlled transactivator.

rogated in mutants that fail to bind p56<sup>lk</sup>. As this molecule appears to perform important kinase-independent functions during T cell activation, it may act as an adapter between CD4 and the TCR-CD3 complex (12).

The possibility that CD4-TCR interaction has an effect on T cell function was recently assessed in a series of hen egg lysozyme (HEL) 52-61-specific T cell hybridomas (13, 14). While CD4<sup>+</sup> hybridomas responded to any peptide containing this epitope regardless of length or NH<sub>2</sub>- and COOH-terminal composition, CD4<sup>-</sup> variants could only respond to peptides containing two additional COOH-terminal tryptophans at positions 62 and 63. Thus, the fine specificity of MHC-peptide recognition by the TCR was dramatically affected by CD4 and by the COOH-terminal peptide composition. Interestingly, peptides that failed to induce IL-2 secretion in the CD4<sup>-</sup> variants nevertheless induced strong tyrosine phosphorylation of CD3 $\zeta$  (15). Thus, TCR-recognition of certain peptides failed to induce the full array of signals required for IL-2 secretion, and CD4 restored this defect. This partial signaling was also seen in other systems using antagonist peptides (16, 17).

The aim of the present study was to determine which portion and function of CD4 was responsible for restoring T cell reactivity in CD4<sup>-</sup> T cell hybridomas. The distal D1/D2 domains, which bind to MHC class II molecules (18), or the cytoplasmic tail, which transduces signals into the T cell via p56<sup>lk</sup> (6), are possible candidates. However, the proximity of the membrane proximal D3/D4 domains to the TCR-CD3 complex, together with data suggesting that CD4 can interact with this complex, raised the possibility that these domains may be involved.

## Materials and Methods

**Construction of CD4 Mutants.** The mutant CD4 molecules made are detailed in Fig. 1. The mCD4. $\Delta$ CY construct was made by substituting Gln103 (19) for a stop codon (T for C at bp 1312) using site-directed mutagenesis (Promega Corp., Madison, WI). This leads to a cytoplasmic tail of only five amino acids (RCRH), which is required to retain CD4 in the membrane. The mutant mCD4.MM4 is a MHC contact mutant that has had the D2-A strand mutated (residues 101-107: KVTFSRG to GLTTTT; kindly provided by Dan Littman, Howard Hughes Medical Institute [HHMI], New York University, New York) (12). The chimera, mCD4.MM4/ $\Delta$ CY was made by subcloning the 5' terminal half of pSM-L3T4-MM4 into mCD4. $\Delta$ CY.pAlter at the unique SexA1 restriction site.

The remaining constructs involved substituting the mCD4 D3/D4 domains with segments from either hCD4, human intercellular adhesion molecule 1 (hICAM-1), or mCD8 (Fig. 1). A mCD4 template was first constructed using site-directed mutagenesis by inserting restriction enzyme sites at the junction of these domains (mCD4.*mutD3/D4*), and then generating the required donor fragments by recombinant PCR using primers with comparable restriction sites. The strategy for producing these constructs took advantage of the numerous blunt cutting restriction enzymes currently available. All mutations gave rise to blunt fragments between the second and third base pair in a codon to ensure constructs remained in frame (division at this point was chosen as it did not give rise to any unwanted amino acid mutations).

These mutations were made in murine CD4 by site-directed mutagenesis and the resultant construct, mCD4.*mutD3/D4*, was used as a recipient for mutant fragments produced by PCR. The division between the D2 and D3 domains occurred within the conserved Phe186 (bp 660-665; 19), which is the second amino acid after the proposed D2/D3 junction, by insertion of a HpaI site. The division between D4 and TM occurred within Glu371 (bp 1203-1208), which is the penultimate amino acid before the D4/TM junction, by insertion of a SmaI site. Information regarding the junctions between mCD4, hCD4, and hICAM-1 domains was provided by Steven Harrison (personal communication, Harvard University, Cambridge, MA).

The following domains were produced by PCR: hCD4.D3/D4 domains, HpaI site to cleave Phe181 (bp 684-689; 20), second amino acid after D2/D3 junction, and SmaI site to cleave Pro370 (bp 1260-1265), last amino acid before the D4/TM junction (last nine COOH-terminal amino acids in hCD4.D4 domain contains three prolines that may be structurally important, so all three were retained); hICAM1.D3/D4 domains, HpaI site to cleave Ala189 (bp 702-707; 21), last amino acid before D2/D3 junction, and PmlI site to cleave Pro369 (bp 1242-1247) between the D4 and D5 domains; hICAM1.D4/D5 domains, HpaI site to cleave Pro287 (bp 975-980), last amino acid before D3/D4 junction, and PmlI site to cleave Glu453 (bp 1494-1499), last amino acid before the D5/TM junction; hICAM1.D4-CY domains (includes the D4, D5, TM, and CY domains), HpaI site to cleave Pro287 (bp 975-980), last amino acid before D3/D4 junction, and EcoRI site (bp 1686-1691) introduced 12 bp after the stop codon at bp 1674; and mCD8 stem, HpaI site to cleave Ser124 (bp 462-467) (22) and NruI site to cleave Arg154 (bp 552-557). This latter construct also included a substitution of Cys151 for Ser (T544A) as this prevented intracellular retention (Vignal, D., and B. Chang, unpublished results). In producing this construct it was assumed that CD4 and CD8 extend the same distance from the cell surface, thus the CD8 stem is analogous to three CD4 domains. Therefore, two thirds of the stem was used starting from the base of the Ig-like domain of CD8.

All PCR reactions were performed with pfu DNA polymerase which has 3'-5' exonuclease capability, thus substantially reducing base error incorporation (Stratagene Inc., La Jolla, CA; used according to the manufacturer's instructions with the addition of 10% DMSO). PCR products were first blunt end subcloned into pGEM-7Zf(+) for complete bi-directional sequencing (Sequenase; United States Biochemical Corp., Cleveland, OH). After sequence verification, the fragments were excised with the restriction enzymes encoded within the PCR primers and ligated into mCD4.*mutD3/D4* to produce the complete construct. The correct junctions were verified by DNA sequencing. Recombinant PCR and other standard molecular biology techniques were employed.

All constructs were subsequently subcloned into a eukaryotic expression vector containing the human  $\beta$ -actin promoter, SV40 poly A and a neomycin resistance cassette (pH $\beta$ Apr-1neo; 23). Some mutants were subcloned into a new vector, pH $\beta$ Apr-II $puro$ , which differs from pH $\beta$ Apr-1neo in possessing a puromycin resistance cassette and expanded polylinker. Efficient surface expression of the constructs was tested using transient Cos transfection and immunocytoLOGY, before transfection of T cell hybridomas.

**mAbs.** A panel of mAbs was obtained and used to stain the transfecteds for subsequent analysis by flow cytometry. The anti-mCD4 mAbs and the domains they recognize are indicated (see Fig. 2). They are as follows (all rat IgG unless stated): GK1.5 (24), YTS 191.1, and YTA 3.1 (25) were already present in this labora-

tory; YTS 177, H129.19 (26), KT6, and KT9 were kindly provided by Kathryn Wood (Oxford University, Oxford, England); YT4.1 and YT4.2 (27) by Charles Janeway (Yale University School of Medicine, New Haven, CT); RL172.4 (28; IgM) by Johnathan Sprent (Scripps Research Institute, La Jolla, CA); and 2B6 (29; IgM) by Ethan Shevach (National Institutes of Health, Bethesda, MD). hCD4 mAbs were as follows (all mouse IgG) and domain(s) recognized are in square brackets: OKT4 was already present in this laboratory [D4]; Q425 [D3] (30) was kindly provided by Peter Kwong (Columbia University, New York); L120 [D4] (30) by David Buck (Becton Dickinson & Co., San Jose, CA); and MT429 [D3-4] by Peter Rieber (Munich University, Munich, Germany). hICAM-1 mAbs were as follows (all mouse IgG): P3.58-BA-19 [D1], P3.58a [D3-4], P3.58-BA-14 [D4-5], P3.58-BA-23 [D4-5], P3.58-BA-3 [D5], and P3.58-BA-11 [D5] (31, 32) were kindly provided by Judy Johnson (Munich University), and CL203-4 [D2 or 4] (33) by Soldano Ferrone (New York Medical College, Valhalla, NY).

**Murine T Cell Hybridomas and Transfectants.** CD4<sup>+</sup> and CD4<sup>-</sup> variants of 3A9 (P4 and N49, where P denoted CD4<sup>+</sup> and N denotes CD4<sup>-</sup>), and A2.2B2 (P2, N22) were isolated as previously described (13, 34). Both are HEL 52-61 specific and restricted by H-2A<sup>k</sup>. Peptides used were HEL 48-61WW (DGSTDYGILQIN-SRWW) and HEL 48-61FF (DGSTDYGILQINSRFF). BW.D10-TCR(9.3).Null(115.3) (BW.D10) and BW.D10-TCR(9.3).CD4 (8.12).Null(5.3) (BW.D10.mCD4) are BW5147 transfectants expressing the D10 TCR +/− CD45.Null and recognize the conalbumin peptide (HRGAIEWEGIESG) in the context of H-2A<sup>k</sup> (kindly provided by Dave Leitenberg and Kim Bottomly, HHMI, Yale University School of Medicine) (35). 171.3 [herein termed 171] (CD4<sup>-</sup>), 171.mCD4, 171.hCD4, and 171.MM4 recognize HEL 74-88 (NLANIPASALLSSDI) in the context of H-2A<sup>b</sup> (kindly provided by Mark Hill and Dan Littman, HHMI, New York University) (12). Crude peptides 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 (36).

The 3A9 and A2.2B2 CD4<sup>-</sup> hybridomas were transfected with the constructs detailed above, hCD4-pH $\beta$ Apr-1neo, and mCD8-pH $\beta$ Apr-1neo (provided by Jane Parnes, Stanford University, Stanford, CA) (20 mg of PvU1 linearized DNA by electroporation; Gene Pulser, Bio-Rad Laboratories, Hercules, CA) and selected with G418 (GIBCO BRL, Gaithersburg, MD). The BW.D10 and 171 hybridomas were transfected with constructs subcloned into pH $\beta$ Apr-1puro and selected with puromycin. Resistant transfectants were cloned by FACS®. Cells were double labeled with GK1.5 biotin (anti-CD4) followed by streptavidin-phycerythrin (Caltag Laboratories, South San Francisco, CA) and anti-CD3-FITC (GIBCO BRL). Positive cells with equivalent TCR-CD3 and CD4 expression as the parental CD4<sup>+</sup> hybridoma were sorted at one cell per well (via EPICS 750 Series with autoclone attachment, Coulter, Hialeah, FL, or FACS Star Plus®, Becton Dickinson & Co.). After 2 wk, ~24 clones were tested for CD4 and TCR, and selected clones for equivalence of CD2, LFA-1, CD45, CD5, LFA-1, and CD28 expression by flow cytometry (FACScan®, Becton Dickinson & Co.). At least six of these were then tested for their equivalence of sensitivity to immobilized anti-TCR (H57.157).

**Tetracycline-regulated Expression of the m/hCD4 Chimera.** m/hCD4 was expressed under the regulation of a tetR/VP16 fusion protein that acts as a potent tetracycline-controlled transactivator (tTA) when bound to the tetO upstream of a TATA box. To establish the system used in this study, two new plasmids were constructed

from those originally developed by Gossen and Bujard (37). First, a new reporter plasmid, UHD.2neo, was made that contains a neomycin resistance gene and a new polylinker cassette. The starting plasmid was pUHD.10S (kindly provided by Maarten Fornerod and Gerard Grosfeld, St. Jude Children's Research Hospital) which differs from the original vector in containing a 5' SV40 double stop and a different plasmid backbone (38). A neomycin resistance cassette, containing a SV40 early promoter and poly A site, was subcloned from pMAMneo (Clontech, Palo Alto, CA) into a unique NgoMI site in the opposite orientation and 3' of the 7tetO-TATA-SV40 poly A cassette. Finally, a new polylinker was inserted by ligating two 94-bp annealed oligonucleotides between the EcoRI and XbaI sites. This contained the following elements and unique restriction sites: downstream T7 promoter-EcoRV-EcoRI-XbaI-SalI-MscI-upstream SP6 promoter. For this study, the m/hCD4 chimera was subcloned into the EcoRI/SalI sites to create m/hCD4.UHD.2neo.

The second plasmid, UHD-Tet/VP16puro, involved a modification of the original construct containing the tTA driven by a constitutive cytomegalovirus promoter. We and others (39) have found that the majority of transfectants fail to express the tTA, possibly due to the toxic effects of overexpression. To obtain a more sensitive and regulated system, a new plasmid was developed following an idea originally proposed by David Schatz (HHMI, Yale University School of Medicine; 39) in which the tTA regulates its own expression by using the same 7tetO-TATA promoter system that drives expression of the reporter in UHD.2neo. First, an EcoRI/HindIII fragment from pUHD15-1 (37) containing the tTA construct was subcloned into pPGKpurobpA (kindly provided by Ramiro Ramirez-Solis, Baylor College of Medicine, Houston, TX) 5' to a puromycin resistance gene driven by the phosphoglycerate kinase promoter. Then, the tTA/PGK promoter/puromycin cassette was subcloned into the EcoRI/XbaI sites of UHD10S, such that the 7tetO-TATA promoter drives expression of the tTA, and the SV40 poly A site in UHD10S is placed 3' to the puromycin gene. This generates the new plasmid UHD-Tet/VP16puro.

The original wild-type mCD4-positive 3A9 hybridoma (kindly provided by Paul Allen, Washington University, St. Louis, MO) was first transfected by electroporation with the UHD-Tet/VP16puro plasmid. Puromycin-resistant clones were tested for regulated expression of the tTA by PCR. One clone, 3A9.V12 [herein referred to as 3A9.V], was chosen and transfected with m/hCD4.UHD.2neo. Several clones were tested by flow cytometry with Q425, which recognizes the D3 domain of human CD4, and in antigen presentation assays as described below. Transfectants were cultured for 24 h before the experiment with 0.1–1  $\mu$ M tetracycline ( $LD_{50}$ , 0.5 mM) which was found to silence transcription of the m/hCD4 chimera.

**Coimmunoprecipitation of CD4 and p56<sup>ck</sup>.** Approximately  $10^7$  cells were lysed with 500  $\mu$ l lysis buffer (1% NP-40 [Fluka Chemical Corp., Ronkonkoma, NY], 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5% glycerol, and enzyme inhibitors [2 mM Pefablock from Centerchem, Stamford, CT, 25  $\mu$ M aprotinin, and 25  $\mu$ M leupeptin]). Lysates were left at 4°C for 1 h, spun for 15 min in a microcentrifuge and precleared twice with 50  $\mu$ l of a 10% suspension of Pansorbin (Calbiochem-Novabiochem Corp., San Diego, CA). Protein G-Sepharose beads (Pharmacia, Piscataway, NJ) were precoated for 2 h at 4°C with either GK1.5 (anti-mCD4) or 53.6.7 (anti-mCD8). Beads (25  $\mu$ l) were added to lysate and rocked at 4°C, overnight. 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 a 12% SDS-

PAGE gel and transferred onto nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). Blots were blocked with 5% nonfat dry milk in TBS-T (0.2% Tween 20, 10 mM Tris/HCl, pH 8, 150 mM NaCl) at 4°C overnight. p56<sup>lk</sup> was detected with a rabbit polyclonal antisera (from Joseph Bolen, Bristol-Myers Squibb, Princeton, NJ), followed by a 1:40,000 dilution of goat anti-rabbit horseradish peroxidase, and developed using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

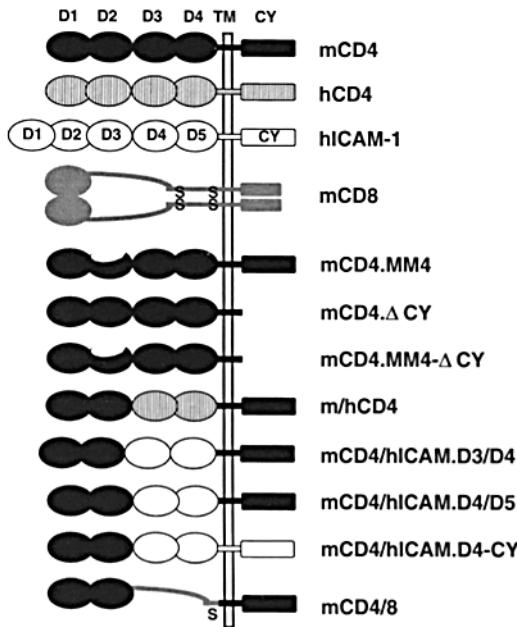
**Antigen Presentation Assays.** Assays were performed essentially as described elsewhere (34, 40). Briefly, T cell variants ( $5 \times 10^4$ ; 100  $\mu$ l) were cultured with  $2.5 \times 10^4$  (100  $\mu$ l) LK35.2 (murine B cell lymphoma; H-2A<sup>k</sup>) in flat-bottom, 96-well microtiter plates with synthetic peptides or HEL (Sigma Chemical Co., St. Louis, MO) at the concentrations indicated. Supernatants (50  $\mu$ l) were removed after 24 h for estimation of IL-2 secretion by culturing with the IL-2-dependent T cell line CTLL-2. Two protocols were used: (a)  $5 \times 10^4$  cells in 50  $\mu$ l were used and proliferation was determined by an MTT assay according to the manufacturer's instructions (Promega Corp.) (see Fig. 4); or (b)  $10^4$  cells in 100  $\mu$ l were used and proliferation was determined by pulsing with [<sup>3</sup>H]thymidine as previously described (34) (see Figs. 5 and 6). IL-2 concentration was determined by using murine recombinant IL-2 (Genzyme Corp., Cambridge, MA) as a standard. EC<sub>50</sub> values were determined by titrating peptides 10-fold from 10  $\mu$ M to 100 pM, calculating the number of IL-2 units for each dilution from a recombinant IL-2 standard, and then evaluating the concentration of peptide required to give 50% stimulation.

**FRET.** Hybridomas ( $10^6$ ) in RPMI 1640 plus 10% FCS were stained with 5  $\mu$ g/ml each of F23.1-FITC (anti-TCR-V $\beta$ 8) and GK1.5-tetramethylrhodamine isothiocyanate on ice for 30 min. Cells were then washed and analyzed as previously described (9).

## Results

**Production and Characterization of Murine CD4 Mutants.** A series of murine CD4 mutants was constructed (Fig. 1). They consisted of CD4 molecules that either lacked a cytoplasmic tail (mCD4. $\Delta$ CY), possessed mutations in the A strand of the D2 domain, which results in an abrogation of MHC class II binding (mCD4.MM4) (12), or a combination of the two mutations (mCD4.MM4- $\Delta$ CY). In addition, the membrane proximal D3/D4 domains of murine CD4 were replaced in five mutants with either the homologous domains from human CD4 (hCD4), domains from the Ig supergene family relative, hICAM-1, or part of the membrane proximal stemlike structure of murine CD8 (mCD8). One of these also possessed the hICAM-1 transmembrane and cytoplasmic segments.

These mutants were transfected into CD4 loss variants derived from 3A9 and A2.2B2, two H-2A<sup>k</sup>-restricted, HEL 52-61-specific T cell hybridomas (13, 15). In some experiments, certain mutants were transfected into two further CD4 loss variants, BW.D10, which recognizes a conalbumin peptide in the context of H-2A<sup>k</sup> (35), and 171.3 [herein termed 171], which recognizes HEL 74-88 in the context of H-2A<sup>b</sup> (12). Stable transfectants were cloned by single cell sorting to derive cells that had comparable CD4 and TCR-CD3 expression as the parental CD4<sup>+</sup> T cell hybridoma. The structural integrity of the mutants was tested in two ways: first by flow cytometry using a panel of mAbs,

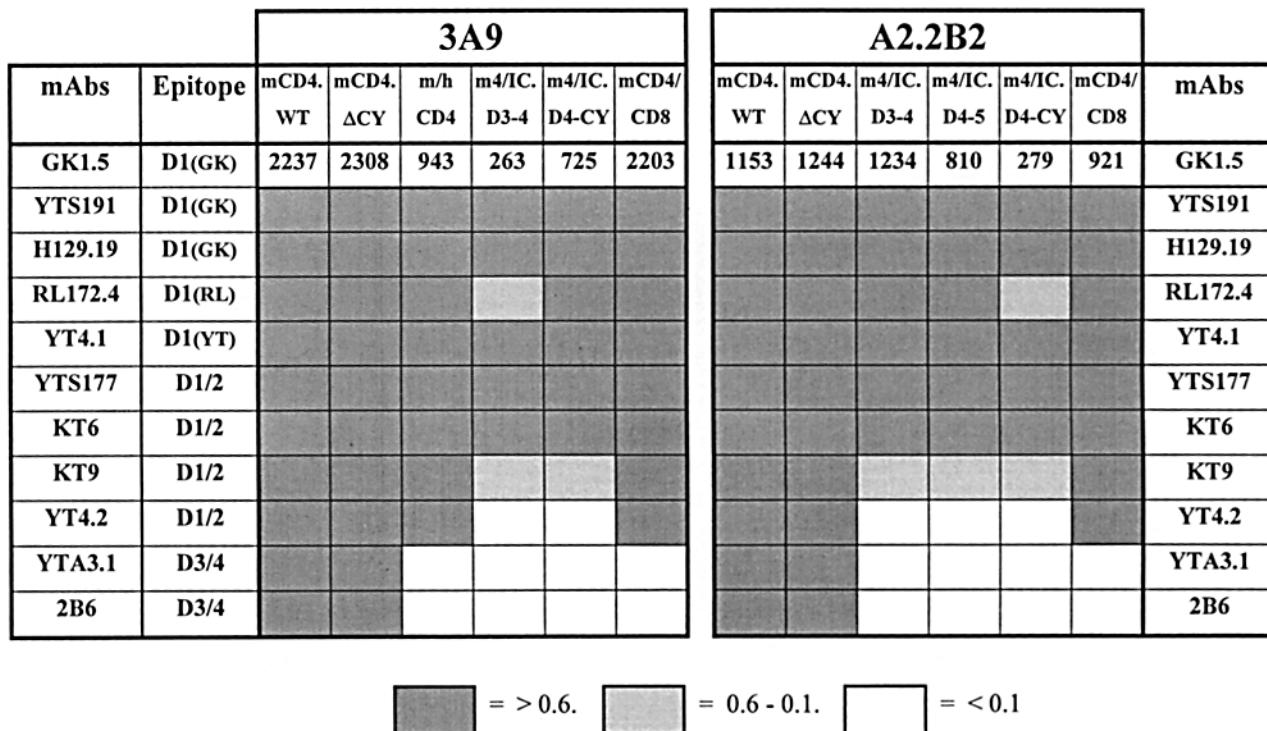


**Figure 1.** Diagrammatic representation of the CD4 mutants used in these studies. (D1–D5) Extracellular domains of CD4 or ICAM-1; (TM) transmembrane domain; (MM4) mCD4 containing a mutation in the D2 domain resulting in failure to bind to MHC class II molecules (12); ( $\Delta$ CY) cytoplasmic domain deleted.

and second by assessing the ability of the mutants to bind the *scc*-family tyrosine kinase, p56<sup>lk</sup>.

Epitope analysis of the CD4 mutants with a panel of anti-CD4 mAbs demonstrated that there were no gross structural perturbations (Fig. 2). Most of the mAbs gave binding patterns that could have been predicted from the domains they recognized (41). Thus, GK1.5 and YT4.1, which recognize distinct epitopes in the D1 domain, recognized all the mutants whereas YTA3.1 and 2B6, which recognize epitopes in the murine D3/D4 domains, only recognized molecules that possess these domains. However, two exceptions were noted: KT9 and YT4.2, antibodies specific for epitopes in the D1/D2 domains, stained all the mutants except those that included domains from hICAM-1. This could be due to either a conformational distortion of these, but not other mAb epitopes, or steric hindrance of these epitopes caused either by the distinct positioning of the ICAM-1 domain loops or additional glycosylation sites (mCD4 D3 has one site, hICAM-1 D3 and D4 have two sites each) (19, 21). The two instances of partial staining with RL172.4 are not thought to be significant as they involved two different constructs and were with transfectants that had the lowest levels of GK1.5 staining. The mCD4.MM4 mutant was recognized by all the anti-mCD4 mAbs except YT4.2 (data not shown), suggesting that this antibody binds to an epitope that encompasses residues on the A strand of the D2 domain.

The T cell transfectants were also tested with mAbs that recognize either hICAM-1 or hCD4 (data not shown). All but one of the seven anti-hICAM-1 mAbs recognized the CD4 mutants as expected. P3.58a, which is reported to



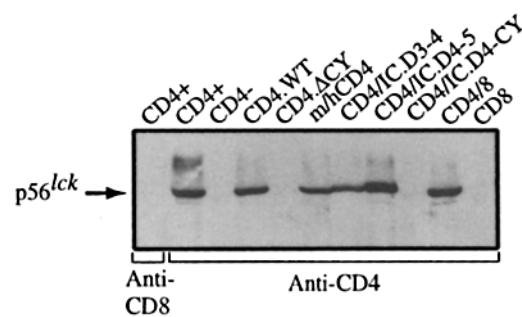
**Figure 2.** Analysis of CD4 mutant molecules by flow cytometry. Epitopes were as deduced in reference 41 and by the data presented here (GK, RL, and YT are separate epitopes in D1 named after the principle antibody). Data are representative of two separate experiments. GK1.5 data are represented as mean log fluorescence. The remaining data were normalized to GK1.5 data of each cell to account for variable cell surface expression and the intensity of each mAb with 3A9.P4/A2.2B2.P2 to 1.00. Data in (solid boxes)  $>0.6$ ; (stippled boxes) 0.2–0.6; and (open boxes)  $<0.1$ . Controls stained with GK1.5 were: 3A9.P4, 2,188; 3A9.N49, 5; A2.2B2.P2, 1,163; A2.2B2.N22, 8; negative control, 4.

recognize an epitope in the D3-4 domains of hICAM-1 (32), failed to recognize hICAM.D3/4. The reason for this is unclear but could be due to a defect in the m4/IC.D3/4 mutant. The binding of all four anti-hCD4 D3/D4 domain mAbs to the m/hCD4 mutant and hCD4 transfectants was identical.

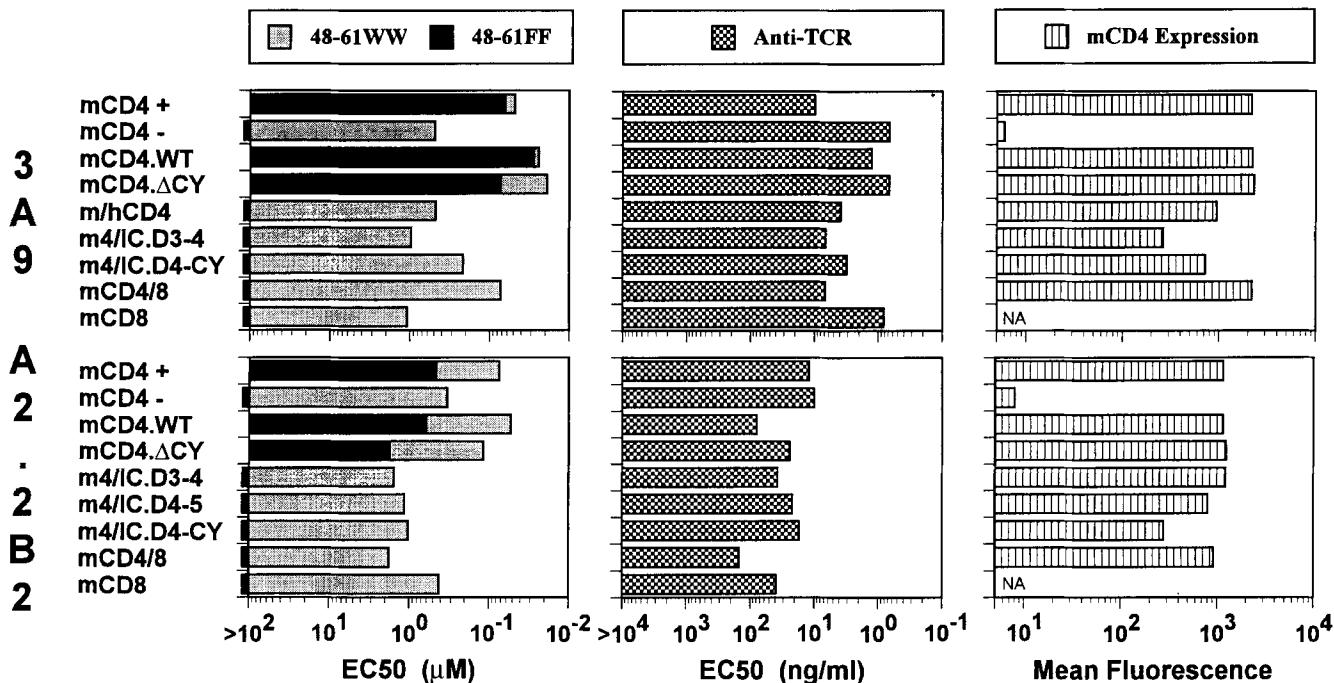
The mutant molecules were also tested for their ability to bind p56<sup>lk</sup>, which intrinsically associates with the cytoplasmic tail of CD4 (6). All the constructs, except as expected mCD4.ΔCY and mCD4/hICAM.D4-CY, bound to p56<sup>lk</sup> with comparable efficiency (Fig. 3).

**The Influence of the D3/D4 Domains of CD4 on TCR Recognition.** The recognition of HEL 52-61-derived peptides that lack the two COOH-terminal adjacent tryptophanes (Trp62/63;WW) was previously shown to be totally dependent on the presence of CD4, whereas the presence of these residues negated the requirement for CD4 (15). Peptides in which these residues were substituted with phenylalanine (Phe62/63;FF) failed to stimulate CD4<sup>+</sup> T cell hybridomas. The effect of the CD4 mutants on T cell function was assessed by comparing the response of 3A9.N49 (CD4<sup>-</sup>) transfectants to HEL 48-61WW and 48-61FF (Figs. 4 and 5). Ligation by immobilized anti-TCR mAbs was used as a control. As expected, all the transfectants responded to the anti-TCR mAb and HEL 48-61WW, albeit with some variation. However, their response to HEL

48-61FF was dramatically different. Despite several reports demonstrating a substantial loss of function in the absence of p56<sup>lk</sup>-CD4 interaction (8, 11, 12, 42), the mCD4.ΔCY transfectants were as responsive to HEL 48-61FF as the wild-type transfectant. Binding of CD4 to MHC class II molecules has also been shown to be critical for the function of some T cell hybridomas (12). However, the response of the mCD4.MM4 transfectant of 3A9 cells to HEL 48-61FF was



**Figure 3.** Ability of CD4 mutants to bind p56<sup>lk</sup>. Anti-CD8 or anti-CD4 immunoprecipitates from the indicated transfectants were probed by Western blot for the coassociation of p56<sup>lk</sup>. (CD4<sup>+</sup> and CD4<sup>-</sup>) Sorted 3A9 variants; (CD4.WT) 3A9 CD4 loss variant transfected with wild-type mCD4; (D3-D5) extracellular domains of ICAM-1; (IC) hICAM1; and (ΔCY) cytoplasmic domain deleted. Data are representative of three experiments.



**Figure 4.** Effect of CD4 mutants on T cell responses to peptide and anti-TCR antibody. Peptides ( $10^2$ – $10^{-4}$   $\mu$ M) and anti-TCR antibody (H57.157;  $10^4$  to  $10^{-2}$  nM) were titrated and the data presented as  $EC_{50}$ , which is the concentration that gives 50% stimulation. Bar charts (right) depict the cell surface expression of mCD4 determined by flow cytometry using GK1.5. (NA) Not applicable. Data are representative of five experiments performed in duplicate. (m4/IC) mCD4/hICAM.

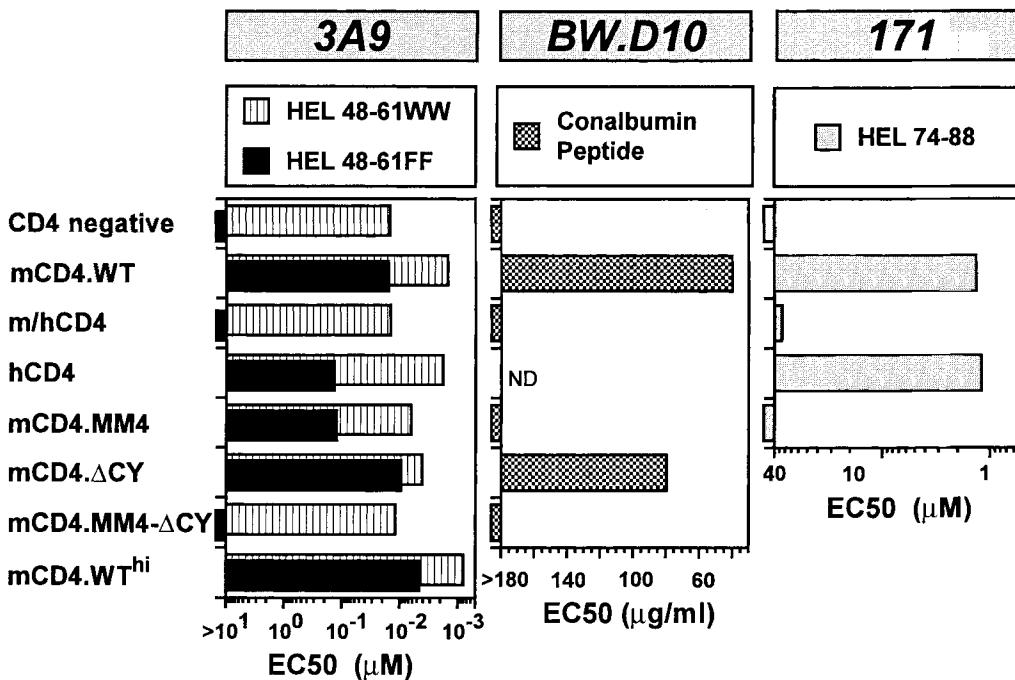
reduced by only one  $\log_{10}$ , despite the complete inability of this mutant to restore any function in the 171 and BW.D10 T cell hybridomas (Fig. 5). Thus, the function of CD4 in the recognition of HEL 48-61FF by 3A9 was distinct from its ability to bind MHC class II molecules or p56<sup>lk</sup>, as neither of these interactions alone was critical. However, if these two mutations were combined (mCD4.MM4-ΔCY), no restoration of function was observed. Thus at least one, but not both, of these sites of interaction was required for the function of CD4 in 3A9 cells. Furthermore, mCD8 failed to restore reactivity to HEL 48-61FF; thus, increasing cell-cell adhesion in this way was insufficient to restore function.

Surprisingly, any mutant in which the D3/D4 domains of mCD4 were replaced with domains from hCD4, mCD8, or hICAM-1 failed to restore responsiveness to HEL 48-61FF, implying a unique role for this region of CD4 (Figs. 4 and 5). Even the homologous substitution of domains between murine and human CD4 abrogated function in all the hybridomas tested. Possibly all CD4-dependent hybridomas require the D3/D4 domains for function, even though their dependence on CD4 interaction with p56<sup>lk</sup> and/or MHC class II molecules may vary.

hCD4 can functionally replace mCD4 both in vitro and in vivo (42–45). In 3A9 transfectants, hCD4 was reproducibly one  $\log_{10}$  less efficient than mCD4 at restoring reactivity to HEL 48-61FF, despite an equivalence of function in 171 transfectants (Fig. 5). This difference has also been observed with another hybridoma, A167 (34). Although hCD4 did not fully replace mCD4 in 3A9, it was far better than

the m/hCD4 chimera. This discrepancy could be explained on the basis of affinity or structural integrity. While hCD4 has an affinity of  $3 \times 10^6$  M<sup>-1</sup> for HLA-DR4 (46), mCD4 has an affinity of  $\leq 10^4$  M<sup>-1</sup> for H-2A/E<sup>d</sup> (47). Furthermore, hCD4 appears to have significantly higher affinity for H-2A<sup>d</sup> than mCD4 (44). Thus, the m/hCD4 chimera may have a lower affinity for H-2A<sup>k</sup> than hCD4. Second, the construction of this mutant may have affected some subtle structural feature and its ability to interact with the TCR-CD3 complex, MHC class II molecules, and/or p56<sup>lk</sup> for signal transduction.

**The m/hCD4 Chimera Can Act as a Potent Dominant Negative.** Neither the interaction of CD4 with MHC class II molecules nor that with p56<sup>lk</sup> was totally essential for its function in 3A9; one or the other appears to suffice for at least partial activity. The failure of the m/hCD4 chimera to function at all was anomalous and it was, therefore, important to demonstrate that the m/hCD4 chimera was structurally intact. Were it able to interact with MHC class II molecules and p56<sup>lk</sup> with an efficiency comparable to wild-type CD4, but unable to interact with the TCR-CD3 complex, it would actively compete with wild-type CD4; 50% inhibition might be seen when the mutant and wild-type CD4 are present in equal amounts. To evaluate this hypothesis, the m/hCD4 chimera was expressed in the wild-type CD4<sup>+</sup> 3A9 T cell hybridoma under the regulation of tTA. Strikingly, the m/hCD4 chimera completely outcompeted wild-type CD4 (Fig. 6 A) even though both were present at comparable levels (Fig. 6 B). In the presence of tetracycline, the expression of the m/hCD4 con-



**Figure 5.** Comparison of mutant CD4 function in three different hybridomas. A series of CD4 mutants were transfected into three different CD4<sup>-</sup> T cell hybridomas specific for the antigens indicated above. (3A9) Restricted by H-2A<sup>k</sup>. (BW.D10) A BW5147 transfectant expressing the D10 TCR and CD45.Null (35) is restricted by H-2A<sup>k</sup> and specific for a conalbumin peptide (HRGAIEWEGIESG). (171) (12) Restricted by H-2A<sup>b</sup> and specific for HEL 74-88 (NLANIPASALLSSDI). For explanation of the CD4 mutants, see legends to Figs. 1 and 4. For the 3A9 transfectants, mCD4.WT serves as the control for m/hCD4 and hCD4 which all express comparable levels of CD4, whereas mCD4.WT<sup>hi</sup> is the control for mCD4.MM4, mCD4.ΔCY, and mCD4.MM4-ΔCY. All transfectants of a given hybridoma responded comparably to immobilized anti-TCR antibody. Data are representative of three to five independent experiments.

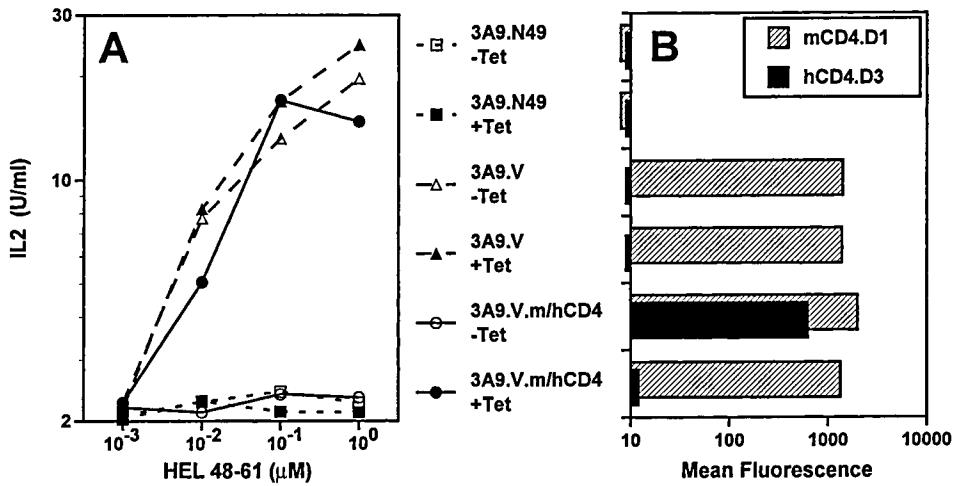
struct was abrogated and the response of 3A9 to HEL 48-61 restored to wild-type levels. These data strongly suggest that the m/hCD4 chimera is fully capable of binding to both MHC class II molecules and p56<sup>kk</sup> in a manner functionally comparable to wild-type CD4. Thus, the m/hCD4 chimera acts as a potent dominant negative in the presence of wild-type CD4.

**Reduced Physical Interaction between the m/hCD4 Chimera and the TCR-CD3 Complex.** Since the D3/D4 domains of CD4 can influence TCR recognition, the possibility that these domains may directly interact with the TCR-CD3 complex was next explored. FRET, in which molecular association is determined by energy transfer from a FITC-labeled antibody bound to the TCR-CD3 complex to a TRITC-bound antibody attached to CD4, was employed (9, 11). As expected, the wild-type CD4 transfectant of 3A9.N49 displayed good levels of energy transfer, whereas essentially insignificant levels were observed with the mCD4/hICAM.D4-CY transfectant (Fig. 7). This construct lacks both the D3/D4 domains and cytoplasmic tail of mCD4. No energy transfer was observed with the mCD4/8 transfectant even though it retains the CD4 cytoplasmic domain. As this mutant has had the two bulky Ig-like domains of CD4 replaced with part of the stemlike structure from CD8, which is normally expressed as a dimer, it is possible that the juxtaposition of the mCD4 D1 domain has been altered so that energy transfer cannot occur or that

dimerization of the CD8 stem, which cannot occur in this mutant, is required for structural integrity. Although the mCD4.ΔCY transfectant showed reduced energy transfer, this level was significantly above background levels (compared with mCD4/hICAM.D4-CY), implying the presence of another interaction site. Similarly, only half the energy transfer observed with the wild-type transfectant was seen in hybridomas expressing m/hCD4. Thus, the interaction of CD4 with the TCR-CD3 complex appears to involve both the D3/D4 domains and the cytoplasmic tail, although only the former is functionally significant.

## Discussion

There is now substantial evidence that CD4 physically interacts with the TCR-CD3 complex (3–5). This colocalization may be required to bring the CD4-associated tyrosine kinase, p56<sup>kk</sup>, into close proximity to targets within the TCR-CD3 complex or associated molecules (6). However, the responsiveness of a CD4-negative T cell hybridoma could also be restored with a CD4-p56<sup>kk</sup> chimera that lacked the kinase domain, suggesting that p56<sup>kk</sup> may have functioned purely as an adapter to cross-link CD4 with the TCR-CD3 complex (12). Similarly, although CD4 was first described as an adhesion molecule, the affinity of its interaction with MHC class II molecules is very low (8, 18, 47). Thus, CD4 is more likely to play a role in the



absence of 1  $\mu$ M tetracycline. All respond comparably to HEL 48-63 and immobilized anti-TCR (data not shown). Phenotype seen with HEL 48-61 is comparable to that seen with HEL 48-61FF (data not shown). (B) Expression of mCD4 plus m/hCD4 (anti-mCD4.D1 - GK1.5), and m/hCD4 only (anti-hCD4.D3 - Q425) is presented as mean fluorescence. Data are representative of several clones tested in three independent experiments.

colocalization of a given TCR with the same MHC molecule, rather than as a "classical" adhesion molecule (3, 5).

Clearly activation of the HEL 48-63-specific T cell hybridoma used here, 3A9, was not dependent solely on interaction of CD4 with either MHC class II molecules or p56<sup>kk</sup>. However, when both sites of interaction were altered, function was completely abrogated. CD4 appears not to be required solely for providing the kinase activity of p56<sup>kk</sup> in 3A9 cells; otherwise, the cytoplasmic tail deletion alone would have abrogated function. Similarly, CD4 is not needed solely for interaction with MHC class II proteins through its D1/D2 domains; otherwise the MM4 mutation in the D2 domain alone would have abrogated function. Additionally, physical and functional experiments presented here using mCD4 D3/D4 mutants suggest that these domains may represent a third functionally relevant

site of interaction for CD4. Whereas some loss of CD4-TCR interaction using the D3/D4 mutants was evident in FRET experiments, it was only partial, indicating that the D3/D4 domains were responsible for only a portion of the interaction. However, substitution mutations of the D3/D4 domains abolished the ability of CD4 to restore T cell function. The functional effects mediated by the D3/D4 domains may require a second site of interaction to aid in the colocalization of CD4 with the TCR. Thus, removal of either the D1/D2 or cytoplasmic domains would have only a marginal effect, but removal of both would abrogate function.

Strikingly, the m/hCD4 mutant acted as a potent dominant negative in the presence of wild-type CD4. Since the levels of CD4 expression can fall 20-fold with only a minimal effect on T cell function, these data are surprising. Two explanations present themselves. First, the m/hCD4 mutant may interact either with the wild-type CD4 molecule directly, or with an oligomeric complex containing CD4, resulting in functional inactivation. Structural and mutagenesis studies on CD4 and MHC class II molecules have provided, at least in part, a possible basis for the dimerization/oligomerization of these molecules with one another (48–51). However, if construction of the m/hCD4 mutant had affected its ability to dimerize with CD4 directly, it would be unable to inactivate the wild-type molecule and would therefore have no effect on function. Alternatively, functional interaction with MHC class II molecules and/or p56<sup>kk</sup> could require participation of all CD4 molecules within the dimer or oligomer. A single m/hCD4 mutant within an oligomer could result in the inactivation of the whole complex. Such a result was recently obtained using a CD4 mutant that cannot bind to MHC class II molecules in the T cell hybridoma 171, which is known to be dependent on this interaction (12, 51). However, it should be noted that the 3A9 hybridoma used in the studies described

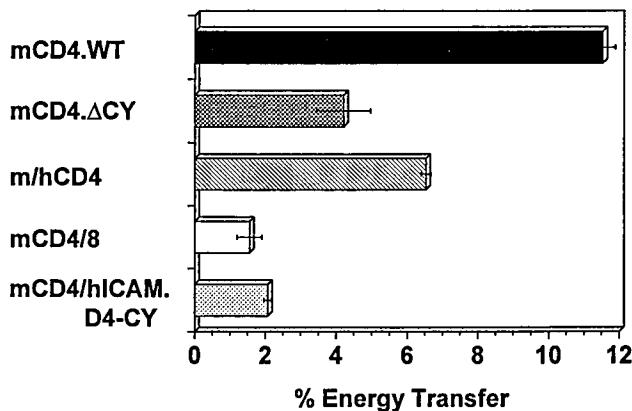


Figure 7. Physical interaction between CD4 mutants and the TCR-CD3 complex as determined by FRET. Data are expressed as the mean of three separate experiments  $\pm$  standard error. GK1.5-FITC (anti-CD4) and F23.1-TRITC (anti-TCR-V $\beta$ 8) (9, 11).

here, does not appear to be totally dependent on CD4 interaction with either MHC class II molecules or p56<sup>lk</sup>.

The second possibility is that MHC-TCR colocalization occurs normally with the chimera, but signal transduction may be affected in a manner that inactivates the complex. A functional parallel could provide a clue to the mechanism by which the dominant negative phenotype is manifest. Substitution of MHC-bound peptide residues that contact the TCR can lead to analog peptides that compete for TCR binding and antagonize the response to the natural ligand (for reviews see references 52, 53). Such altered peptide ligands can block T cell stimulation at concentrations similar to the agonist, and are in essence acting as dominant negatives. A phenotypically similar effect may occur with the m/hCD4 mutant, such that interaction with the TCR-CD3 complex could induce an incomplete or negative signal. Incomplete signals can be manifest by phosphorylation of some proteins but not others (15-17).

What physiological benefit might result from CD4/TCR-CD3 interaction? MHC class I- and II-mediated responses are distinguished in several ways. First, epitopes presented by class I occur as "single" peptides, whereas those bound to class II occur as large nested sets (36, 40, 54). Second, the structures of CD4 and CD8 are completely different, despite the fact that the two molecules perform related functions. It is possible that because MHC class I-restricted TCR only have to recognize a single peptide bound within the MHC class I protein, an extracellular interaction between CD8 and the TCR is not required. However, as MHC class II-restricted TCR recognize a large number of peptides derived from a single epitope and these peptides are not entirely within the MHC protein, CD4 may have evolved to transfer a signal through the extracellular portion of the TCR in order to increase the level of tolerance to peptide variation.

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