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CD4 Dimers Constitute the Functional Component Required for T Cell Activation[1]

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The CD4 molecule plays a key role in the development and activation of helper T cells. Dimerization and oligomerization is often a necessary step in the function of several cell surface receptors. Herein, we provide direct biochemical evidence confirming the presence of CD4 as dimers in transfected cells from hemopoetic and fibroblastic origin as well as in primary T cells. Such dimers are also observed with murine CD4 confirming selective pressure during evolution to maintain such a structure. Using a series of point mutations, we have precisely mapped the dimerization site at residues K318 and Q344 within the fourth extracellular domain of CD4. These residues are highly conserved and their mutation results in interference with dimer formation. More importantly, we demonstrate that dimer formation is essential for the coligand and coreceptor functions of CD4 in T cell activation. These data strongly suggest that CD4 dimerization is necessary for helper T cell function. *The Journal of Immunology,* **2002, 169: 6261–6268.**

The CD4 molecule is composed of an N-terminal extracellular portion, a transmembrane part and a short cytoplasmic tail. This molecule plays an important role in the development and the activation of T cells by increasing the affinity/avidity of TCR for the peptide/class II molecule of MHC II and/or by transducing signals through the associated tyrosine kinase $p56^{lck}$ (1–4). The extracellular part of CD4 consists of four domains (D1–D4) with Ig-like structures and is involved in the interaction with class II molecules of the MHC and other ligands (1, 2, 5).

Previous reports have suggested that CD4 may exist as a dimer. Several of the CD4 ligands have been described as multimers, including MHC II as a dimer of $\alpha\beta$ heterodimers, the chemoattractant factor IL-16 as a homotetramer, and the HIV gp160 as a trimer (6–10). Moreover, the superdimer in the crystal structure of the human class II DR1 isotype can hypothetically interact with two CD4 molecules (11). Additionally, we have reported that both sides of CD4 are involved in CD4-mediated T cell activation, suggesting that one side of CD4 may play a role in MHC II interaction, while the other side may be involved in CD4 self-association (12).

In 1997, the crystal structure of the four extracellular domains of human CD4 has been determined. It shows that CD4 molecules can form dimers through the D4 domain (13). This is clearly different from the conclusion proposed by others who have used peptide analogs to show that CD4 dimerizes through the CDR3 loop of its membrane distal domain D1. Others, using chimeras of CD4 and CD2 have suggested that D3 and D4 could mediate CD4 dimer formation (10, 14–16). Dimers of CD4 have recently been demonstrated on the surface of lymphocytic cell lines, in lysates from primary T cells and from lymphocytic and monocytic cell lines (17). However, these reports have not addressed the functional importance of these dimers nor have they identified the CD4 dimerization site (17).

Herein, we confirm the presence of CD4 dimers on the surface of T cells and we provide direct evidences that residues in the D4 domain, present at the dimer interface as shown in the crystal, are required for dimer formation. More interestingly, we clearly demonstrate that CD4 dimerization is required for CD4-mediated T cell activation.

Materials and Methods

Cells

A2.01 is a human CD4 negative T cell line, generated by limiting dilution of the A3.01 variant of the CEM T cell line (18). MA15 cells were generated by stable transfection of the A2.01 cells with wild-type (WT)[4] CD4 as described below. KR3 is a CD4 negative murine T cell hybridoma whose TCR is specific to the male Ag H-Y (19). 3DT52.5.8 is a murine CD4 negative T cell hybridoma obtained by single-cell cloning of 3DT52.5 hybridoma (20–22). DAP-D^d, DAP-D^dDR4, and DAP-DR1 were generated by stably transfecting DAP-3 cells (12, 23) with the gene encoding the murine MHC class I isotype D^d alone or together with cDNAs encoding the α- and β-chains of the human class II isotypes DR4 or with cDNA encoding the isotype DR1, respectively (12, 24).

Antibodies

L-68 and OKT4 are mouse mAbs specific to human CD4 (12, 23). The rabbit anti-human CD4 polyclonal serum was a gift of Dr. A. Truneh (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). H129 is a rat anti-murine CD4 mAb (25). The rabbit anti-hemagglutinin (HA) polyclonal Ab Y-11 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the mouse anti-glutamic acid-proline (EP) mAb CLP001A

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[4] Abbreviations used in this paper: WT, wild type; IAA, iodoacetamide; NEM, *N*-ethylmaleimide; SEB, staphylococcus enterotoxin B; HA, hemagglutinin; EP, glutamic acid-proline; sCD4, surface CD4.

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from Cedarlane Laboratories (Hornby, Ontario, Canada). KJ12 is an mAb specific to the D^d alloantigen-specific TCR (12). The mouse F23.1 mAb is specific to the mouse TCR bearing Vβ8 and was provided by Dr. A. Veillette (McGill Cancer Center, Montreal, Quebec, Canada; Ref. 26). Abs anti-human Fc, DR, and NK were prepared in our laboratory from the hybridoma IV.3, D1.12, and 129, respectively (American Type Culture Collection, Manassas, VA). The CD8-specific Ab was purchased from Pierce (Rockford, IL); the peroxidase-conjugated anti-rabbit Ab was from Jackson ImmunoResearch Laboratories (Bar Harbor, ME); the anti-mouse Ig was from Pierce; and anti-rat Ig was from Santa Cruz Biotechnology.

Western blot analysis

Cell pellets were lysed in 0.5% digitonin (Sigma-Aldrich, Oakville, Ontario, Canada), 150 mM NaCl, 20 mM triethanolamine, and 1 mM EDTA. Laemmli sample buffer lacking SDS and 2-ME (60 mM Tris (pH 6.8), 20 μg/ml bromophenol blue, and 6% glycerol) was added to the supernatant. Whole-cell lysates were then fractionated in 9% SDS-PAGE gels and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Blots were probed with the CD4-specific polyclonal Ab, followed by peroxidase-conjugated anti-rabbit Ab or with the CD4-specific mAb L-68 followed by peroxidase-conjugated anti-mouse Ab. Blots were then developed using ECL (NEN, Guelph, Ontario, Canada) and autoradiography (Eastman Kodak, Rochester, NY).

Cell metabolic labeling and immunoprecipitation

Forty-eight hours post transfection (27) of the eukaryotic expression vector SVCMV expressing WT CD4 or mutated (C4202A) CD4 and/or WT $p56^{lck}$ (28, 29), COS-7 cells were cultured in methionine- and cysteine-free DMEM media (ICN Pharmaceuticals, Costa Mesa, CA); then, the translabel mix ([^{35}S]methionine and [^{35}S]cysteine 200 μCi; ICN Pharmaceuticals) was added for 5 h. Immunoprecipitations were conducted on cell lysates using OKT4 hybridoma supernatant in the presence of protein A-Sepharose beads, then resuspended in nondenaturing and nonreducing Laemmli buffer and fractionated in 8% SDS-PAGE gels. Dried gels were autoradiographed. Alternatively, total cellular proteins were prepared in 0.5% digitonin and protein inhibitors mixture (2 mM PMSF, 10 μg/ml leupeptin, and 0.1 μM pepstatin). Protein G-Sepharose beads (Amersham Pharmacia Biotech) were incubated with rabbit anti-HA polyclonal Ab Y-11, and immunoprecipitations were then conducted by adding the coupled Y-11 Ab to the cell lysate supernatants. Immunoprecipitates were resuspended in denaturing, but nonreducing Laemmli buffer to prevent the dissociation of the Ig chains H and L, and then resolved in 9% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech) and blotted with the mouse anti-EP mAb CLP001A (10 μg/ml) or the mouse anti-CD4 mAb L-68 (1 μg/ml) followed by anti-mouse peroxidase-conjugated secondary Ab and developed using ECL and autoradiography.

Generation of cells expressing tagged CD4 or mutant CD4 molecules

The CD4-tagged molecules were generated by PCR using a pair of oligonucleotides corresponding to the complementary coding sequences for the HA epitope (TGAATTCACAGGCTTGCGTAGTCTGGTACATCGTAT GGATACCCGGGAATGGGGCTACATGTCTT) or a 6-fold repeat of the EP motif (TGAATTCAAGGTTCAGGTTCTGGTTCAGGTTCTGGTTCT GGCTCCCCGGGAATGGGGCTACATGTCTT). Mutagenesis was confirmed by dideoxy sequencing. The cDNAs encoding the tagged molecules were then cloned into the eucaryotic expression vector SRα neomycin (30) and used to transfect A2.01 cells by electroporation (18). The dimerization mutants were made by overlapping PCR, introduced into the SRα-puromycin vector (30), and transfected into A2.01 and 3DT52.5.8 cells (18, 20–22) as described previously (12). Stable transfectants were generated by adding the selection agent, puromycin (Sigma-Aldrich) or G-418 (Life Technologies, Burlington, Ontario, Canada), at concentrations of 0.8 μg/ml and 1.5 mg/ml, respectively.

Preparation of $CD4^+$ T cells

Peripheral blood PBMCs were isolated from human blood by Ficoll gradient (Amersham Pharmacia Biotech). Peripheral blood collection from healthy human volunteers was obtained following approval of institutional ethical review board. $CD4^+$ T cells were then purified by negative selection using Abs specific to Fc, DR, NK, and CD8 according to the manufacturer's recommendations (Pierce columns). The purity of the $CD4^+$ population was then tested by staining with Simultest CD4/CD8 (BD Biosciences, Mountain View, CA) and flow cytometry analysis. Purity of sorted populations was always >90% $CD4^+$ T cells.

Stimulation of T cells and measurement of IL-2 production

The coligand and coreceptor assays have been described previously (12, 23). After overnight incubation at 37°C, supernatants were then harvested and levels of IL-2 production were assessed using the IL-2-dependent cell line CTLL.2 and the hexosaminidase colorimetric assay as previously described (23).

Determination of EC_{50} ratio

These ratios were calculated as described previously (12). In brief, EC_{50} were determined as the amount of staphylococcus enterotoxin B (SEB) resulting in the half maximal response of stimulated T cells as measured by IL-2 production. The log concentrations of SEB used were plotted on the *x*-axis and the corresponding IL-2 production on the *y*-axis. Curve fitting was then performed using the Sigma Plot program (Jandel Scientific, San Rafael, CA). The EC_{50} ratios were calculated according to the following formula: EC_{50} obtained for cells expressing mutant CD4/EC_{50} obtained for cells expressing WT CD4.

Results

Homodimers of CD4 are present on the cell surface of T cells

We developed a biochemical assay to assess the presence of CD4 dimers in A2.01 CD4-transfected T cells (18). Following gentle cell lysis and sample preparation in the absence of SDS and 2-ME, immunoblots with either CD4-specific polyclonal or mAb (L-68) Abs enabled us to detect two bands with approximate molecular mass (≈) of 55 and 110 kDa, corresponding to the estimated sizes of monomeric and dimeric forms of CD4 (Fig. 1*A* and data not

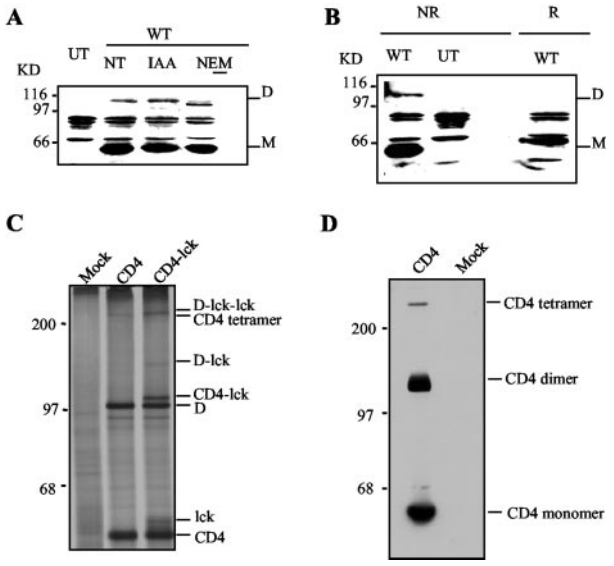


FIGURE 1. CD4 molecules exist as monomers, dimers, and tetramers. Cell pellets were lysed with 0.5% digitonin and analyzed by Western blot using a CD4-specific polyclonal Ab. Lanes are as follows. *A*, Untransfected (UT) or transfected A2.01 cells with WT CD4 (WT) untreated (NT) or treated with the sulfhydryl blocking reagents 10 mM IAA (IAA) or 8 mM NEM (NEM). B, UT or transfected A2.01 cells with WT CD4 (WT) whose total lysates were prepared using Laemmli buffer containing neither SDS nor β-ME (NR) or containing 2% SDS and 1% 2-ME (R). *C*, COS-7 cells were transiently transfected with control plasmid (Mock), SVCMC CD4wt (CD4) or cotransfected with SVCM CD4wt and SVCMV $p56^{lck}$ (CD4-*lck*). Cell labeling and immunoprecipitations with the anti-CD4 mAb OKT4 were conducted as described in *Materials and Methods*. The mobilities (in kilodaltons) of molecular mass markers are indicated to the left of the autoradiogram. D refers to dimer; M refers to monomer. *D*, COS-7 cells were transfected with SVCMV-CD4wt (CD4) or control plasmid (Mock). Forty-eight hours posttransfection, cells were lysed in digitonin buffer and cellular lysate immunoprecipitated with anti-CD4 mAbs. Immunocomplexes were separated by gel electrophoresis using nondenaturing conditions, transferred to nitrocellulose, and analyzed by Western blot using a rabbit polyclonal anti-CD4 Ab.

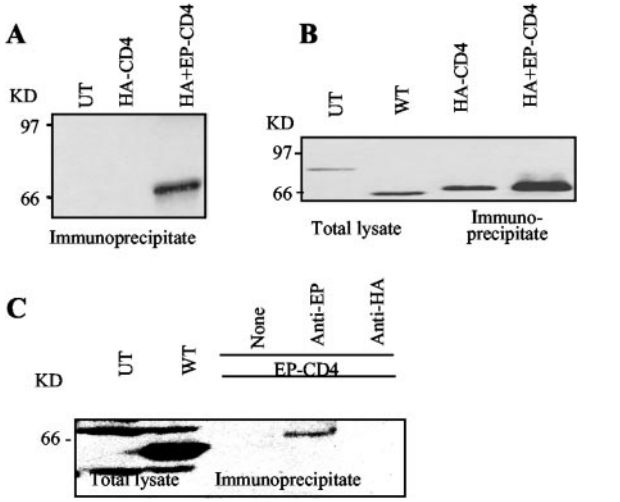


FIGURE 2. CD4 molecules form homodimers. *A*, Immunoprecipitations, with the HA-specific Ab Y-11 followed by Western blot using the anti-EP mAb CLP001A, on lysates from A2.01 untransfected (UT) or transfected with HA-tagged CD4 alone (HA-CD4) or together with EP-tagged CD4 (HA+EP-CD4). *B*, Western blot using the CD4-specific mAb L-68 and total lysates of A2.01 (UT) or MA15 (WT), the anti-HA immunoprecipitate from the lysates of A2.01 transfected with HA-tagged CD4 alone (HA-CD4), or together with EP-tagged CD4 (HA+EP-CD4). *C*, Western blot using anti-CD4 polyclonal Ab and total lysate of A2.01 cells untransfected (UT) or transfected with WT CD4 (WT). Immunoprecipitates obtained from lysates of A2.01 cells transfected with EP-tagged CD4 using protein G-Sepharose beads alone (None), the anti-EP mAb (Anti-EP), or the anti-HA polyclonal Ab (Anti-HA).

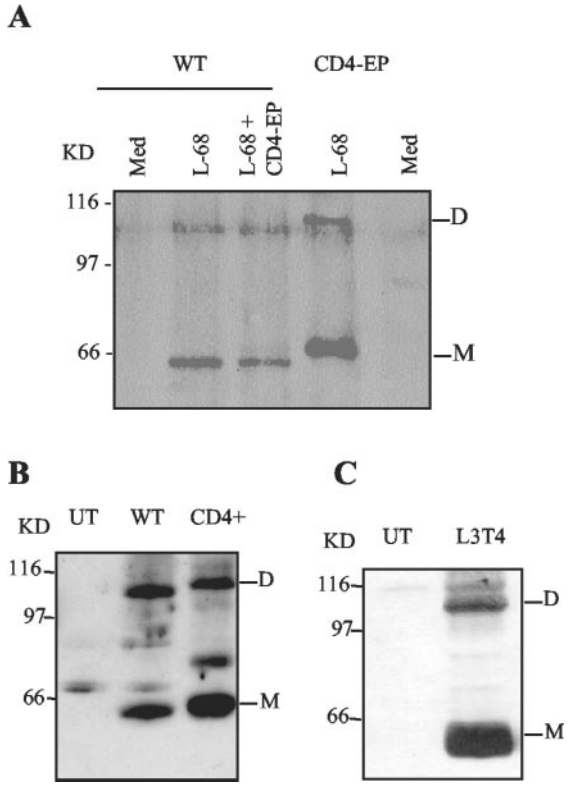


FIGURE 3. CD4 dimers are present on the cell surface of transfected cells, in primary T cells, and are conserved across species. *A*, A2.01 cells transfected with WT CD4 (MA15) (WT) or EP-tagged CD4 (EP-CD4) were treated with either medium (NT) or the CD4 specific mAb L-68 (L-68). After washing out unbound Ab, cells were lysed and immunoprecipitations with protein G-Sepharose beads were performed as described for Fig. 2*B*. In lane L-68/EP, after incubation with the CD4-specific mAb L-68 and washing out unbound Ab, the cells were lysed in the presence of 5-fold excess amounts, as measured by cell equivalence, of lysates prepared from EP-CD4$^+$ cell expressing similar levels of CD4 molecules. Subsequently, immunoprecipitations with protein G-Sepharose beads were performed as described for Fig. 2*B*. The immunoprecipitates were then analyzed by Western blot as described in Fig. 1*A*. *B*, Western blot was conducted, as in Fig. 1*A*, on total proteins prepared from A2.01 cells (UT), MA15 cells (WT), or from primary CD4$^+$ T cells (CD4$^+$). *C*, Cells from the KR3 T cell hybridoma untransfected (UT) or transfected with the murine CD4,L3T4 (L3T4) were lysed and analyzed by Western blot as described above except that we used the L3T4-specific mAb H129 followed by peroxidase-conjugated anti-rat Ab for blot revelation.

shown). The L-68 mAb maps to the CDR1 and CDR3 loops of D1 of CD4 (23); it does not bind the dimerization site and hence should not affect CD4 self-association. As expected, CD4 molecules migrate faster under these conditions than in reducing and denaturing conditions (Fig. 1*B*). A similar pattern was obtained in COS-7 cells transfected with CD4, demonstrating that CD4 oligomer formation occurs in the absence of lymphocyte-specific proteins (Fig. 1, *C* and *D* and data not shown). The OKT4 mAb used in this study to immunoprecipitate CD4 complexes maps to the D3 domain and does not encompass the dimerization site; notably, OKT4 was shown to inhibit homodimerization of soluble recombinant CD4 molecules, but not of membrane-bound CD4 molecules (17). Interestingly, upon cotransfection of CD4 with p56^{lck}, additional bands corresponding to CD4 dimers associated with one *lck* molecule (D-*lck*) or with two *lck* molecules (D-*lck*/*lck*) were observed. These protein bands were not detected when a mutant CD4 that is unable to bind *lck* was cotransfected (Fig. 1, *C* and *D* and data not shown). CD4 dimerization is not due to nonspecific interactions through free cysteines, as demonstrated by the detection of this dimer even in the presence of the alkylating reagents iodoacetamide (IAA) or *N*-ethylmaleimide (NEM) (Fig. 1*A*). The small difference in migration of dimers following treatment with IAA and NEM could be due to differences in their interaction with thiol groups within CD4, which could induce different structural constraints on CD4 dimers. Two additional bands, migrating at ~70 and 85 kDa that appear in some blots are most likely to be unspecific since they are also observed in lysates of untransfected cells (Figs. 3*B*, and 5, *A* and *B*, and data not shown).

To demonstrate that the ≈110-kDa band corresponds to the CD4 homodimer, two constructs involving the C-terminal addition to CD4 of the influenza HA epitope or the EP repeat were generated and stably cotransfected into A2.01 cells (31). Immunoprecipitations with the HA-specific polyclonal Ab Y-11 followed by Western blot with the EP repeat-specific mAb CLP001A revealed a band corresponding to the EP-tagged CD4, indicating the formation of a complex that includes EP and HA-tagged CD4 molecules (Fig. 2*A*). Control Western blots performed using the CD4-specific mAb L-68, detected CD4 in immunoprecipitates from both transfectants (Fig. 2*B*). Furthermore, HA-tagged CD4 molecules transfected into A2.01 cells were not revealed by the CLP001A mAb (Fig. 2*A*). Finally, a signal was observed only in the immunoprecipitate obtained with anti-EP mAb when lysates from cells expressing EP-CD4 were used for immunoprecipitation with protein G Sepharose alone or complexed to either anti-EP or anti-HA Abs followed by Western blot with a CD4-specific polyclonal Ab (Fig. 2*C*). Altogether, these results demonstrate that CD4 can form homodimers and that these dimers migrate at ~110 kDa.

To demonstrate that CD4 dimers are present on the cell surface, intact A2.01 WT CD4$^+$ transfectants were first incubated with the CD4-specific mAb L-68, and then lysed and cell surface immunocomplexes analyzed by Western blot using a rabbit polyclonal

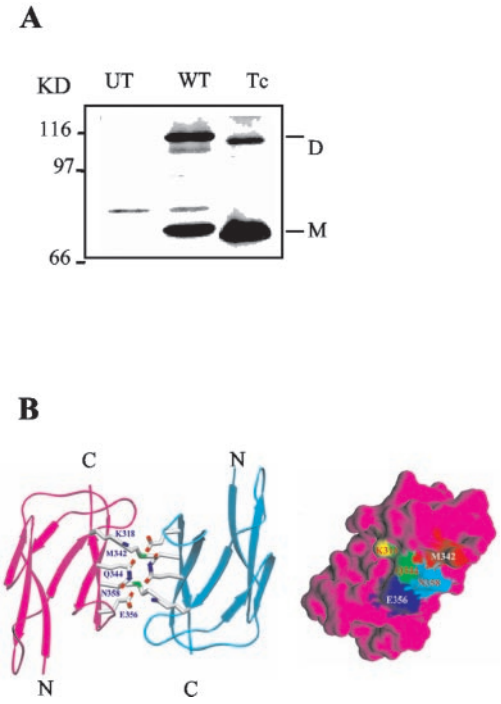


FIGURE 4. Identification of the CD4 dimerization site. *A*, Total cell lysates from untransfected (UT) or transfected A2.01 cells with WT CD4 (WT) or CD4 deleted in its cytoplasmic portion (Tc) (41) were analyzed by Western blot as described in Fig. 1*A*. *B*, *left*, Ribbon diagram of the D4 domain dimer structure, in red and blue, respectively, looking down the 2-fold axis. The side chains of dimer-interface residues are shown. *Right*, Molecular surface of the D4 protomer in red, rotated by 90 degrees along *y*-axis. Dimer-interface residues are labeled.

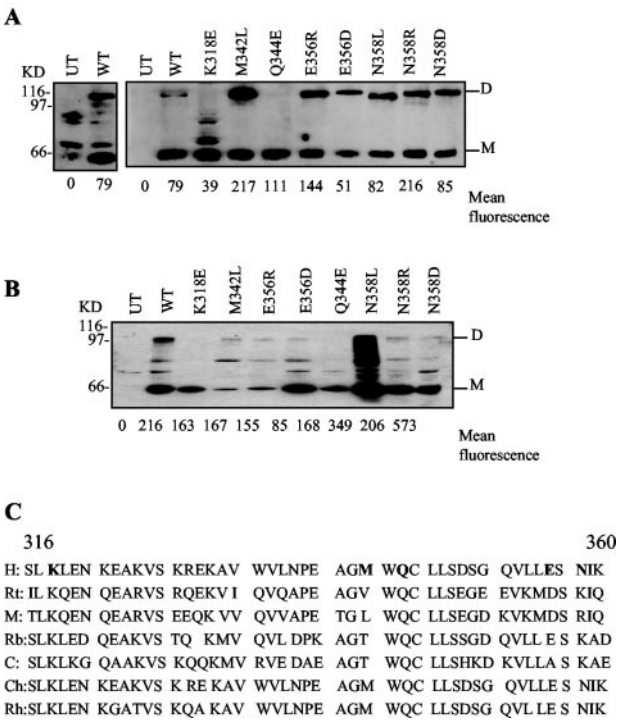


FIGURE 5. Biochemical analysis of the CD4 dimerization site. *A*, Western blot as described in Fig. 1*A* on total proteins from A2.01 cells untransfected (UT) or transfected with either WT CD4 (WT) or different CD4 mutants. Each mutant is designated by the letter symbol of the amino acid followed by the number of its position in the CD4 sequence and the substituted residue. Mean fluorescence values obtained by flow cytometry for each CD4 transfectant are indicated below the corresponding transfectant. *B*, Western blot as described in Fig. 1*A* on total proteins from 3DT52.5.8 cells untransfected (UT) or transfected with either WT CD4 (WT) or different CD4 mutants. Each mutant is designated by the letter symbol of the amino acid followed by the number of its position in the CD4 sequence and the substituted residue. Mean fluorescence values obtained by flow cytometry for each CD4 transfectant are indicated below the corresponding transfectant. *C*, Sequence alignment of primary structure of CD4 molecules originating from different species including human (H), rat (Rt), mouse (M), rabbit (Rb), cat (C), chimpanzee (Ch), and rhesus macaque (Rh). Shown are residues between S316 and K360, and those present at the dimerization site are boldface.

anti-CD4 Ab. Results of a representative experiment ($n = 3$) are illustrated in Fig. 3*A* and clearly show the presence of cell surface CD4 monomers (band at ≈55 kDa) and cell surface CD4 dimers (band at ≈110 kDa) (Fig. 3*A*). We performed the following experiment to ensure that the surface-bound Ab does not bind any intracellular material released postlysis and that all Ab sites are saturated following their binding to the cell surface CD4 molecule. We took advantage of the fact that we had generated EP-tagged CD4 molecules and incubated lysates of cells expressing such molecules with immunoprecipitates of cell surface CD4. A 5-fold excess of lysates from cells expressing EP-CD4 was used in these experiments. The advantage of using such molecules resides in the fact that they can be easily distinguished from WT CD4 by their molecular mass or from binding to Abs specific to the EP-tag (Fig. 2, *B* and *C* and data not shown no. 2). If Ab sites were not saturated by cell surface CD4 molecules, they would then interact with the excess of EP-tagged CD4 molecules. This should result in the presence of bands migrating at molecular mass corresponding to EP-tagged CD4 when immunoprecipitations of WT CD4 are conducted in the presence of excess EP-tagged CD4. Results of Fig. 3*A* clearly show the presence of two bands migrating at ≈55 and 110 kDa only (Fig. 3*A*). They fail to demonstrate the presence of EP-tagged CD4 molecules. These results provide an unambiguous demonstration that the CD4 molecules observed in our experimental conditions correspond to cell surface CD4 (Fig. 3*A*). Specificity of the experiment is provided by the absence of any detectable bands in WT $CD4^+$ or EP-$CD4^+$ cells put in the presence of media alone or CD4-negative cells treated with media or the L-68 mAb (Fig. 3*A* and data not shown).

A similar analysis was performed on purified human $CD4^+$ T cells to confirm that this finding could be extended to physiologically relevant T cell subsets. Results of these experiments show that CD4 dimers are present on primary $CD4^+$ T cells. A small difference in the migration pattern of CD4 molecules was observed in $CD4^+$ T cells from some donors ($n = 3$) when compared with transfected cells (Fig. 3*B* and data not shown). This could reflect differences in CD4 glycosylation.

Strong conservation of amino acid sequences of human and murine CD4 (55%) led to suggest that murine CD4 could also dimerize. Using similar experimental conditions to those detailed in Fig. 1*A*, lysates from the KR3 T cell hybridoma transfected with murine CD4 were analyzed. As shown in Fig. 3*C*, the immunoblot, using the mouse CD4 specific mAb H129, also reveals the presence of the ≈55 and 110 KDa forms of CD4, indicating that murine CD4 also homodimerizes. The fact that this homodimerization is conserved through evolution clearly infers the functional relevance of CD4 dimers (Fig. 3*C*).

Characterization of the CD4 dimerization site

To determine the respective contribution of the extracellular and intracellular domains of CD4 to dimer formation in our assay, we

Table I. *Serological analysis of CD4 dimerization mutants*

	CD4 Mutants[b]								
mAbs[a]	WT	K318E	M342L	Q344E	E356R	E356D	N358L	N358R	N358D
Leu3a	+	+	+	+	+	+	+	+	+
L-68	+	+	+	+	+	+	+	+	+
L-206	+	+	+	+	+	+	+	+	+
B66.6.1	+	+	+	+	+	+	+	+	+
L-93	+	+	+	+	+	+	+	+	+
OKT4B	+	+	+	+	+	+	+	+	+
OKT4A	+	+	+	+	+	+	+	+	+
MT408	+	+	+	+	+	+	+	+	+
OKT4	+	+	+	+	+	+	+	+	+
Q425	+	+	+	+	+	+	+	+	+
OKT4E	+	+	+	+	+	+	+	+	+
OKT4D	+	+	+	+	+	+	+	+	+
MT-427	+	+	+	+	+	+	+	+	+
L-120	+	+	+	+	+	+	+	+	+
L-117	+	+/−	−	−	+/−	+/−	−	+/−	+

[a] Murine T cell hybridoma cell line 3DT52.5.8 expressing human CD4 WT or mutant CD4, was subjected to immunofluorescent with all of the Abs listed here at concentration saturating for the CD4WT. Analyses were carried out with FACScan flow cytometer. Results were calculated as follows: ([mt FL (mAb) - mt FL (neg)]/[mt FL (Leu3a) - mt FL (neg)])/ ([WT FL (mAb) - WT FL (neg)]/[WT FL (Leu3a) - WT FL (neg)]). FL indicates mean fluorescence value; mt, a given CD4 mutant; WT, the WT CD4; neg, the negative control. Symbols For binding are: +, >50%; +/−, 30 to 50%; −, <30%.

[b] Mutants are indicated by the name of the residue (one-letter amino acid code) and its position on the sequence, followed by the name of the substituting residue.

first generated cells expressing CD4 molecules deleted in the cytoplasmic domain and analyzed them as described in Fig. 1*A* (Fig. 4*A*). Results obtained show that truncated forms of CD4 retain the ability to form dimers, although to a lesser extent than the WT CD4 molecules. This finding confirms the data of surface CD4 (sCD4) crystal structure implying a major contribution of the extracellular domains of CD4 to dimer formation and suggesting a minor, stabilizing role of the cytoplasmic domain (13).

To precisely define the dimerization site of CD4, we generated a series of point mutations of the residues present in the predicted interface of the CD4 dimer, as deduced from the crystal structure of CD4 (13) (Fig. 4*B*). As illustrated in Fig. 4*B*, five residues within D4 are located within the CD4 dimer interface. Mutations that lead to conservative substitutions (E356D) or changes in residues charge (K318E, Q344E, E356R, N358R, and N358D) or their hydrophobicity (M342L and N358L) were made and stably transfected into the A2.01 cell line. Populations expressing comparable levels of cell surface CD4 were selected. The ability of these mutants to dimerize was tested as described in Fig. 1*A*. Representative results from a series of reproducible experiments ($n = 3$) in two different cell lines show that mutations involving residues K318 (K318E) and Q344 (Q344E) clearly abrogate the capacity of CD4 to dimerize (Fig. 5, *A* and *B*). However, all the other mutants form dimers demonstrating that residues K318 and Q344 are those critical for dimer formation. We should point out that among the five mutated residues, only K318 and Q344 are conserved among at least seven species (human, mouse, rat, rabbit, cat, chimpanzee, and macaque Rhesus monkeys) (Fig. 5*C*) further confirming the evolutionary pressure to maintain this structure and clearly suggesting its importance (32–35). Results obtained in Fig. 3*C* with murine CD4 do indeed support this hypothesis.

Dimers are the functional moieties of the enhancement by CD4 of T cell activation

We then assessed the importance of CD4 dimers in T cell activation. Biochemical analysis of CD4 mutants transfected into the

Table II. *Characterization of CD4 dimerization mutants*

Mean Fluorescence[a]		Epitopes[b]			Mean Fluorescence[a]		Stimulation (EC_{50} ratio mt/wt)[c]	
Cells	Leu3a	C	A	L	KJ12	F23.1	KJ12	F23.1
$CD4^-$	0				19.8	29.4	0.3	0.8
WT	216.1	15	0	0	11.6	22.7	1	1
K318E	163.3	14	1	0	9.7	22.4	0.3	0.8
M342L	167.7	14	0	1	21.6	37.9	0.3	0.9
Q344E	168.3	14	0	1	13.2	18.1	0.5	0.7
E356R	155	14	1	0	17.7	32.5	0.3	0.8
E356D	85.1	14	1	0	21.6	42.6	0.2	0.9
N358L	349.2	14	0	1	16.1	33.6	0.3	0.8
N358R	206.6	14	1	0	19.3	22.8	0.4	0.5
N358D	573.3	15	0	0	11.2	13.7	0.3	0.5

[a] The surface expression levels of CD4 and TCR were determined by flow cytometry using Leu3a and KJ12 or F23.1, respectively, and are presented as mean fluorescence values.

[b] The structure of the CD4 mutants was analyzed by staining them with 15 CD4 specific mAbs directed against the four extracellular domains (Leu3a, L-68, L-206, B66.6.1, L-93, OKT4B, OKT4A, MT408, OKT4, Q425, OKT4E, OKT4D, MT-427, L-120, and L-117). The number of conserved (C), affected (A), or lost (L) epitopes is indicated.

[c] The stimulations by the anti-TCR mAbs KJ12 and F23.1 are presented here as EC_{50} ratios calculated as described previously (23).

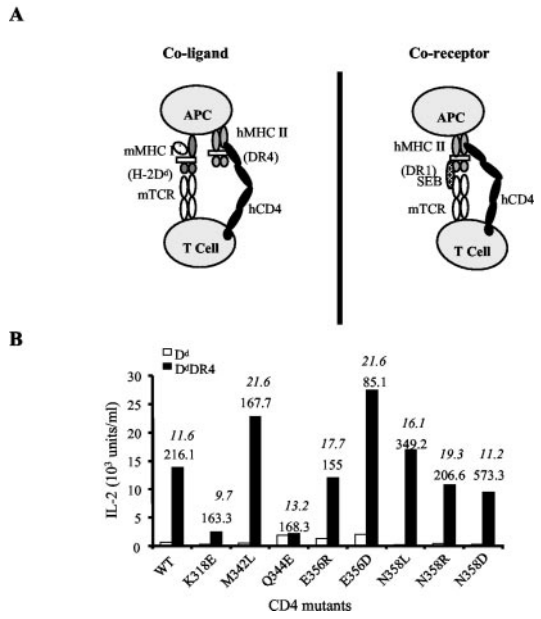
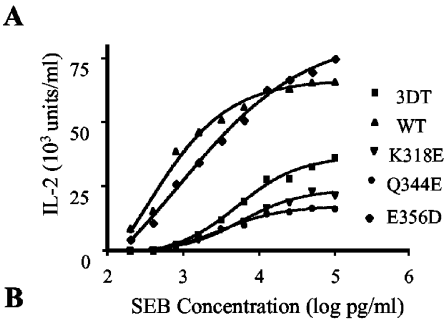


FIGURE 6. Dimer formation is required for CD4 coligand function. *A*, Schematic representation of the two functions of CD4 in the 3DT cell system, coligand vs coreceptor. In the coligand system, CD4 and TCR bind to different classes of MHC molecules. The TCR recognizes the class I cognate alloantigen H-2D^d while the CD4 interacts with the class II MHC molecule HLA-DR4. In the coreceptor system, the TCR and CD4 can both bind to the class II molecule HLA-DR1 in the presence of the bacterial superantigen SEB. In both systems, the interaction of CD4 with the class II molecules enhances the T cell response. *B*, IL-2 production by 3DT52.5.8 cells expressing WT CD4 or different CD4 mutants when cocultured with DAPD^d (□) or DAPD^{d}DR4 cells (■). Mean fluorescence intensity values obtained by staining with the anti-TCR mAb KJ12 (italic) or the CD4 specific mAb Leu3a (Roman) and analysis by flow cytometry are noted on the top of each transfectant column.



Cells	SEB stimulation (EC_{50} ratio mt/wt)	Max IL-2 production (10^3 units/ml)
CD4-	9.3	35.6
WT	1	64.2
K318E	8.6	23.5
Q344E	5.4	17
E356D	1.6	75.6

FIGURE 7. Dimer formation is required for CD4 coreceptor function. *A*, T cell hybridomas were stimulated by increasing concentrations of SEB presented by HLA-DR1 expressed on DAP-3 cells. *B*, Maximal IL-2 level produced by each transfectant in response to SEB stimulation.

CD4$^-$ murine T cell hybridoma 3DT52.5.8 confirms that, as in Fig. 4*B*, only K318E and Q344E show impaired capacity to form dimers (Fig. 5*B*). Flow cytometric analysis indicates that the overall structure of these mutants is well maintained since most epitopes, recognized by a panel of 15 anti-CD4 mAbs specific for residues distributed along the four extracellular domains of CD4, are conserved (Table I). Transfectants were selected to express comparable levels of cell surface CD4 and TCR and to respond with similar efficacy to stimulation with anti-TCR Abs KJ12 and F23.1 (Table II).

In the coligand assay, interaction of WT CD4 with its ligand HLA-DR leads to a significant enhancement of IL-2 production when the TCR recognizes its nominal Ag H-2D^d (21-fold) (Fig. 6*B*) (12, 23). Two independently derived populations of each transfectant were tested ($n = 3$). Representative results are presented in Fig. 6*B*. They show that mutants K318E and Q344E can no longer enhance IL-2 production in the presence of D^d and HLA-DR4. In contrast, cells expressing all other CD4 mutants show similar pattern of reactivity to cells expressing WT CD4 (10- to 40-fold of enhancement in IL-2 production in the presence of HLA-DR4) (Fig. 6*B*). These results indicate that CD4 dimerization is critical for the coligand function of CD4.

In the coreceptor assay, the DAP-DR1 transfectant was used to present SEB to the Vβ8$^+$ 3DT52.5.8 T cell hybridoma TCR (12). The level of IL-2 production in response to SEB stimulation and the SEB concentration required to obtain the half-maximal response (EC_{50}) were determined for each CD4 mutant (12). Cells transfected with mutants K318E or Q344E as well as CD4$^-$ 3DT52.5.8 cells produce significantly lower levels of IL-2 (5-fold), even at the highest concentrations of SEB, when compared with transfectants bearing WT CD4. A control mutant that can still dimerize, E356D, produces comparable levels of IL-2 as the WT CD4 transfectant (Fig. 7, *A* and *B*). These data indicate that CD4 dimerization is also necessary for the coreceptor function of CD4. Altogether, these results clearly demonstrate that CD4 dimers constitute the critical moiety required for T cell activation.

Discussion

Dimerization has been described for a wide variety of transmembrane receptors (36). In this report, we clearly show that monomeric and dimeric forms of CD4 do coexist on the surface of both CD4-transfected and primary T cells. The involvement of CD4 oligomers in MHC class II binding and/or T cell activation has been previously suggested, but not proven, by a panoply of crystallographic studies (13, 37), molecular modeling studies (reviewed in Ref. 10), functional studies (11, 15, 24, 38), and inhibition of IL-2 production using synthetic peptides (39, 40). In addition, CD4 oligomerization has been reported to be critical for an efficient interaction of CD4 with other ligands, such as gp160 or IL-16 (6–10, 41). Moreover, residues within the dimerization site have been shown to be essential for both IL-16 binding and lymphocyte activation (41). Direct evidence for the presence of oligomeric forms of human CD4 in lymphoid and monocytoid cells was provided by Cunningham and colleagues (17). Our finding supports and extends this latter report (17). Using specific point mutations, we identify K318 and Q344, two highly conserved residues within the D4 domain, as critical for CD4 dimer formation, thus confirming the result of the sCD4 crystal structure where those residues are clearly at the interface of the CD4 dimer (13). More importantly, our study demonstrates that such dimers constitute the functional component of CD4 in T cell activation.

Dimerization of CD4 cannot be attributed to nonspecific interactions between free cysteines since treatment with either IAA or NEM does not interfere with the formation of dimers (Fig. 1*A*). The finding that the mutation of a single residue can abrogate CD4 dimer formation suggests that self-association of CD4 is of low affinity and could explain difficulties encountered in detecting CD4

dimers biochemically when classical analytical methods are used. Q344 is highly conserved among species and is present at the center of the CD4 dimer interface. This residue forms an H-bond with the symmetrically related Q344. K318 is also very conserved during evolution and may form a salt bridge with E356 (32–35). CD4 dimerization could not be observed in the cocrystal of CD4 with MHC class II, since the CD4 component included only the two N-terminal domains of CD4. Arguing against CD4 dimerization, this recent study has reported that alanine substitutions of residues K318 and Q344 are without effect on CD4-MHC II binding, measured by rosette formation between CD4-transfected COS-7 cells and MHC II-expressing Raji cells (42). There are several possible explanations for these findings: 1) overexpression of CD4 molecules in COS-7 cells might facilitate cell-cell adhesion and thus bypass the need for CD4 dimerization in rosette formation; and 2) CD4 dimerization might be required only for T cell activation, when the TCR recognizes its nominal Ag on the MHC. The latter hypothesis is supported by previous studies that showed that CD4 accumulation at the immunological synapse is dependent on TCR binding and/or signaling (Ref. 43, and M.-C. Moldovan, unpublished observations).

The unexpected result obtained with the E356R mutation, which does not have any effect on dimer formation, can be explained by the presence of E320 in its vicinity, which leaves the possibility for the formation of a salt bridge between residues R356 and E320. Dimers involving the mutation of residue N358 lead to a slightly different migration pattern when compared with WT CD4 (Fig. 5, *A* and *B*). Indeed, the N358L substitution results in a faster migration rate of this dimer, while the N358D substitution has the opposite effect. As for mutant N358R, it migrates at the same rate as WT. Interestingly, basic residues are found at this position in all other species (Fig. 5*C*). These differences in migration patterns could be due to the effect of mutations on compactness of dimers. Thus, the substitution of N358 for the hydrophobic amino acid leucine would promote its interaction with residues in its vicinity, thereby increasing the compactness of the resulting dimers and allowing their faster migration. With the knowledge that the CD4-associated tyrosine kinase $p56^{lck}$ can dimerize, our results also demonstrate that CD4 homodimerizes mainly through its extracellular domains, especially the D4 domain, and that CD4 dimers are not a result of the $p56^{lck}$ dimerization (44). This idea is confirmed by our observation that in T cells CD4 truncated in its cytoplasmic portion conserves its ability to dimerize (Fig. 4*A*). Moreover, in the COS-7 cell reconstitution system, we show that CD4 dimers can be observed in the absence of $p56^{lck}$ and that a mutant CD4 that fails to associate with $p56^{lck}$ retains its dimerization ability (Fig. 1*C*) (45). Similar findings have been previously reported by Lynch et al. (17) using peptides encompassing sequences within the cytoplasmic portion of CD4. Although believing that the extracellular D4 domain is the key player in CD4 dimerization, we cannot rule out a stabilizing effect provided by the associated $p56^{lck}$ kinase (Fig. 4*A*). According to the crystal structure of sCD4, it has been suggested that $p56^{lck}$ *trans* autophosphorylation could ensue the dimerization of CD4 (13). The fact that CD4 dimers can associate with two $p56^{lck}$ molecules further confirms the possibility that CD4 dimerization could lead to $p56^{lck}$ *trans* phosphorylation (Fig. 4*A*).

We also show that dimerization is required for CD4 functions as a coligand of the TCR where CD4 and TCR interact with distinct MHC II ligands on APCs and as a coreceptor where the same MHC II molecule is recognized by both TCR and CD4. There are many examples in the literature reporting that dimerization is required for the function of receptors, including receptors for growth factors, cytokines, and TCR (46–48). Indeed, prior crystallographic, biochemical, and functional data have indicated the necessity of coreceptor dimerization for an efficient TCR/Ag/MHC interaction and subsequent full T cell activation. In fact, the CD8 coreceptor molecule is expressed on the T cell surface as either $\alpha\alpha$ homodimers or $\alpha\beta$ heterodimers (49). More importantly, it has been reported that the affinity of the TCR for its specific ligand is enhanced through a decreased "off" rate in the presence of either CD8 $\alpha\alpha$ homodimers or $\alpha\beta$ heterodimers (50). Thus, the CD8 dimers may modulate TCR/Ag/MHC interactions (51).

The CD4 coreceptor molecule crystallizes as a dimer (13) and its functional significance could be explained by earlier observations reporting that the interaction between CD4 and MHC II is observed only when CD4 molecules are oligomeric (immobilized on matrix), but not monomeric (in solution) (38, 52). In that context, it is important to note that dimers of MHC II constitute the minimal oligomer size necessary for T cell activation (53).

Recently, video microscopic analyses of interactions between T cells and APCs indicate that formation of a stable central cluster within the immunological synapse is a critical event for T cell activation (43, 54). In that context, it has been shown that CD4 is important for the triggering of synapse formation between T cells and APCs, while in the later stages CD4 is excluded from the central core of the synapse. Interestingly, the recruitment of CD4 at the synapse seems to depend solely on its extracellular part (43). Although the CD4 molecule does not appear to be involved in the initial recognition by the TCR of specific peptide-MHC complexes, its association at the synapse is essential in allowing activation of Ag-specific T cells, probably by augmenting the T cell contact with the APC and by facilitating receptor cross-linking at the contact junction. As a matter of fact, a recent study has demonstrated that CD4 allows efficient recruitment of $p56^{lck}$ and the transient autophosphorylation of lck at the synapse (55). In light of these experiments, it can be inferred that in our system, either monomeric or dimeric forms of CD4 are being recruited to the engaged TCR/Ag/MHC complexes. It can be surmised that because of their different avidities, dimers only would persist much longer in their interaction with MHC molecules as compared with monomers and would allow the formation of a stable immunological synapse and the induction of a complete stimulatory signal, resulting in IL-2 production. Indeed, it has been recently shown that monomeric CD4 possesses poor peptide-MHC binding (K_d = 200 μM) with no detectable affinity for the TCR, leading to the hypothesis that the weak monomeric CD4-MHC affinity could be overcome by augmenting the avidity of the interaction through CD4 dimerization/oligomerization (56). The latter hypothesis could explain our findings that only cells bearing CD4 molecules that can form dimers produce IL-2 upon antigenic stimulation. Also, lack of detection of the dimeric form correlates with the loss of physiological activity of the CD4 molecule (Figs. 5*B*, 6*B*, and 7, *A* and *B*). We show in this study that there is a basal level of CD4 dimers in the absence of any ligand. Upon the transition of the TCR/Ag/MHC II/CD4 to the center of the synapse, more CD4 molecules may dimerize. This would lead to efficient initial TCR signaling allowing for the formation of the mature immunological synapse and the subsequent full T cell activation and effector functions (57, 58). Altogether, these data suggest that dimerization of CD4 is a critical event in T cell activation.

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