CD4-mediated Enhancement or Inhibition of T Cell Activation Does Not Require the CD4:p56^{lck} Association

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Summary

CD4 is the coreceptor molecule expressed on the surface of T cells specific for or restricted by class II molecules of the major histocompatibility complex (MHC). Its expression on T cells is required for an optimal response to antigen (Ag). Three mechanisms have been invoked for the involvement of CD4 in T cell activation. First, it was shown that CD4 binds to MHC class II molecules on antigen presenting cells (APCs) thereby favoring an adhesion between effector cells and APCs. Association of CD4 to the T cell receptor and to the tyrosine kinase p56^{lck} have also been shown to be critically involved in the positive function of CD4. Here, we demonstrate that the interaction of CD4 with p56^{lck} is not required to enhance the response of two CD4-dependent, Ag-specific T cell hybridomas. Mutant forms of CD4 (TCD4), which lose association to p56^{lck}, were expressed in these T cells and were shown to enhance the Ag-specific response as efficiently as the wild-type CD4. Moreover both CD4-dependent and independent T cell responses were inhibited by CD4-specific mAbs even when CD4 was not associated with p56^{lck}. These results indicate that mechanisms distinct from sequestration of p56^{lck} and/or negative signaling operate in these inhibitions. Results demonstrating enhancement of TCR-mediated signaling by the coaggregation of TCD4 mutant to the TCR further confirm that the association of p56^{lck} to CD4 is not absolutely required for the regulatory functions of CD4. Our results suggest that the mechanisms implicated in the enhancement of T cell stimulation via CD4 depend solely on the extracellular and transmembrane domains of CD4.

The CD4 molecule is a cell surface glycoprotein of 55,000 mol wt, expressed on the T cell subset that recognizes its Ag in the context of the MHC class II molecules (reviewed in 1). It belongs to the Ig superfamily and possesses four Ig-like domains, a transmembrane region, and a cytoplasmic tail (2) that allows association with the tyrosine kinase p56^{lck} (3). This association involves cysteines 420 and 422 of CD4 and cysteines 20 and 23 of p56^{lck} that may contribute to the formation of a metal ion coordination complex (4, 5).

The requirement for CD4 in thymocyte maturation and in Ag-specific responses of mature T cells is well established (1). CD4 has been shown to have an adhesion function mediated through its interaction with MHC class II molecules (6), thereby increasing the stability of the TCR/Ag/MHC complex (7). The amino acids involved in this interaction have been mapped to the first and second domain of the CD4 molecule (8, 9). Although mouse CD4 (L3T4) and human CD4 only show 55% homology in their extracellular domain, human CD4 has been demonstrated to fully replace its murine counterpart in Ag-specific stimulation (10) and

in thymocyte differentiation (11). Conversely, residues on MHC class II molecules that interact with CD4 are highly conserved throughout evolution and are located in the nonpolymorphic region of the β2 domain (12).

CD4 can also affect TCR-mediated stimuli; aggregation of CD4 with the TCR by CD4 and TCR-specific mAbs leads to the enhancement of TCR-mediated stimulation whereas cross-linking of CD4 molecules with specific mAbs before activation through the TCR results in the loss of T cell responses (13, 14). The association of CD4 with the tyrosine kinase p56^{lck} was demonstrated, providing a mechanism whereby CD4 could now play in addition to its adhesion function a receptor role by transducing a signal through this kinase. Indeed the tyrosine kinase activity of p56^{lck} is enhanced upon cross-linking of CD4 with specific mAbs; furthermore, one of the phosphorylated substrates is the ζ subunit of the TCR complex (15). On the other hand, sequestration of p56^{lck} from the TCR complex when CD4 is not recruited to the vicinity of the TCR has been shown to inhibit anti-TCR–mediated T cell activation (16).

Several reports have indicated that CD4 can interact with other cell surface molecules on T cells, i.e., the CD3/TCR complex (17) CD45 (18); and suggested that these interactions are critical for T cell activation. The role of CD4 in T cell activation can thus be mediated by binding to MHC class II molecules, colocalization of CD4 with TCR or other cell surface molecules, and activation of p56^{lck}. These events would lead to optimal T cell stimulation.

In this report, we have used two different T cell hybridomas in order to determine the importance of CD4 interaction with its multiple ligands; we demonstrate, using two Ag-specific systems, that the enhancement by CD4 of an Ag-specific response or other TCR-mediated stimuli occurs even if CD4 is not associated with p56^{lck}. This function is totally abrogated by CD4-specific mAbs even when a mutated form of CD4, not associated with p56^{lck}, is used. These results reduce the importance of the CD4:p56^{lck} association in CD4-mediated enhancement of T cell activation. Moreover, they emphasize the role of the interactions of the extracellular domain of CD4 with its different ligands.

Materials and Methods

Cells. The male (H-Y) Ag-specific T cell hybridoma KR3 and the beef and pork insulin-specific T cell hybridoma BI-83/141 have been already described (19, 20). They were maintained in RPMI 1640 medium, supplemented with 10% heat inactivated FCS, 5×10^{-5} M β-mercaptoethanol, 1 mM sodium pyruvate, 10 mM Hepes, and 2×10^{-5} M gentamicine (all purchased from GIBCO BRL, Burlington, Ontario, Canada).

The FT5 7H2 L cells transfected with the murine MHC class II I-A $\alpha^b\beta^k$ molecules (gift from N. Braunstein, Columbia University, New York) were used as APCs for the beef insulin–specific (BI)[1] hybridoma and were maintained in DMEM 5% FCS (GIBCO BRL). APCs for the KR3 hybridoma are splenocytes from male or female C57Bl/6 or DBA/2 mice (Charles River Canada, St-Constant, Québec, Canada). The IL-2–dependent cell line CTLL.2 was maintained in complete RPMI with 10 IU/ml of recombinant human IL-2 (Cetus Corp., Emoryville, CA). DAMP is a dog amphotropic packaging cell line maintained in culture in DMEM supplemented with 5% FCS.

Reagents. Beef and pork insulin were purchased from Sigma Chemical Co. (Canada, Mississauga, Ontario, Canada). G418 from GIBCO BRL, mitomycin C from Sigma Chemical Co. (St. Louis, MO), [^{3}H]thymidine methyl (81.3 Ci/mmol) from Du Pont Canada (Mississauga, Ontario, Canada).

Antibodies. The mAb F23.1, a specific anti–mouse TCR Vβ8 was provided by P. Marrack (National Jewish Center, Denver, CO). mAbs L68 and L93, two human CD4-specific mAbs, were provided by D. Buck (Becton Dickinson, Monoclonal Antibody Center, San Jose, CA), OKT4 by American Type Culture Collection (ATCC; Rockville, MD), Q425 by Q. Sattentau (Institut d'Immunologie Marseille-Luminy, Marseille, France). GK1.5 is an anti–mouse L3T4 mAb (ATCC), IM7 is an anti–mouse PGP-1 mAb (ATCC).

Plasmids. The CD4 constructs were subcloned in the pMNC recombinant retroviral vector. It confers resistance to the selective agent G418 and allows stable expression of CD4 molecules in infected cells (21). The wild-type (wt) human CD4 is a gift from

A. Peterson and B. Seed (Massachusetts General Hospital, Boston, MA), the truncated CD4 molecule from E. Long (Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD), C420 2A CD4 was obtained from S. Gratton (Institut de Recherches Cliniques de Montréal, Montréal, Canada) (22).

DNA-mediated Gene Transfer. DAMP cells expressing the different forms of human and murine CD4 were obtained by the calcium phosphate coprecipitation technique (23). T cell hybridomas expressing CD4 were obtained by coculturing the CD4$^-$ T cell hybridoma (10^6 cells) and mitomycin C (50 μg/ml, 45 min, 37°C) treated DAMP cells (70% confluent flask) for 24 h with polybrene at 4 μg/ml. T cells were then washed and incubated for another 24 h before selecting transfectants by adding G418 (1 mg/ml for BI and 2.5 mg/ml for KR3). Stable populations were obtained by aseptic cell sorting on a FACstar® Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA) or single cell cloning (for KR3 cells) and further analyzed for CD4 expression.

Flow Cytometry Analysis. Expression of the CD4 and TCR molecules were monitored by staining the cells with either an anti–human CD4-mAb (L93) or with an antimurine TCR Vβ8 mAb (F23.1). 0.5–1 $\times$ 10^6 cells were incubated on ice for 30 min with indicated mAbs, washed in PBS, and subsequently incubated with goat anti–mouse Ig (GAM) FITC (Becton Dickinson, Mississauga, Ontario, Canada) for 30 min on ice and washed. Cells were then analyzed for fluorescence with a FACScan® flow cytometer (Becton Dickinson, Immunocytometry Systems). For each histogram, live cells gated by propidium iodide exclusion were analyzed on a log scale. As negative control, cells were stained with the secondary FITC-labeled antibody alone. Results are expressed in arbitrary units of fluorescence.

Ag Stimulation of the T Cell Hybridoma

BI Hybridoma. 5 $\times$ 10^4 T cells were stimulated by incubation with 10^4 APCs and various concentrations of either beef or pork insulin for 24 h, at 37°C. Supernatants from the cocultures were assayed for IL-2 production as described below.

KR3 Hybridoma. 5 $\times$ 10^4 T cells were incubated for 24 h at 37°C with various concentrations (6 $\times$ 10^6 to 10^4) of splenocytes from either C57Bl/6 male mice expressing the H-Y Ag in the context of I-A^b or splenocytes from female as a negative control or female DBA/2 mice expressing Mls-1^a. Supernatants were assayed for IL-2 production.

IL-2 Assay. 100 μl of supernatants obtained from the T cell stimulation assays (or serial dilutions 1:2 of supernatant) were added to 5 $\times$ 10^3 CTLL.2 and incubated for 48 h at 37°C. CTLL.2 proliferation was measured by adding 0.25 μCi/well of [^{3}H]thymidine for 16 h. Wells were harvested and thymidine incorporation was counted with a β-plate counter (LKB Pharmacia Canada, Baie d'Urfé, Québec, Canada). cpm were converted to IL-2 units using a standard curve of rIL-2.

Stimulation with Coated Antibodies. Plates were coated overnight with serial dilutions of anti-TCR mAb in PBS at 4°C. Plates were then washed twice with PBS and 5 $\times$ 10^4 T cells were added per well and incubated for 16 h at 37°C. IL-2 production was then measured. When GAM (The Jackson Laboratory, Bar Harbor, ME) was used to cross-link TCR and CD4 mAbs, 10 μg/ml of GAM diluted in PBS were coated on plates, incubated overnight at 4°C, and wells were washed twice with PBS. 5 $\times$ 10^4 cells were then incubated on ice for 60 min with TCR and CD4 mAbs, washed twice with PBS, and resuspended in medium. Cells (5 $\times$ 10^4) were added to the GAM-coated wells and incubated for 16 h at 37°C. IL-2 production was then measured.

Protein Analysis. 5 × 10⁵ T cell hybridoma were lysed in 100 µl loading buffer (2% SDS, 40 mM Tris HCl, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue) and boiled for 10 min. Supernatants were run on a gradient (5–10%) SDS polyacryl-amide gel. After transfer onto nitrocellulose (S & S NC™, pore size 0.2 µm; Mandel Scientific Co., Guelph, Ontario, Canada) binding of the antiphosphotyrosine mAb was performed for 2 h in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) with 2% gelatin and was revealed with ¹²⁵I-GAM (Amersham Corp., Oakville, Ontario, Canada) on XAR film (Eastman Kodak Co., Rochester, NY).

Results

Cytofluorometric Analysis of KR3 and BI Hybridomas Trans-fected with the wt and Mutated Human CD4 Molecules. The human CD4 can fully replace L3T4 in the BI hybridoma and restore its response to low concentrations of Ag (reference 10 and data not shown). cDNAs encoding the human CD4 molecule and two different mutants, TCD4 and C420, were stably introduced by infection into two L3T4 negative hy-bridoma variants 83/141 (BI) and KR3⁻ (specific for H-Y). The TCD4 mutant lacks the COOH-terminal 31 amino acids of the cytoplasmic tail while the C420 mutant has been ob-tained by substituting for alanines, the two cysteines at posi-tions 420 and 422. Cell surface expression of the different molecules was verified by FACScan® analysis using an anti–human CD4 (L93) mAb and an antimurine TCR Vβ8 (F23.1) mAb (Fig. 1) or an antimurine CD4 (GK1.5). Popu-lations expressing similar levels of the CD4 and TCR mole-cules were retained for functional and biochemical analysis.

Analysis of the Intrinsic Capacity of Each Population to Pro-duce IL-2. Aggregation of the TCR by the TCR-specific F23.1 mAb adsorbed on plates provides an independent stimulus to control the capacity of each line to secrete IL-2. Cells expressing wt CD4 (BICD4.3, KR3CD4.H8), TCD4, and C420 (in KR3 and BI) were as efficient as wt CD4 ex-pressing populations to respond to F23.1 mAb (Fig. 2, *A* and *B*, for BI and KR3, respectively) indicating that these populations had the same intrinsic capacity to produce IL-2 upon aggregation of their TCRs.

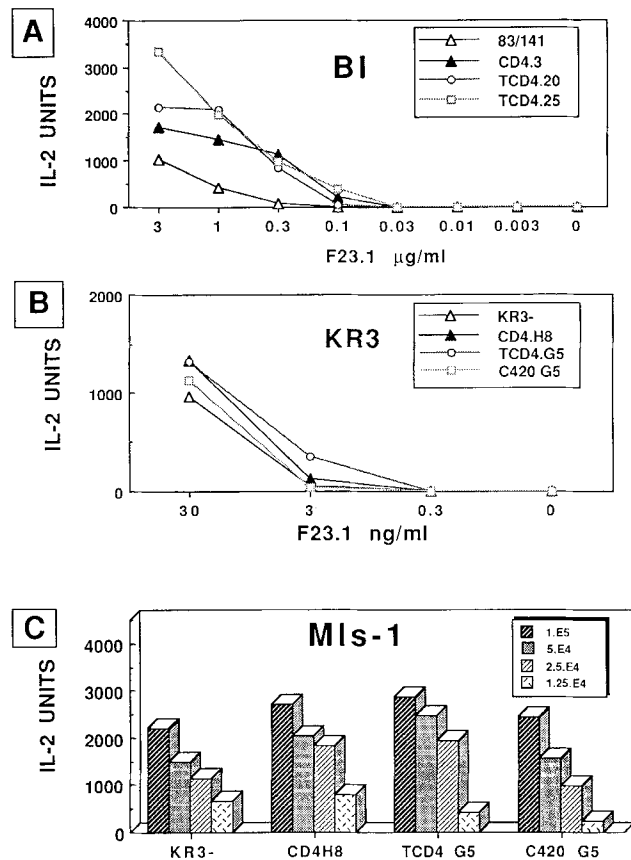The Mls-1ª superantigen (SAG) can strongly stimulate the KR3 hybridoma (19). To further confirm that these popula-

Figure 2. T cell hybridomas transfected with wt and mutant CD4 mol-ecules show a similar capacity to produce IL-2 upon a non-CD4–depen-dent stimulus. (*A*) Each BI transfectant (5 × 10⁴ cells/well) was stimulated with decreasing concentrations of F23.1 coated on plates and incubated at 37°C for 24 h. Supernatants were assayed for IL-2 production by measuring [³H]thymidine incorporation of the IL-2–dependent CTLL.2 line; IL-2 units are obtained using a standard curve generated with rIL-2. (*B*) KR3 transfectants (5 × 10⁴ cells/well) were stimulated as in *A* and IL-2 production was measured using the IL-2–dependent CTLL.2 line. (*C*) KR3 hybridomas were stimulated with decreasing concentrations of Mls-1ª–expressing splenocytes for 24 h and IL-2 production was then quantified as described above.
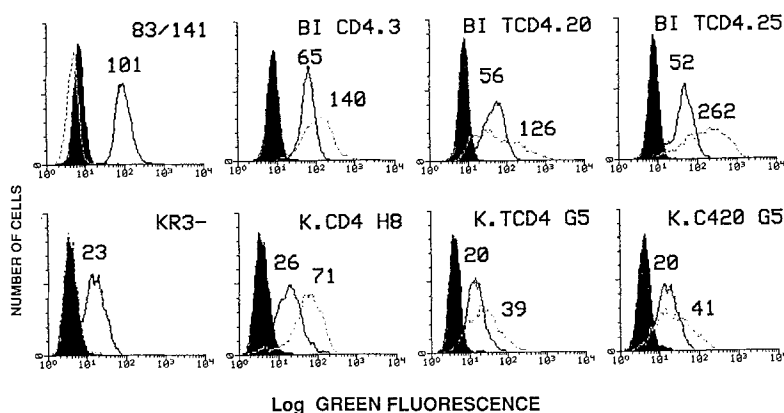
Figure 1. Fluorescence analysis of the different popu-lations for the expression of TCR and CD4 molecules. Cells were stained with the F23.1 (10 µg/ml) mAb specific for the murine TCR Vβ8 chain (—) and with the anti–human CD4 L93 (5 µg/ml) (---). As a nega-tive control, cells were only stained with GAM-FITC and are represented in all panels by a histogram in bold; staining with the GK1.5-FITC mAb (anti-L3T4) is over-lapping with the negative control. Mean fluorescence is indicated on the top of each curve. Fluorescence is plotted on a four decade log scale (x-axis) against number of cells (y-axis). For each Ag-specific system, transfected populations were chosen to express comparable levels of cell surface expression of CD4 and TCR molecules.

tions are comparable in terms of IL-2 production, KR3⁻, KCD4.H8, KTCD4, KC420 were stimulated by DBA/2 splenocytes. This response was CD4-independent since the KR3⁻ population showed the same capacity to respond to Mls-1ᵃ as wt CD4, TCD4, or C420 CD4 transfected cells. As shown in Fig. 2 C, all the clones tested produced equivalent amounts of IL-2, even in presence of the lowest concentration of Mls-1ᵃ expressing cells (1.25 × 10⁴ Mls-1ᵃ-expressing splenocytes). These results provide another evidence that these different populations have the same intrinsic capacity to produce IL-2 upon cross-linking of their TCR and can be further compared for their capacity to respond to a CD4-dependent antigenic stimulation.

Lack of Association of CD4 with p56ᵏᵏ Does Not Alter the Capacity of CD4 to Enhance Ag-specific Stimulation. The requirement for the CD4:p56ᵏᵏ association in Ag specific responses was verified in each one of these systems. BI cells respond to beef or pork insulin when presented by the murine MHC class II molecule I-A αᵇβᵏ. The BI L3T4 negative variant (83/141) loses partially (with beef insulin) or completely (with pork insulin) its capacity to respond to its Ag when compared to the L3T4⁺ parental cell line (24). BI populations expressing wt CD4 or TCD4 were stimulated by increasing concentrations of beef or pork insulin in the presence of DAP-3 cells expressing the I-A αᵇβᵏ murine MHC class II molecules. Both cell types were capable of producing high levels of IL-2 at 40 μg/ml of beef insulin whereas the CD4 negative cell line (83/141) never produced comparable levels of IL-2 even at the higher concentrations of beef insulin (Fig. 3 A). Interestingly, TCD4 was perfectly capable of restoring a pork insulin specific response with the same efficacy as wt CD4. The half maximal IL-2 response for the BICD4.3 and BITCD4.20 populations (Fig. 3 B) occurred at the same pork insulin concentration (160 μg/ml). To further confirm this result we derived from independent infections several TCD4⁺ populations expressing levels of TCD4 and TCR comparable to wt CD4⁺ cells. These cells produced significant amount of IL-2 when stimulated with 160 μg/ml pork insulin and appropriate APCs, as compared with cells transfected with wt human or murine (L3T4) CD4 (Fig. 3 C). These results demonstrate the lack of requirement for the CD4:p56ᵏᵏ association in a CD4 dependent response; they emphasize the role of the extracellular domain of the CD4 molecule.

To confirm this statement, we studied the effect of this mutation on the response to Ag of a second CD4-dependent T cell hybridoma. The murine T cell hybridoma KR3 is specific for the H-Y Ag when presented by the murine MHC class II I-Aᵇ. The KR3 L3T4 negative variant (KR3⁻) loses the capacity to respond to cells expressing its specific antigen H-Y, whereas it retained the ability to be stimulated by Mls-1ᵃ (19). KR3 wt CD4, TCD4, and C420 populations were stimulated with decreasing concentrations of male BL/6 splenocytes (6–0.75 × 10⁵ cells) or 6 × 10⁵ female BL/6 splenocytes (H-Y⁻). The wt human CD4 molecule was perfectly capable of restoring the H-Y-specific response (Fig. 4 A). Moreover the TCD4 molecule enhanced the reactivity
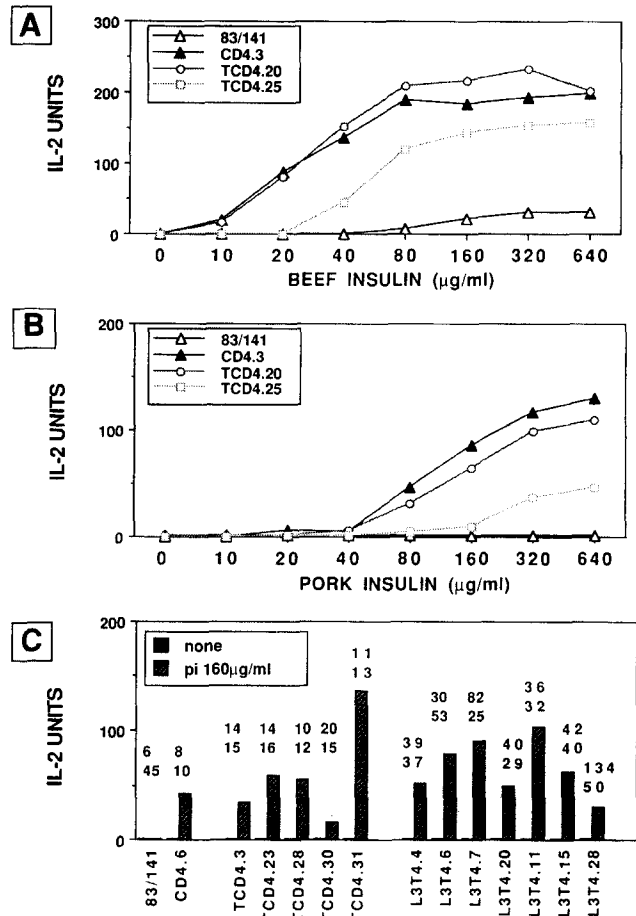
Figure 3. Pork insulin–specific stimulation of the T cell hybridomas is dependent upon CD4 expression but association of CD4 with p56ᵏᵏ is not required. (*A* and *B*) BI hybridomas were stimulated with antigen as described in Materials and Methods. Increasing concentrations of beef or pork insulin were incubated with APCs (10⁴ cells) and T cells (5 × 10⁴) for 24 h at 37°C and supernatants from the cocultures were assayed for IL-2 by measuring [³H]thymidine incorporation of the IL-2–dependent CTLL.2 line. (*C*) Comparison of IL-2 release between TCD4- or L3T4-expressing populations stimulated with 160 μg/ml pork insulin. The 83/141 T cell hybridoma was infected with retroviruses containing the TCD4 or full-length L3T4 cDNA as described in Materials and Methods. G418-resistant cells were selected. Each T cell hybridoma expressing wt CD4, TCD4, or L3T4 was analyzed by flow cytometry for its expression of TCR and CD4. Mean values of fluorescence for CD4 (*top*) and TCR (*bottom*) molecules are indicated on top of each histogram. Cell surface expression of CD4 and TCR was obtained by flow cytometry as described in Fig. 1. Cocultures of APCs with the different T cell hybridomas were carried out as described in A. IL-2 units were obtained using the IL-2–dependent CTLL.2 assay.

to H-Y as efficiently as wt CD4 (Fig. 4 A). Similar results were obtained with the C420 mutant with a dose response curve to H-Y⁺ cells overlapping with the one obtained with wt CD4. Several clones of TCD4 and C420 cells expressing comparable levels of CD4 and TCR were screened for their response to H-Y (Fig. 4 B); they all demonstrated the same capacity to respond to H-Y⁺ APCs as cells expressing wt CD4.
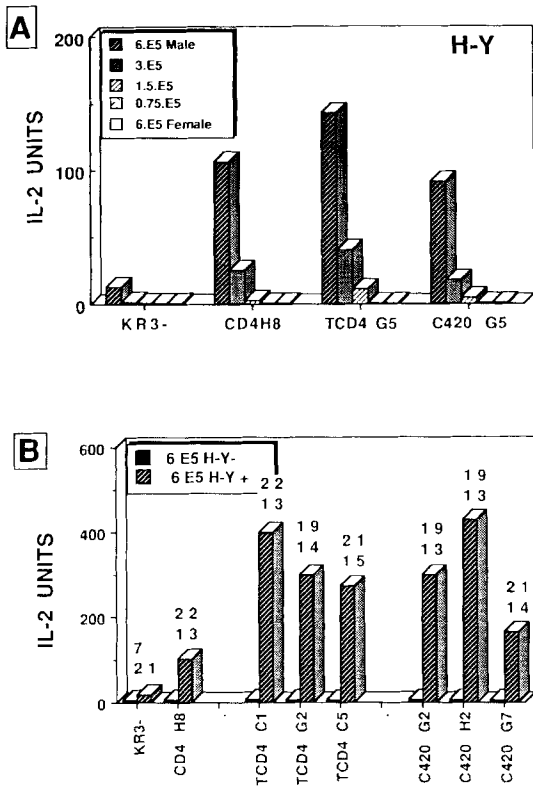
Figure 4. H-Y-specific stimulation of the KR3 hybridoma is dependent upon CD4 expression but does not require CD4:p56^{lck} association. (*A*) KR3 hybridomas were stimulated with several concentrations of male (H-Y$^+$) I-A^b Bl/6 splenocytes or 6 × 10^5 female Bl/6 splenocytes for 24 h at 37°C. Supernatants were assayed for IL-2 production by measuring [^{3}H]thymidine incorporation in the IL-2–dependent CTLL.2 line. (*B*) Screening of TCD4 and C420 expressing populations after stimulation with 5 × 10^6 male splenocytes. The KR3$^-$ hybridoma was infected with retroviruses containing TCD4 or C420 mutants cDNAs as described in Materials and Methods. G418-resistant populations were selected and each one of these hybrids were analyzed by flow cytometry for their expression of CD4 and TCR. Mean fluorescences for each T cell hybridoma used in these experiments are indicated on top of each histogram: CD4 (*top*) and TCR (*bottom*). Cell surface expression of CD4 and TCR was obtained as described in Fig. 1. Cocultures of different T cell hybridomas with splenocytes were carried out as in (*A*). IL-2 units were obtained using the IL-2–dependent CTLL.2 assay.

Figure 5. Specific inhibition of the pork insulin stimulation with anti-CD4 antibodies. T cell hybridomas (5 × 10^4) were incubated for 30 min on ice with decreasing concentrations of either the human CD4 (L68) mAb (5 – 0.005 μg/ml) or the murine CD4 (GK1.5) mAb (2–0.002 μg/ml) or with the anti-PGP-1 mAb (5 μg/ml). Cells were then incubated with 160 μg/ml of pork insulin in the presence of APCs (10^4 cells) for 24 h and supernatants were assayed for IL-2 production as already described: (*A*) BICD4.3; (*B*) BITCD4.20; (*C*) BIL3T4.

Taken together, these experiments confirm in two independent antigenic systems that mutants of CD4 that are not associated with p56^{lck} have the same capacity as wt CD4 to rescue a CD4-dependent response. These results suggest that the enhancement of T cell stimulation resulting from the expression of CD4 in those cells, is only due to the involvement of the extracellular/transmembrane region of CD4.

Inhibition of a CD4-dependent Ag Stimulation by Human CD4-specific mAbs. Several reports have shown that CD4-dependent responses are inhibited by CD4-specific mAbs (25, 26). To assess the specific involvement of CD4-associated p56^{lck} in this inhibition, cells were incubated with a CD4 mAb (L68) or L3T4 mAb (GK1.5) or an Ab recognizing an irrelevant cell surface protein (PGP-1) before triggering with APCs
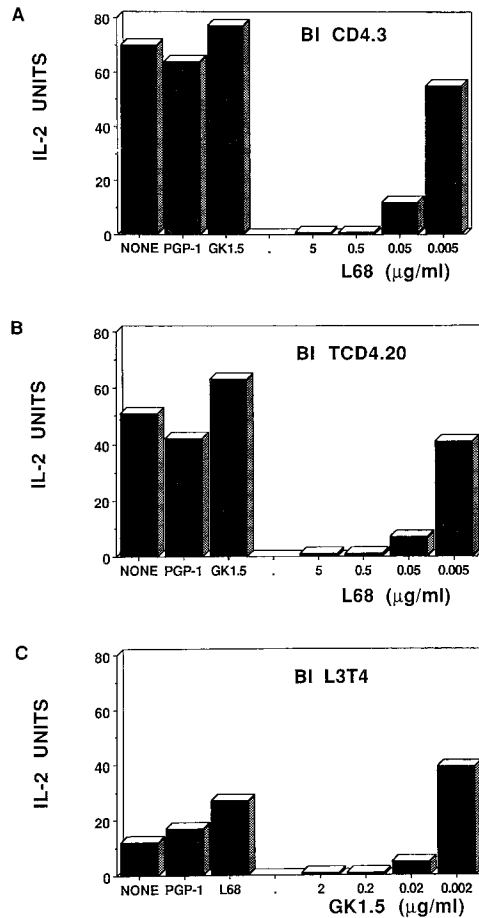
and specific Ag. Fig. 5 *A* shows that L68 inhibited the response to pork insulin of cells expressing wt CD4 in a dose-dependent fashion. Interestingly, a similar pattern of inhibition, with an identical dose response, was obtained for cells expressing TCD4 (Fig. 5 *B*) indicating that the CD4:p56^{lck} association is not required for these inhibitions. Stimulation of control cells expressing L3T4 was not inhibited by L68 but was abrogated by mAbs specific for L3T4 (Fig. 5 *C*). Similar observations were made using KR3 cells expressing wt or mutant CD4 molecules. The CD4-specific mAb L68 inhibited the H-Y-specific response of hybridomas expressing wt CD4 (Fig. 6 *A*), TCD4 (Fig. 6 *B*) and C420 CD4 (Fig. 6 *C*). Control cells expressing L3T4 were inhibited with L3T4 mAb but not with a human CD4-specific mAb (Fig. 6 *D*). Inhibition of T cell response was never observed with the irrelevant anti-PGP-1 mAb.
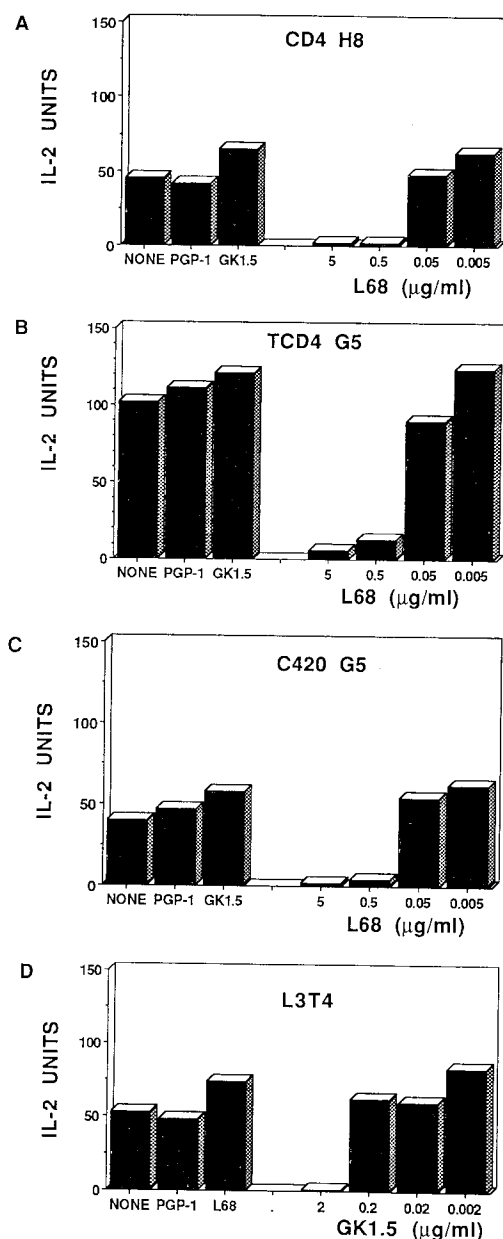
Our results demonstrate that the CD4 molecule enhances

Figure 6. Specific inhibition of the H-Y stimulation with an anti-CD4 mAb. KR3 hybridomas were incubated with the different mAbs, as in Fig. 5, before incubation with 3 × 10⁵ H-Y⁺ splenocytes for 24 h; supernatants were assayed for IL-2 production as previously described. (*A*) KCD4 H8, (*B*) KTCD4 G5, (*C*) KC420 G5, (*D*) KL3T4.

Ag stimulation without being associated with the tyrosine kinase p56^{lck}. This enhancement is entirely blocked by human CD4-specific mAbs, even when mutant forms of CD4 that do not interact with p56^{lck} are expressed, confirming that the interaction of CD4 with its external ligands is critical for CD4 to exert its functions.

Inhibition of a CD4-independent Ag Response with Human CD4-specific mAbs. In a CD4-dependent Ag-specific system, activation absolutely requires the extracellular domain of CD4 interacting with MHC class II, thus making it difficult to

assess whether the CD4:p56^{lck} association is critical per se for the inhibition with CD4-specific mAbs. We thus studied the effect of CD4-specific mAbs on a response to Ag that is independent of the expression of CD4. As shown in Fig. 2 *C*, KR3 CD4⁺ and L3T4⁻ populations responded as efficiently to Mls-1ª SAG. KR3⁻, KCD4, KC420, KTCD4, and KL3T4 populations were incubated with decreasing concentrations of CD4-specific mAbs before stimulation by Mls-1ª-expressing splenocytes. Three different mAbs were used: L68, which maps to the V1 domain of the CD4 molecule; GK1.5 mAb, which is specific for L3T4; and IM7 mAb, which is specific to PGP-1.

As illustrated in Fig. 7, CD4-specific mAbs were very efficient in inhibiting the Mls-1ª response although CD4 expression is not required for an optimal T cell activation. Quantitative differences were however observed in the degree of inhibition with the various forms of CD4. Whereas wt CD4-expressing cells were reproducibly totally inhibited with a saturating concentration of the CD4-specific mAb L68 (5 µg/ml), inhibition of the TCD4 (Fig. 7 *C*) and C420 populations (Fig. 7 *D*) varied between 50 and 100% at the same concentrations of L68 (Fig. 7 and data not shown). This inhibition was not due to cross-linking of CD4-specific mAbs by Fc receptors expressed on splenocytes, since the same pattern of inhibition was obtained after a stimulation with Fc receptor negative Mls-1ª/DR1-expressing DAP cells (data not shown). Altogether these results indicate that both CD4-dependent and CD4-independent responses are inhibited by CD4-specific mAbs by a mechanism in which the CD4: p56^{lck} association is not involved.

Enhancement of IL-2 Production by Cross-linking CD4 to the TCR Can Be Observed in Absence of p56^{lck} Associated with CD4. Experiments were then set up to determine the role of the CD4:p56^{lck} association in the enhancement of anti-TCR specific responses by co-cross-linking the TCR with CD4 using specific mAbs. Such a system enables us to dissociate the adhesion from the signaling function of CD4. Cells were first incubated with a suboptimal concentration of anti-TCR together with or without a CD4- or a PGP-1-specific mAb. As shown in Fig. 8, a 10-fold enhancement of IL-2 production (varying from 3- to 14-fold depending on the experiment) was noted in the BI system when wt CD4 molecules were cross-linked to the TCR, as compared with aggregation of the TCR alone. A similar enhancement, although weaker (ranging from three- to sixfold depending on experiments), was obtained when the TCD4 mutant molecule was co-cross-linked to the TCR. No enhancement was obtained by co-cross-linking PGP-1 to the TCR. Similar results were obtained with the KR3 hybridoma expressing the wt or TCD4 or C420 mutant molecules (data not shown). Our data demonstrate that an additional mechanism independent of CD4:p56^{lck} association is involved in the above-described enhancement of TCR-mediated stimulation and requires the extracellular domain of CD4.

Enhancement of IL-2 Production Occurs Independently of the CD4:p56^{lck}-mediated Increase in Tyrosine Phosphorylation. We then verified if the IL-2 enhancement observed after cross-
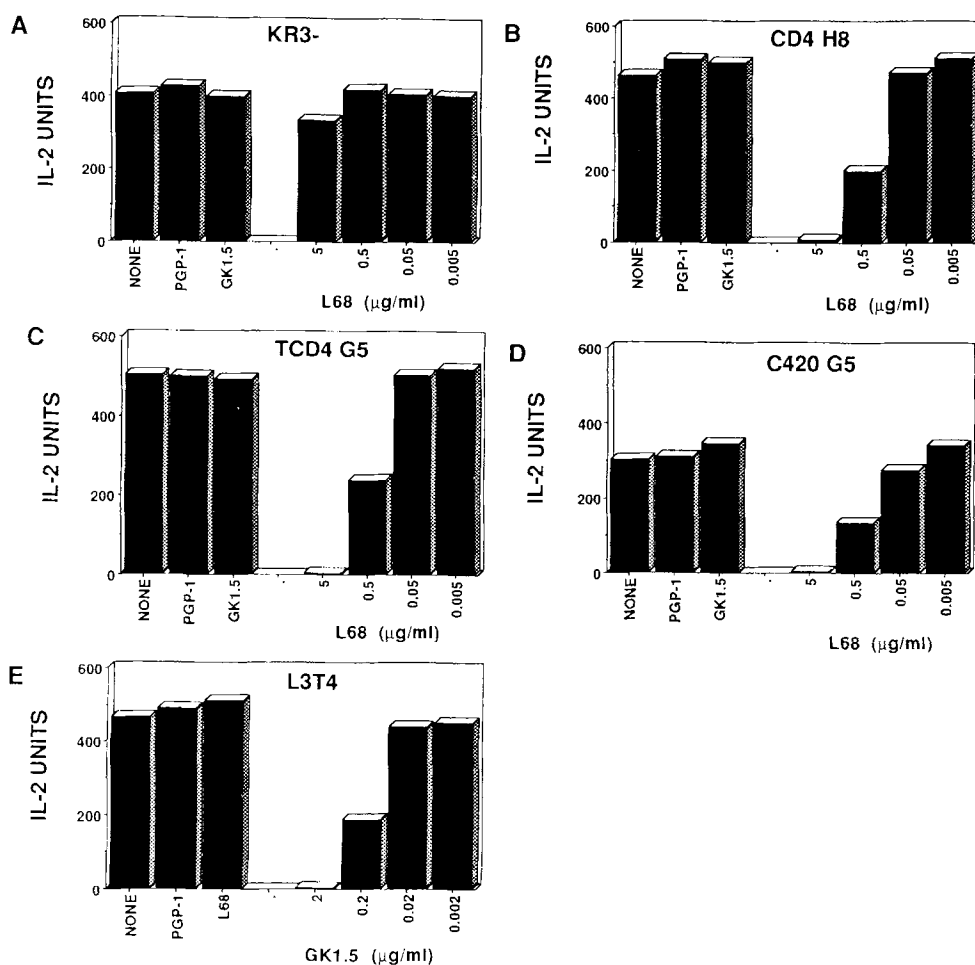
Figure 7. Inhibition of a CD4-independent stimulation with anti-CD4 mAbs. KR3 hybridomas (5 × 10⁴ cells) were incubated for 60 min at 4°C with the various mAbs, as in Figs. 5 and 6, before incubation with 10⁵ Mls-1ᵃ -expressing splenocytes for 24 h at 37°C. IL-2 production was then measured. (*A*) KR3⁻, (*B*) KCD4 H8, (*C*) KTCD4.G5, (*D*) KC420 G5, (*E*) KL3T4.
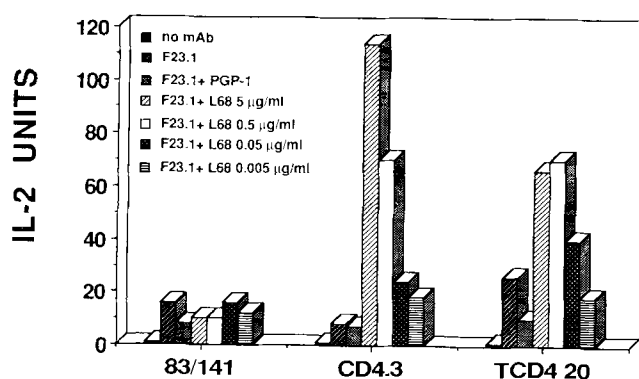

Figure 8. Cross-linking CD4 to the TCR complex enhances a suboptimal anti-TCR Ab stimulation. 83/141, BICD4.3, BITCD4.20 cells (5 × 10⁴) were incubated with a suboptimal dose of anti-TCR alone (F23.1, 0.75 μg/ml) or together with varying concentrations of L68 or an anti-PGP1 mAb (5 μg/ml) for 1 h on ice. Cells were washed twice, resuspended in medium, and incubated for 24 h in wells precoated with GAM (10 μg/ml). Supernatants were assayed for IL-2 production as already described. (The three populations express high level of PGP-1 and the GAM Ab binds to the anti-PGP-1 very efficiently as illustrated by FACScan® analysis by staining the cells with the anti-PGP-1 + GAM-FITC. Cells are 100% positive with a mean value of: 204 (83/143), 361 (TCD4.20), 477 (L3T4) vs. a mean of 8 for GAM-FITC alone).

linking of the different CD4 molecules and TCR with specific mAbs was paralleled by an increase in the phosphotyrosine protein content of such activated T cells. Immunoblots with a phosphotyrosine-specific mAb (Fig. 9) showed overlapping patterns of tyrosine phosphorylated substrates when comparing BICD4.3 (Fig. 9 *A*) and BITCD4.20 (Fig. 9 *B*) stimulated with an anti-TCR; an increase in tyrosine phosphorylation of four cellular substrates (160, 97, 70, and 42 kd) was noted with a peak occurring at five min after the cross-linking of TCR molecules. An identical pattern of tyrosine phosphorylation was obtained when TCR and CD4 molecules were co-cross-linked in BITCD4.20 (Fig. 9 *D*). In contrast, as already described (27), tyrosine phosphorylation of cellular substrates in cells expressing the wt CD4 molecule was significantly increased after the coaggregation of wt CD4 and TCR molecules. This increase was detected as soon as 30 s with a peak occurring at 1 min. Phosphorylation of a new substrate at 66 kD was noted under these conditions. These results suggest that this increase in tyrosine phosphorylation content is not associated with the enhancement of IL-2 production observed after the coaggregation of CD4 and TCR molecules. Conversely high levels of IL-2 production do not require these qualitative and quantitative modifica-
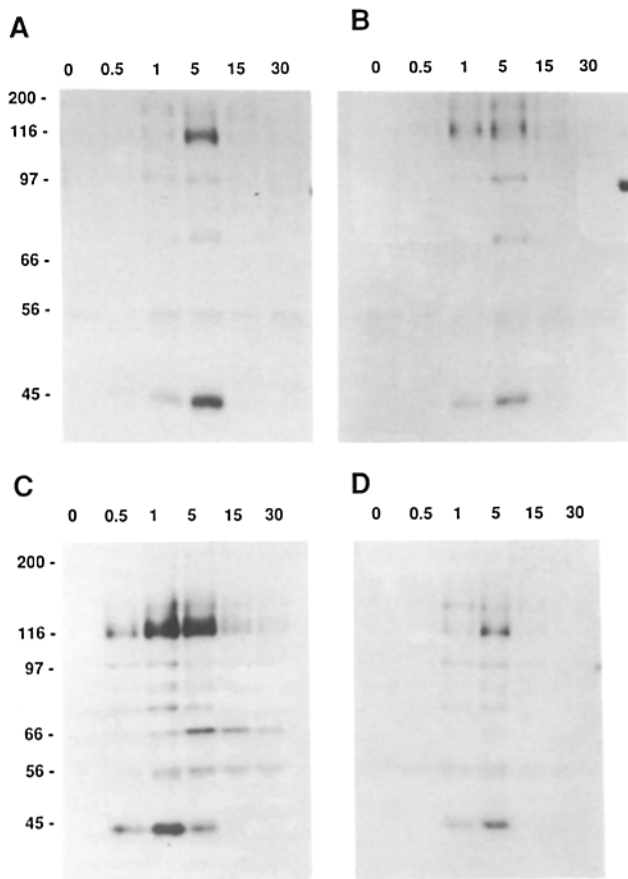
Figure 9. Enhancement of IL-2 production occurs independently of the CD4-p56^{lck}–mediated increase in tyrosine phosphorylation. (*A* and *B*). 0.5 × 10^6 cells (BICD4.3 and BITCD4.20) were incubated with F23.1 (0.75 μg/ml) on ice for 1 h. cells were washed twice in PBS and TCR were cross-linked with GAM (5 μg/ml) at 37°C for 0, 0.5, 1, 5, 15, and 30 min. Cells were then directly lysed in sample buffer and lysates were run on SDS-PAGE. Immunoblots were performed by incubating with an antiphosphotyrosine mAb (1:800) and revealed with a ^{125}I-GAM. (*C* and *D*) 0.5 × 10^6 cells (BICD4.3 and BITCD4.20) were incubated with 0.75 μg/ml F23.1 and 10 μg/ml L68 and samples were processed as in *A*.

tions in tyrosine phosphorylation of cellular substrates. Our results suggest that the activation of the tyrosine kinase activity of p56^{lck} occurring after the cross-linking of CD4 is not required for this enhancement.

Discussion

The BI and KR3 hybridomas are both dependent on the expression of CD4 for an optimal response to antigen. Transfection into these cells of CD4 mutants, which do not associate with the tyrosine kinase p56^{lck}, has enabled us to demonstrate that this interaction is not required for CD4-dependent enhancement of Ag-specific T cell activation. The use of the human CD4 molecule was justified since it can fully replace its murine counterpart (10), even during T cell differentiation (11). The response of the BI hybridoma to low concentrations of Ag (beef insulin) was fully restored after expression of the TCD4 mutant as well as the wt CD4 molecule (Fig. 3 *A*, *BI CD4.3*, *BI TCD4.20*). Moreover stimulation by pork insulin, which is entirely dependent on CD4

expression, was as efficient in TCD4 as in wt CD4–expressing cells. This enhanced response in TCD4-expressing cells was not due to a clonal variation, since several independently derived populations exhibited a similar pattern of response to both antigens. Furthermore the lack of requirement for the CD4:p56^{lck} association could not be attributed to aberrantly high levels of expression of TCD4 or TCR on these cells (Figs. 1 and 3 *C*). Reexpression of the murine CD4 molecule cannot explain these results since cell surface expression (Fig. 1) could not be detected. Moreover the Ag-specific responses were not inhibited by murine CD4-specific mAbs (Figs. 5, 6, and 7). As expected, the tyrosine phosphorylation pathway is activated after TCR cross-linking and this pathway is enhanced after the coaggregation of wt CD4 and TCR molecules (Fig. 9 and reference 27). These results suggest that wt CD4:p56^{lck} association is functional in our hybridoma.

Our results contrast with those previously reported by Miceli et al. (28), who showed that the response of the BI hybridoma to low concentrations of beef insulin was dependent on the association of CD4 with p56^{lck}. This hybridoma expresses at least two different TCR α chains (29), since the fusion partner of beef insulin–specific T cell blasts has two productive rearrangements of the TCR α chain. Qualitative and quantitative differences in TCR expression caused by different TCR α/β combinations could account for the differences observed between the two laboratories. Hence, levels of TCR complex specific for pork or beef insulin are likely to be overestimated when these cells are stained or stimulated by a TCR mAb (F23.1) that recognizes the Vβ chain of the specific TCR. This point is best illustrated with the BITCD4.25 population (Figs. 2 *A* and 3, *A* and *B*) and other wt CD4 populations (data not shown). BITCD4.25 responded very efficiently to low concentrations of F23.1 (Fig. 2 *A*) but very poorly to Ag (Fig. 3, *A* and *B*). This point is further illustrated with KR3, the H-Y–specific MHC class II–restricted hybridoma that expresses only one TCR α/β combination on its surface. There was always a perfect correlation between the responses to Ag, Mls-1^a SAG, and TCR mAb stimulations (Figs. 2 and 4), further confirming that these cells had the same intrinsic capacity to produce IL-2 upon triggering through their TCR. As in the BI system, we could not show a difference in the response to TCD4, C420, and wt CD4–expressing cells to H-Y stimulation in a quantitative assay dependent on the concentration of male splenocytes (Fig. 4 *A*). These results demonstrate that the critical role of CD4 in these assays is restricted to its extracellular domains (transmembrane included).

The requirement for the association of CD4 and CD8 with p56^{lck} in Ag-specific responses is largely debated. Three groups have clearly demonstrated that T cell hybridomas expressing mutated forms of CD4 or CD8 that do not associate with p56^{lck} will respond less efficiently to Ag or SAGs (30–32). In contrast the above-mentioned results and other reports in the literature (33, 34) show that the CD4 or CD8 extracellular domain is sufficient to enhance Ag-specific T cell activation.

As already mentioned, the use of hybridomas derived from

the $\alpha^+\beta^+$ variant of BW5147, such as BI, expressing several TCR combinations, provides a possible explanation for these discrepancies. However, other explanations must be evoked to explain differences between our results and those obtained by Glaichenhaus et al. (30) who used T cell hybridomas expressing only one combination of TCR α/β chains and observed a requirement for the CD4:p56^{lck} association for optimal response to Ag and SAGs. The affinity of the TCR for its specific Ag could account for the requirement of the CD4:p56^{lck} interaction or lack thereof in T cell hybridomas. In this context, it is important to note that both 83/141 (35) and KR3 (Fig. 2) hybridomas are CD4 independent for their response to SAGs, compatible with the notion that this response is a high affinity interaction. However both hybridomas require CD4 for their response to specific Ag. Moreover the 83/141 variant that was derived from L3T4$^+$ cells obtained after immunization with beef insulin, is less dependent on CD4 for its response to beef insulin than the variant pork insulin peptide, confirming that the affinity of the TCR for its specific Ag dictates the requirement for CD4 or CD8. Whereas it appears that the affinity of TCR for pork insulin is very low, we cannot exclude that a TCR with an even weaker affinity for its Ag needs in its vicinity, the presence of p56^{lck} associated with CD4.

Variations in the intrinsic levels of p56^{lck} among the different hybridomas could also explain the differential requirement for the CD4:p56^{lck} association. p56^{lck} expression has been shown to be required for T cell stimulation through the TCR (36). Furthermore, this molecule can exert its role in enhancing T cell activation even without being associated with CD4 (27). It is conceivable in this case that a minimal level of the p56^{lck} molecule is required for TCR-mediated stimulation. Coaggregation of CD4 with TCR by MHC class II molecules or by mAbs would yield the required local concentration of p56^{lck} in the vicinity of the TCR in cells expressing low levels of the tyrosine kinase.

CD4-dependent, Ag-specific stimulation is inhibited by CD4-specific mAbs. Three nonexclusive mechanisms have been proposed: (*a*) perturbation of the interactions of CD4 with its external ligands (MHC and/or TCR) (7); (*b*) sequestration of p56^{lck} from the TCR complex (16); and (*c*) transduction of a negative signal through the activation of p56^{lck} (14). Availability of mutant forms of CD4 that are not associated to p56^{lck} has allowed us to show that the latter two mechanisms do not play a major role in this inhibition. Sequestration of p56^{lck} (when CD4 does not cocluster with the TCR) or negative signaling (when p56^{lck} is activated by CD4 cross-linking) could play a role in the inhibition observed with cells expressing wt CD4. However, these mechanisms cannot be put forward for cells expressing CD4 molecules that are not associated with this kinase. Our results clearly show that CD4-specific mAbs are capable of abrogating the CD4-dependent responses to beef insulin (Fig. 5) and to H-Y (Fig. 6) by T cell hybridomas transfected with truncated or mutated forms of CD4 that are not associated with p56^{lck}. This inhibition is as efficient with all the mutants of CD4 that have been tested, suggesting that the mechanism involved in this phenomenon requires solely the

external domain of CD4. Since these cells most likely require a CD4-MHC class II interaction for an optimal response to Ag, it becomes difficult to determine if these mAbs affect the T cell response by disrupting a CD4-class II or a CD4 -TCR interaction.

The use of Mls-1^a stimulation, which is independent of the expression of CD4, has enabled us to address the role of CD4 interactions with ligands other than MHC class II in the inhibition by CD4-specific mAbs. As previously shown for CD4 dependent systems, we have been able to demonstrate that CD4-specific mAbs inhibit the activation by Mls-1^a of T cells expressing either wt CD4 or mutated CD4 (Fig. 7) in a dose-dependent fashion. These observations further indicate that sequestration or negative signaling through p56^{lck} or inhibition of the CD4-MHC class II interaction are not the only mechanisms involved in the inhibition of T cell activation by CD4-specific mAbs.

The fact that the synergistic function of CD4 in T cell activation occurs even when TCD4 or C420 CD4 are crosslinked with the TCR (Fig. 8) confirms the above statements. Hence, it is possible that the interaction of CD4 with the TCR modifies the conformation of the TCR complex, thereby increasing the affinity of the TCR for its ligand. This extracellular association would occur equally well with the different mutants. Indeed it was shown that specific sets of TCR mAbs can induce an association with CD4 that results in changes in TCR conformation (17). Alternatively, it is possible that CD4 is associated with other molecule(s) involved in T cell stimulation such as CD45 (18), which would be recruited equally well to the complex by wt or mutated forms of CD4. Aggregation of these molecules with the TCR complex could be sufficient to enhance T cell activation. On the other hand, disruption of these interactions by CD4-specific mAbs could explain the inhibition observed with CD4 mAbs in CD4-independent stimulation.

p56^{lck} involvement in T cell activation (36) and maturation has been clearly demonstrated (37). The experiments described here show that in these T cell hybridomas p56^{lck} associated to CD4 is not required for CD4 to enhance Ag-specific responses. However this does not exclude the involvement of non-CD4-associated p56^{lck} in T cell stimulation. Indeed, thymocytes in mice lacking p56^{lck} are arrested at a stage of differentiation which precedes the expression of CD4 and CD8 (38), further confirming the role of p56^{lck}, which is not associated with CD4. Our results demonstrate that the enhancement by CD4 of Ag-specific responses is not due to the enzymatic activity of the CD4-associated *lck* (28, 38). Rather, it is dependent on the interaction of CD4 with its externals ligand. Other mechanisms similar to those proposed by Xu and Littman (39) and Killeen and Littman (38) could also be responsible for this enhancement. In addition to increasing adhesion between APCs and T cells by binding to MHC class II molecules, CD4 can augment the affinity of the TCR for its ligand by stabilizing the conformation of the TCR when these two molecules are in close contact. Structure-function analysis of CD4 interaction with other ligands are currently being carried out to further delineate the importance of each one of these interactions.

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