Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Interactions of CD4 with MHC Class II Molecules, T Cell Receptors and p56^{lck}

Dario A. A. Vignali, Carolyn Doyle, Michael S. Kinch, Jaekyoon Shin and Jack L. Strominger

Phil. Trans. R. Soc. Lond. B 1993 **342**, 13-24
doi: 10.1098/rstb.1993.0130

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right-hand corner of the article or click **here**

To subscribe to *Phil. Trans. R. Soc. Lond. B* go to: **http://rstb.royalsocietypublishing.org/subscriptions**

This journal is © 1993 The Royal Society

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Interactions of CD4 with MHC class II molecules, T cell receptors and $p56^{lck}$

DARIO A. A. VIGNALI[1], CAROLYN DOYLE[2], MICHAEL S. KINCH[2], JAEKYOON SHIN[3] AND JACK L. STROMINGER[1,3]

[1]*Department of Biochemistry & Molecular Biology, Harvard University, Cambridge, Massachusetts 02138, U.S.A.*
[2]*Department of Immunology, Duke University Medical Center, Durham, North Carolina 27710, U.S.A.*
[3]*Division of Tumor Virology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, U.S.A.*

SUMMARY

CD4 and CD8 are members of the immunoglobulin supergene family of proteins, and function as co-receptors with the T cell receptor (TCR) in binding MHC class II or class I molecules, respectively. Within this multimeric complex, CD4 interacts with three distinct ligands. CD4 interacts through its D1 and D2 domains with MHC class II proteins, through its D3 and D4 domains with T cell receptors, and through its cytoplasmic tail with $p56^{lck}$, a *src*-related, protein tyrosine kinase. Each of these interactions is important in the function of CD4 and will be discussed in turn.

1. INTRODUCTION

T cells recognize antigens in the form of short peptides bound in the cleft of major histocompatibility complex (MHC) molecules (Babbitt *et al.* 1985; Bjorkman *et al.* 1987; Buus *et al.* 1987; Brown *et al.* 1993). CD4 and CD8 are transmembrane glycoproteins that function as co-receptors with the T cell receptor (TCR) during T cell development and peripheral activation (Rudd *et al.* 1989; Janeway 1992; Julius *et al.* 1993). Selection of CD4 or CD8 is dictated by TCR specificity and directed by their affinity for MHC class II or class I molecules, respectively (Swain 1983; Parnes 1989). CD4 binds to non-polymorphic regions on the MHC molecule thereby enhancing the adhesion between T cells and antigen presenting cells (APC) (Doyle & Strominger 1987; Gay *et al.* 1988). CD4 has been shown to physically associate with the TCR during T cell activation (Janeway 1992) which, upon binding to the appropriate MHC-peptide complex, results in signal transduction through the CD4 molecule due to its association with $p56^{lck}$ (Veillette *et al.* 1988; Rudd *et al.* 1989).

2. INTERACTION OF CD4 WITH MHC CLASS II MOLECULES

Initial antibody blocking studies suggested that CD4+ T cells recognize APC expressing MHC class II molecules whereas CD8+ T cells recognize cells expressing MHC class I molecules (figure 1) (Engleman *et al.* 1981; Krensky *et al.* 1982; Meuer *et al.* 1982; Biddison *et al.* 1983). This selective MHC restriction was further supported by the demonstration that antibodies could inhibit these interactions (Greenstein *et al.* 1984; Rogozinski *et al.* 1984; Swain *et al.* 1984; Wassmer *et al.* 1985) or that transfection of CD4 into T lymphocytes bearing a class II-restricted T cell receptor could enhance the T cell response by lowering the threshold of antigen required for stimulation or by increasing the T cell response, i.e. lymphokine secretion (Dembic *et al.* 1987). These observations suggested a physical interaction between CD4/CD8 and MHC proteins and led to the speculation that CD4 binds to a monomorphic class II determinant, thereby augmenting low avidity TCR interactions (Marrack *et al.* 1983; Greenstein *et al.* 1985; Gay *et al.* 1988). However, the complex mechanism of this interaction is not fully understood. Whereas the CD4-class II association could simply function as a T cell receptor-independent adhesion pathway, it is likely that CD4 plays an important role in antigen-specific T cell activation requiring both CD4 and the TCR to contact the same MHC molecule (Janeway 1991, 1992).

Direct evidence for the interaction of CD4 and MHC class II molecules was obtained in our laboratory using a cell binding assay (Doyle & Strominger 1987). In these studies, the human CD4 protein was expressed at high levels in simian CV-1 cells using an SV40-derived viral vector. Human B lymphocytes which expressed MHC class II antigens bound to monolayers of CD4-expressing cells, whereas class II negative B cells did not. Antibodies directed against CD4 and class II were able to inhibit cell binding, whereas anti-MHC class I reagents did not. These initial experiments provided the first evidence that CD4 and class II proteins can interact to mediate cell adhesion, even in the absence of the T cell receptor. Although other investigators have reproduced these results qualitatively (Clayton *et al.* 1989; Lamarre *et al.* 1989; Piatier-Tonneau *et al.* 1991), transient expression of CD4 does not accommodate long-term studies of its interactions. Thus, we have expressed the

Phil. Trans. R. Soc. Lond. B (1993) **342**, 13–24
Printed in Great Britain

© 1993 The Royal Society

The Royal Society is collaborating with JSTOR to digitize, preserve, and extend access to *Philosophical Transactions: Biological Sciences.* www.jstor.org

Downloaded from rstb.royalsocietypublishing.org

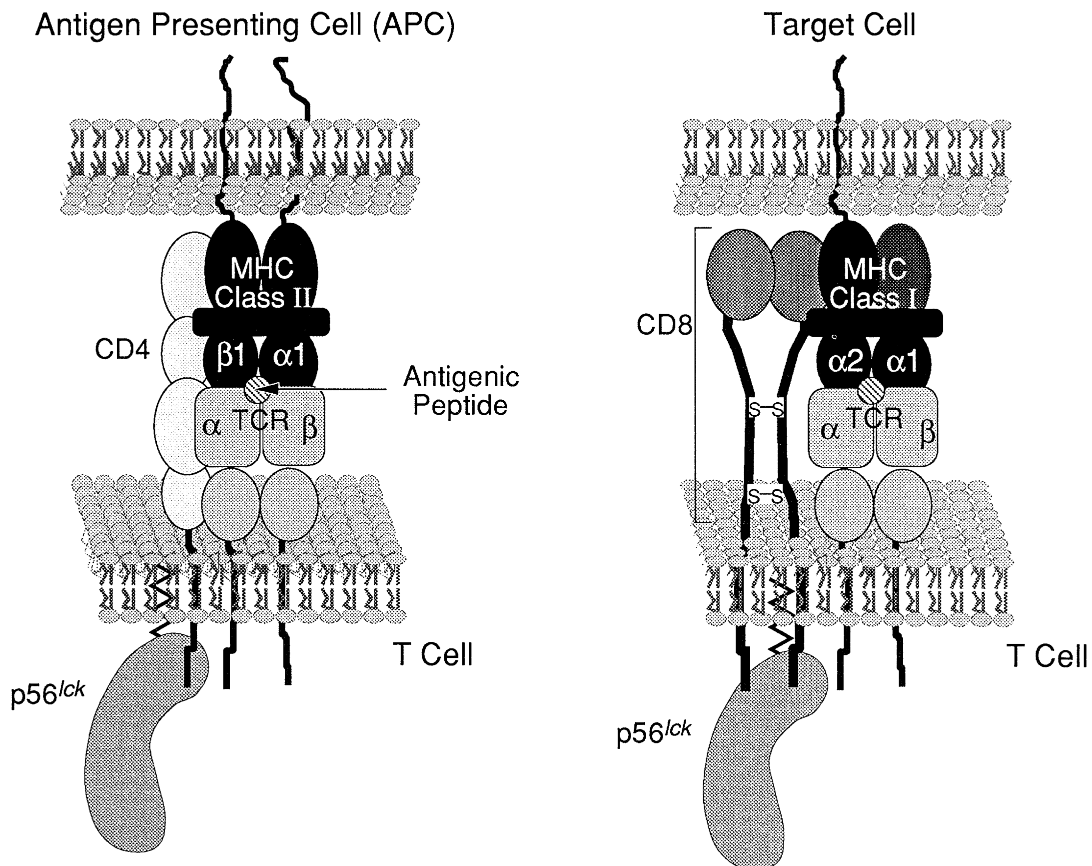


Figure 1. Schematic representation of the multiple interactions between CD4 and the TCR, MHC class II and p56*lck*.

human CD4 protein in Chinese hamster ovary (CHO) cells for use in the cell adhesion assay (Kinch *et al.* 1993). These CHO-CD4 cells, which express levels of CD4 which are comparable to physiologic levels observed in human T lymphocytes, are more convenient and reliable and provide a useful method for the analysis of CD4-class II interactions. CHO cells expressing the human CD8α protein were used similarly to show that CD8 interacts with MHC class I molecules (Norment *et al.* 1988). High level expression of CD4 (or CD8) was a critical parameter in these experiments.

The discovery that CD8 binds to the α3 domain of the MHC class I molecule (Potter *et al.* 1989; Salter *et al.* 1989, 1990) led to the speculation that CD4 might bind in an analogous manner to the membrane proximal β2 domain of the MHC class II molecule. To test this hypothesis, Konig and co-workers engineered mutations in non-polymorphic residues contained within the β2 domain of the murine H-2A^d molecule (Konig *et al.* 1992). Murine L cells, co-transfected with H-2Aα^d and mutant H-2Aβ^d cDNAs, were tested for the ability to induce activation of an antigen-specific CD4+ T cell hybridoma, as measured by lymphokine production. Mutations in the region of the β2 domain encompassing residues 137–143 resulted in the most profound defects in T cell stimulation. Similar results were obtained by Cammarota and co-workers using a solid phase binding assay in which soluble recombinant HLA-DR4 molecules or HLA-DR-derived peptides bind to immobilized recombinant soluble CD4 (Cammarota *et al.* 1992). A DRβ2-derived peptide corresponding to amino acids 134–148, and to a lesser extent peptide 138–152, were shown to bind efficiently to soluble human CD4. These results suggest that it is likely that CD4 interacts with MHC class II molecules in a manner similar to the CD8-class I interaction.

However, other studies using hybrid molecules suggest that both the β1 and β2 domains of class II may interact with CD4. In the experiments of Golding *et al.*, a chimaeric molecule consisting of the β1 domain of the murine H-2A^k class II molecule linked to the α3 domain of the class I molecule, H-2D^d, was constructed and expressed in murine L cells (Golding *et al.* 1985). The recombinant protein was efficiently expressed at the surface of transfected cells, in association with β2-microglobulin, and was recognized by class II allospecific cytotoxic T cells. This response could be inhibited by antibodies directed against the L3T4 (CD4) molecule on the effector cell. These data are consistent with the β1 domain serving as the target for L3T4 (CD4) interaction and allorecognition. Similar studies by Lombardi *et al.* (1991) and Vignali *et al.* (1992*b*) demonstrated a species specificity for human or murine CD4 which correlates with usage of the same species β2 domain in the target cell antigen. Thus, there are reasons to suspect that one or both regions of the β chain polypeptide may interact with CD4.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(*a*) *CD4 binds to epitopes on both the DRβ1 and β2 domains*

In a series of preliminary experiments aimed at identifying the region of the MHC class II molecule which interacts with CD4, we have used synthetic peptides to inhibit the CD4-class II lymphocyte binding described above. These peptides, each of which is 15 amino acids in length, are comprised of overlapping sequences from the entire length of the DR2β polypeptide (Atassi *et al.* 1987; Ulrich *et al.* 1987). For these experiments, the CHO-CD4 cells were pre-incubated with peptide for 12 h, at which time, radiolabelled Raji (class II+) B cells were added. After 4 h, the monolayers were washed and Raji B cells bound in the presence or absence of a given peptide were determined. A number of peptides appeared to have some inhibitory effects, that is, binding of Raji cells was diminished in the presence of peptide. A peptide derived from sequences contained within the β1 domain (DRβ41–55) as well as two peptides derived from the sequence of the β2 domain (DRβ121–135 and DRβ141–155) appeared to exhibit a concentration-dependent inhibitory effect. Notably, these sequences correspond to highly conserved regions of the protein (Kappes & Strominger 1988). Using a class I α2:class II β1 alignment (refer to Brown *et al.* (1988) as a model), the DRβ41–55 peptide corresponds to positions 127–141 on the HLA-A2 structure. This region is contained within a solvent exposed loop and would be highly accessible for intermolecular interactions. Similarly, by aligning the β2 domain of class II with the α3 domain of class I the β2 peptides (DRβ121–135 and DRβ141–155) coincide with the CD8 binding site on HLA-A2. Although the results from these experiments are tentative, they provide important clues for the selection and design of additional site-specific mutations in the class II molecule.

(*b*) *Cell adhesion mediated by CD4 and MHC class II molecules requires active cellular processes*

To ascertain the cellular mechanisms involved in cell adhesion mediated by CD4 and MHC class II molecules, we looked at time-dependent adhesion of class II+ Raji human B lymphocytes to monolayers of CHO-CD4 cells. For these experiments, the B cells were radioactively labelled with ^{3}H-thymidine and incubated with monolayers of CHO-CD4 cells for 1–4 h at 37°C. All experiments were performed in the presence of the TS1/22 or TS1/18 monoclonal antibodies which recognize the LFA-1 α and β chains, respectively (Sanchez-Madrid *et al.* 1982). The inclusion of LFA-1 antibodies in the incubations minimized homotypic aggregation of B cells thus allowing a more accurate assessment of CD4-class II mediated adhesion. The level of binding of radiolabelled Raji B cells correlated with the number of B cells added. Maximal binding was observed upon the addition of 1×10^6 B cells to 1×10^5 CHO-CD4 cells (10:1; Raji:CHO-CD4 cells). Similarly, the level of binding

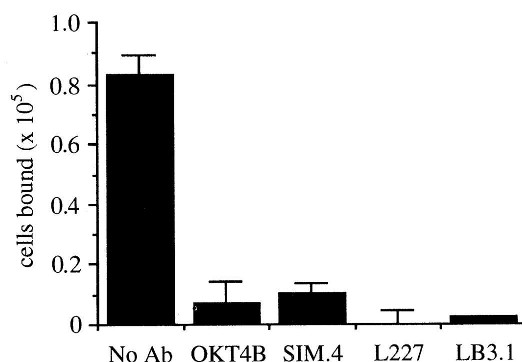


Figure 2. The binding of Raji to the CHO-CD4 cells is inhibited by the addition of CD4 and class II-specific monoclonal antibodies: LB3.1 (anti HLA-DR, culture supernatant) and L227 (anti HLA-DR, -DQ, -DP, 5 μg ml^{-1}); SIM.4 (CD4 monoclonal antibody, culture supernatant); and OKT4B (CD4 monoclonal antibody, 5 μg ml^{-1}). All assays were performed in the presence of 10% normal rabbit serum to prevent F_c receptor binding. All incubations were carried out for four hours at 37°C. After Kinch *et al.* (1993).

correlated with the level of expression of CD4 on the CHO cells. Incubation with antibodies directed against CD4 or MHC class II antigens was performed to demonstrate the specificity of binding (figure 2). Antibodies which interact with the amino-terminal domains of CD4 (OKT4B, SIM.4), effectively inhibited cell adhesion, as did the class II antibodies, LB3.1, which recognizes HLA-DR, and L227, which recognizes HLA-DR, -DQ, and -DP (Gorga *et al.* 1986). The ability of LB3.1 to effectively inhibit cell binding is due to the high level of HLA-DR expression on Raji B cells (data not shown).

We further analysed the effects of temperature, chemical fixation of one or both cell types, and pharmacological agents which disrupt the cytoskeleton. All of these treatments had a significant effect on the formation and maintenance of stable cell conjugates. The CD4-class II mediated cell adhesion was completely abrogated at 4°C (figure 3*a*) or by fixation of the CHO-CD4 cells with 1% paraformaldehyde (figure 3*b*). Fixation of the class II$^+$ B cells resulted in lowered but detectable binding (5% of untreated samples). These data suggest that metabolic activity or lateral mobility within the membrane is required to achieve cell adhesion.

To ascertain whether cytoskeletal interactions are important for CD4/class II-mediated cell adhesion, binding of radiolabelled Raji B cells to CHO-CD4 cells was assessed following the disruption of microtubules or microfilaments with pharmacological agents. No morphologic changes in either cell type were observed following treatment with these reagents at the concentrations used for these studies. Addition of nocodazole ($K_I = 0.2$ mM), colchicine ($K_I = 7.0$ nM) and cytochalasin-D ($K_I = 1.0$ mM) prevented the formation of cell conjugates and although we cannot determine whether an intact cytoskeleton must be maintained by both cell types, these experiments implicate crucial role for the cytoskeleton in the maintenance and stabilization of CD4-class II mediated cells adhesion.

Downloaded from rstb.royalsocietypublishing.org

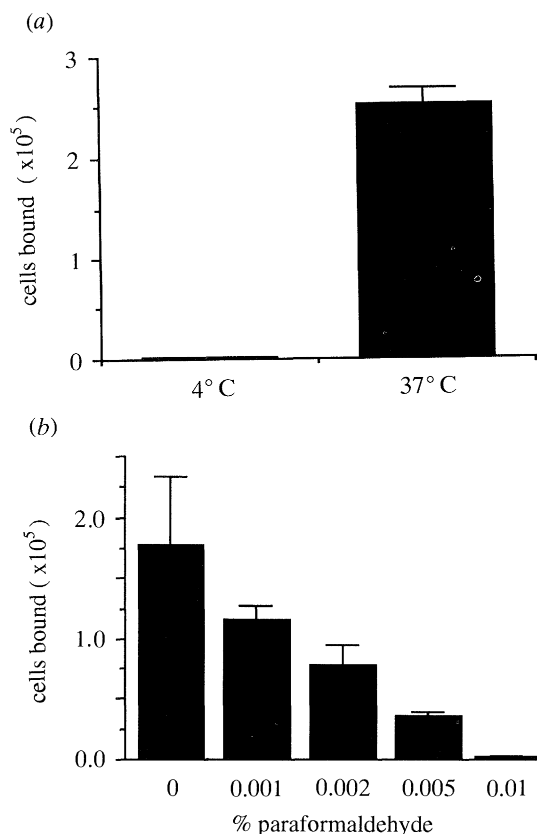


Figure 3. CD4-class II adhesion is energy dependent. (*a*) The binding of Raji to CHO-CD4 cells was assessed following 4 h incubation at 4°C or 37°C. (*b*) CD4/MHC class II adhesion was assessed after 4 h incubation at 37°C following treatment of CHO-CD4 cells with 0.001–0.01% paraformaldehyde for 30 min at 25°C. After Kinch *et al.* (1993).

The temperature dependence of CD4/MHC class II adhesion suggests that metabolic activity or membrane lateral mobility may be involved in establishing and maintaining cell conjugates. Similarly, the effect of agents which disrupt cytoskeletal integrity suggests that CD4/MHC class II adhesion is highly regulated by the mobility of CD4 within the cell membrane and by interactions between CD4 and the cytoskeleton. In support of this, we have observed that CD4 molecules at the cell surface aggregate into adherens-type junctions following their interaction with MHC class II (unpublished data). It is likely that the multi-valency of the aggregated CD4 increases the avidity of the low affinity CD4/MHC class II interaction and thus enhances stable cell–cell binding. Therefore, the energy and cytoskeletal dependence of CD4/MHC class II binding likely reflects the requirement for this redistribution of CD4 at the cell surface.

3. INTERACTION OF CD4 WITH THE T CELL RECEPTOR

There is now a large body of evidence which strongly suggests that a close association between CD4 and the TCR occurs during antigen presentation as a result of their interaction with the same MHC class II molecule (figure 1) (Rudd *et al.* 1989; Janeway 1992). Early speculation that CD4 may play a role in T cell activation came with the observation that co-cross-linking CD4 with the TCR-CD3 complex resulted in a synergistic response (Anderson *et al.* 1987; Eichmann *et al.* 1987). In contrast, crosslinking CD4 alone can induce a negative signal (Bank & Chess 1985; Ledbetter *et al.* 1988).

Initial attempts to identify a direct physical association between CD4 and the TCR-CD3 complex were largely unsuccessful (Brenner *et al.* 1985). However, two recent studies have demonstrated such an interaction using affinity chromatography of ^{125}I-labelled proteins (Gallagher *et al.* 1989), and an *in vitro* kinase assay (Burgess *et al.* 1991). These difficulties are probably due to the fact that this is an unstable interaction (Ratcliffe *et al.* 1992), and that only 5% of the TCR is associated with CD4 on a resting cell (Anderson *et al.* 1988). However, following antigen-specific stimulation up to 30% of the TCR comodulates with CD4 molecules (Rivas *et al.* 1988). There is also substantial evidence for a physical association between CD4 and the TCR from cocapping, comodulation and fluorescence energy transfer experiments (Kupfer *et al.* 1987; Saizawa *et al.* 1987; Mittler *et al.* 1989; Rojo *et al.* 1989; Chuck *et al.* 1990). Indeed certain anti-clonotypic antibodies have been shown to induce the comodulation of CD4 with the TCR in the absence of MHC class II molecules or antibody crosslinking (Saizawa *et al.* 1987; Rojo *et al.* 1989).

Although it has been proposed that CD4/TCR interaction may result in the delivery of a costimulatory signal, the physiological consequences of such an interaction on TCR function are not clear. Vignali and his colleagues have previously addressed this question using CD4 loss variants of three different H-2A^k-restricted murine T cell hybridomas specific for the immunodominant hen egg lysozyme (HEL) peptide 52–61 (Vignali *et al.* 1992*a*). Whereas all the CD4+ and CD4− variants tested responded comparably to immobilized anti-TCR and anti-CD3 mAbs, and naturally processed HEL, only the CD4+ hybridomas responded to the synthetic peptide 46–61. These data suggested that class II-restricted T cells can only recognize a limited repertoire of peptides in the absence of CD4, and that there are at least some peptides that the antigen presenting cell can process from HEL that can stimulate CD4-hybridomas.

To determine which peptides could stimulate the CD4 loss variants, a panel of 53 peptides consisting of every possible 12 to 19-mer which contains the minimal MHC binding epitope, 52–61 (Allen *et al.* 1985*a*, *b*), was produced (figure 4). A CD4+ clone derived from the parental hybridoma, 3A9, responded strongly to all the peptides. In contrast, the 3A9 CD4 loss variant only recognized those peptides which had the C-terminal tryptophans (Trp/W) at positions 62 and 63 (e.g. peptide 52–63). Similar patterns of responsiveness were also observed with CD4+ and CD4-hybridomas from A2.2B2 and 4G4.1.

Although these experiments provide striking data, three questions need to be addressed. First, could the results be explained on the basis of peptide affinity for H-2A^k; second, can these data be reproduced with

Downloaded from rstb.royalsocietypublishing.org

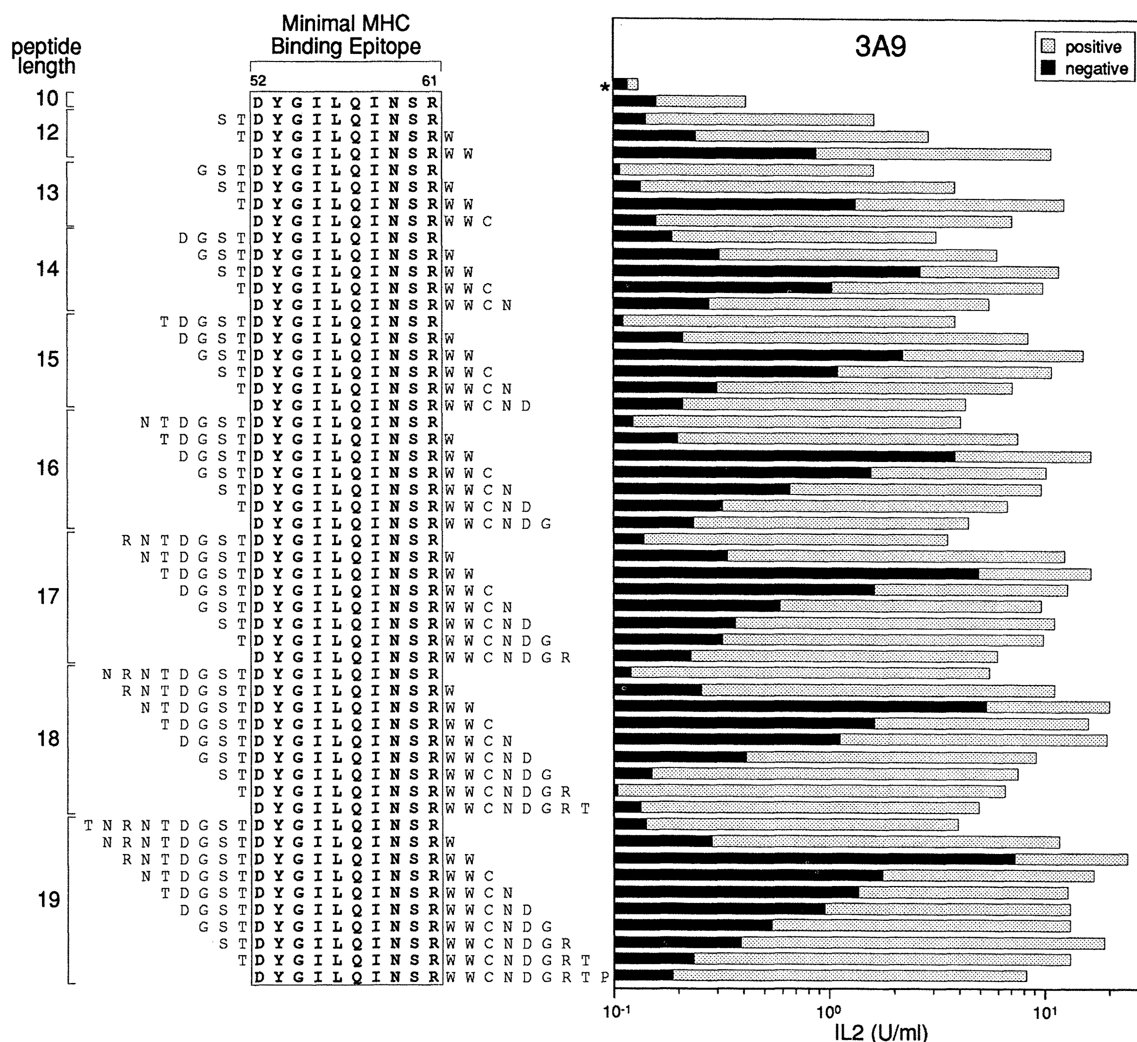


Figure 4. The loss of CD4 has contrasting effects on responsiveness to a panel of HEL 52–61 containing peptides. T cell variants (5×10^4; 100 µl) were cultured with 2.5×10^4 (100 µl) LK35.2 (IA^k) in flat-bottom 96-well microtitre plates with a panel of synthetic peptides (10 µM) (Chiron Mimotopes, Australia). Due to the method of synthesis (pin technology) and peptide quantity, only crude peptides were used. Thus, these data only represent a qualitative assessment. Supernatant (50 µl) were removed after 24 h for estimation of IL2 secretion by culturing with the IL2-dependent T cell line CTLL-2 (5×10^4; 50 µl). Proliferation was determined by using an MTT assay according to the manufacturers instructions (Promega). IL2 secretion determined by using murine recombinant IL2 as a standard. The data presented are representative of three experiments which were found to be highly reproducible.

highly purified peptides; and third, how tolerant is the TCR to substitutions at Trp 62 and Trp 63? These questions were addressed using a second panel of peptides all of which were anchored at the N-terminus at position 48, while varying at the C terminus (table 1). One set consisted of C-terminal extensions of the natural sequence from position 61 to 65, while a second consisted of alanine (Ala/A) and phenylalanine (Phe/F) substitutions of Trp 62 and Trp 63. Importantly, the affinity of these peptides for H-2A^k was found to be comparable as determined using a competition peptide binding assay (table 1).

The original observation was faithfully reproduced using these peptides (figure 5, top). Although CD4+ clones isolated from 3A9, A2.2B2 and 4G4.1 responded to all the analogue peptides, albeit to varying degrees, the CD4 loss variants only responded to C-terminal extensions which contained Trp 63. However, stimulation analysis of the three CD4 loss variants with the alanine and phenylalanine analogues produced some surprising results, with each hybridoma responding differently (figure 5, bottom). The 3A9 CD4 loss variant failed to respond to any of the analogues except for a weak response to 48–61WA, inferring a strict requirement for both tryptophanes particularly Trp 62. On the other hand, the A2.2B2 CD4 loss variant responded strongly to 48–61WF and 48–61FW but to none of the other analogue peptides, suggesting that a single tryptophan in either position plus a hydrophobic amino acid is required for stimulation. In contrast, the 4G4.1 CD4 loss variant is more tolerant to C-terminal variation, responding to six of the ten analogues, although with some preference for Trp 63. It is interesting to note that the response by the CD4+ 4G4.1 hybridoma to 48–61AW was almost 100 times more sensitive than its response to any other peptide including 48–61WW. This clearly shows that single amino acid substitutions

Downloaded from rstb.royalsocietypublishing.org

Table 1. *HEL 48–61 analogue peptides and their affinity for H-2A^k*

(Affinity of the analogues was determined by competition with biotinylated HEL 46–61. Purified H-2A^k (50 nM in 100 μl) was incubated with 300 nM HEL 46–61 biotin and competitor peptide from 10 μM to 30 nM at room temperature for 48 h in McIlvaine citrate/phosphate buffer pH5. 20 μl 1 M Na2HPO4 and 30 μl 10% BSA were added to the reactions and transferred to plates pre-coated with anti-H-2A^k (10.2.16 at 10 μg ml^{-1}). After 90 min, plates were washed and probed with streptavidin-alkaline phosphatase. K_i values of controls were: HEL 46–61, 529; RNase 43–56 (lower affinity for H-2A^k), 4300; HEL 1–18, > 10^4; HEL 105–120, > 10^4 (latter two peptides bind to H-2E^k but not to H-2A^k).)

analogue peptide	peptide sequence	K_i against HEL 46–61 biotin (300 nM)
	48 49 50 51 **52 53 54 55 56 57 58 59 60 61** 62 63 64 65	
48–61	DGST**DYGILQINSR**	511 ± 91
48–61W	DGST**DYGILQINSR**W	477 ± 161
48–61WW	DGST**DYGILQINSR**WW	619 ± 174
48–61WWC	DGST**DYGILQINSR**WWC	491 ± 53
48–61WWCN	DGST**DYGILQINSR**WWCN	546 ± 43
48–61A	DGST**DYGILQINSR**A	518 ± 171
48–61AA	DGST**DYGILQINSR**AA	682 ± 194
48–61WA	DGST**DYGILQINSR**WA	633 ± 73
48–61AW	DGST**DYGILQINSR**AW	634 ± 77
48–61F	DGST**DYGILQINSR**F	753 ± 97
48–61FF	DGST**DYGILQINSR**FF	814 ± 218
48–61WF	DGST**DYGILQINSR**WF	634 ± 166
48–61FW	DGST**DYGILQINSR**FW	583 ± 209
48–61FA	DGST**DYGILQINSR**FA	779 ± 295
48–61AF	DGST**DYGILQINSR**AF	640 ± 917

in flanking regions outside the minimal MHC binding epitope, can have a dramatic effect on T cell responsiveness. Furthermore, the clearly disparate recognition patterns of the three CD4 loss variants infers a TCR rather than MHC based phenomenon.

These data raise two intriguing questions. First, how do Trp 62 and Trp 63 affect TCR recognition, and second, how does CD4 aid this process? As we have already shown the affinity of peptides with and without these residues is the same for MHC class II. Therefore, these two key Trp residues could either be altering the conformation of class II and/or binding directly to the TCR. As T cell responses to these peptides are indistinguishable, the affinity within the TCR-peptide-MHC complex is likely to be similar and reduces the possibility of a peptide-induced conformational change in class II. More importantly, if the Trp residues were affecting either CD4 or class II, one would not expect to see such a dramatic variation in the responsiveness of CD4 loss variants from the three hybridomas.

It is intriguing to speculate whether the TCR directly binds to the two Trp residues. While worthy of examination, this notion runs contrary to both theoretical (Chothia *et al.* 1988; Davis & Bjorkman 1988) and experimental (Jorgensen *et al.* 1992) observations which suggest that the peptide is bound by the CDR3 loop, which is thought to be located in the centre of the TCR. Alternatively, several studies have suggested that the TCR complex may possess the capacity for differential signaling (Sussman *et al.* 1988; O'Rourke *et al.* 1990; Evavold & Allen 1991). Thus, peptides containing the two Trp residues may be more effective at inducing the conformational change required for effective signal transduction. Despite the lack of consensus, it is clear that single amino acid substitutions in the flanking regions of core epitopes can have a dramatic effect on TCR recognition in the absence of CD4.

It is interesting to question why the TCR needs CD4 in order to respond to all the peptides? An affinity requirement for CD4 is unlikely given the huge differences in stimulation by the analogue peptides (e.g. the response of 3A9 to 48–61WW and 48–61FF). It could also be suggested that signal transduction via CD4 is important for effective responsiveness to certain peptides. However, the response of loss variants transfected with CD4 cytoplasmic tailless mutants is indistinguishable from the response of wild-type CD4 transfectants. Taken together, these data raise the possibility that the physical interaction between CD4 and the TCR improves the quality of signaling via the TCR, and that this involves the external CD4 domains.

As present, it remains unclear what the physiological benefit of this interaction might be. There are two distinct observations which distinguish MHC class I and class II-mediated responses that may be relevant here. First, epitopes presented by class I occur as 'single' peptides, while those bound to class II occur as large nested sets. Second, the structure of CD4 and CD8 are completely different despite the fact that the two molecules appear to perform the same function (figure 1). Taken together it could be hypothesized that because MHC class I-restricted TCR only have to recognize a single peptide, CD8 has evolved to avoid extracellular contact with the TCR. However, class II-restricted TCR have to recognize a large number of peptides derived from a single epitope, thus CD4 has evolved to interact with the extracellular

Downloaded from rstb.royalsocietypublishing.org

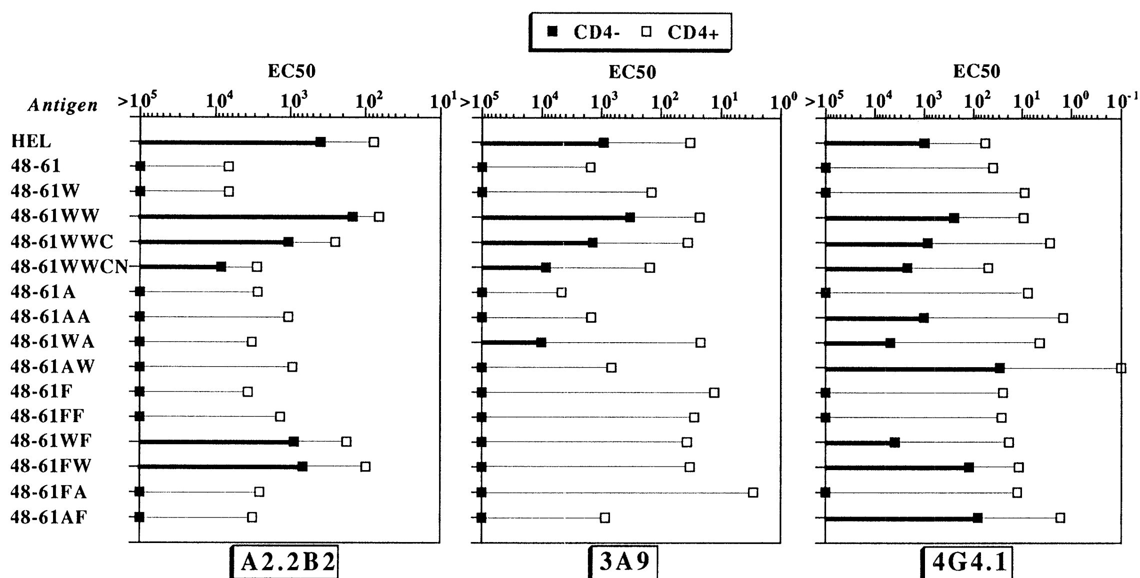


Figure 5. Ability of CD4 loss variants to respond to peptide analogues. Crude peptides were purified to 95%, quantified using amino acid analysis and verified using mass spectrometry. Data are represented as EC50 (the peptide concentration that gives 50% of the maximal response).

portion of the TCR thereby increasing its tolerance to peptide variation. This latter idea is supported, in part, by the data presented here.

4. INTERACTION OF CD4 WITH $p56^{lck}$

CD4 is non-covalently associated with a lymphoid-specific protein tyrosine kinase (PTK) $p56^{lck}$, which is a member of the src family of PTKs (figure 1) (Rudd *et al.* 1989). This association involves cysteine pairs within both the cytoplasmic domain of CD4 and the N-terminal region of the $p56^{lck}$ (Shaw *et al.* 1989, 1990; Turner *et al.* 1990). Upon binding MHC class II molecules on the surface of an APC, CD4 functions to enhance T cell activation. During this process, CD4 physically associates with the T cell receptor complex (Mittler *et al.* 1989; Rojo *et al.* 1989), is phosphorylated on its cytoplasmic serine residues (Maddon *et al.* 1988; Shin *et al.* 1990), dissociates from $p56^{lck}$ (Hurly *et al.* 1989; Sleckman *et al.* 1992), and is down-modulated from the cell surface (Acres *et al.* 1986; Weyland *et al.* 1987). In all of these dynamic features of CD4 regulation, $p56^{lck}$ seems to play a critical role.

CD4 is a type I transmembrane protein with a cytoplasmic domain that is 38 amino acids long. The CD4 cytoplasmic domain can be delineated into two functionally important subregions. A membrane proximal region contains structural motifs required for CD4 down-modulation from the cell surface, while a membrane distal region contains two cysteine residues which are directly involved in the interaction with $p56^{lck}$ (Shin *et al.* 1991).

(*a*) *Role of $p56^{lck}$ in CD4–TCR association during T cell activation*

On resting T cells, only 5% of the TCR/CD3 complex is associated with CD4 (Anderson *et al.* 1988). However, this dramatically increases following T cell activation (Rivas *et al.* 1988). A possible driving force for CD4-TCR/CD3 complex formation would be the interaction of the CD4 cytoplasmic domain with TCR/CD3 via $p56^{lck}$. If this is the case, a CD4 mutant, which could not bind to $p56^{lck}$, should not form complexes with the TCR/CD3 during T cell activation. Wild-type and mutated forms of CD4 containing cysteine to serine point mutations at positions 420, 422 or 430 have been expressed in the murine T cell hybridoma BY155.16. The mutations at positions 420 and 422, but not 430, abolish association with $p56^{lck}$ (figure 6). Fluorescence resonance energy transfer analysis has shown that the CD4 mutants, CS420 and CS422, which fail to interact with $p56^{lck}$, are unable to associate with the TCR/CD3 under conditions in which wild-type CD4 and the CS430 mutant do associate with the TCR/CD3 complex (Collins *et al.* 1992). Thus, $p56^{lck}$ is mediating CD4-TCR/CD3 complex formation possibly by using interaction of $p56^{lck}$ with one of the TCR/CD3 components.

(*b*) *Structural features of the cytoplasmic region of CD4 required for down-modulation and interaction with $p56^{lck}$*

During T cell activation CD4 is phosphorylated on cytoplasmic serine residues and down-modulated from the cell surface by rapid endocytosis and lysosomal degradation (Shin *et al.* 1990, 1991; Ruegg *et al.* 1992). These events may be due to activation of protein kinase C (PKC), as activation of PKC by phorbol esters such as PMA results in serine phosphorylation and internalization of CD4 (Nel *et al.* 1987; Shin *et al.* 1990).

The cytoplasmic features required for CD4 down-modulation have been analysed by expressing CD4 or its mutants in HeLa cells. Mutational analysis includes sequential truncations of the cytoplasmic domain from the C-terminus (at Phe 426, Lys 418,

Downloaded from rstb.royalsocietypublishing.org

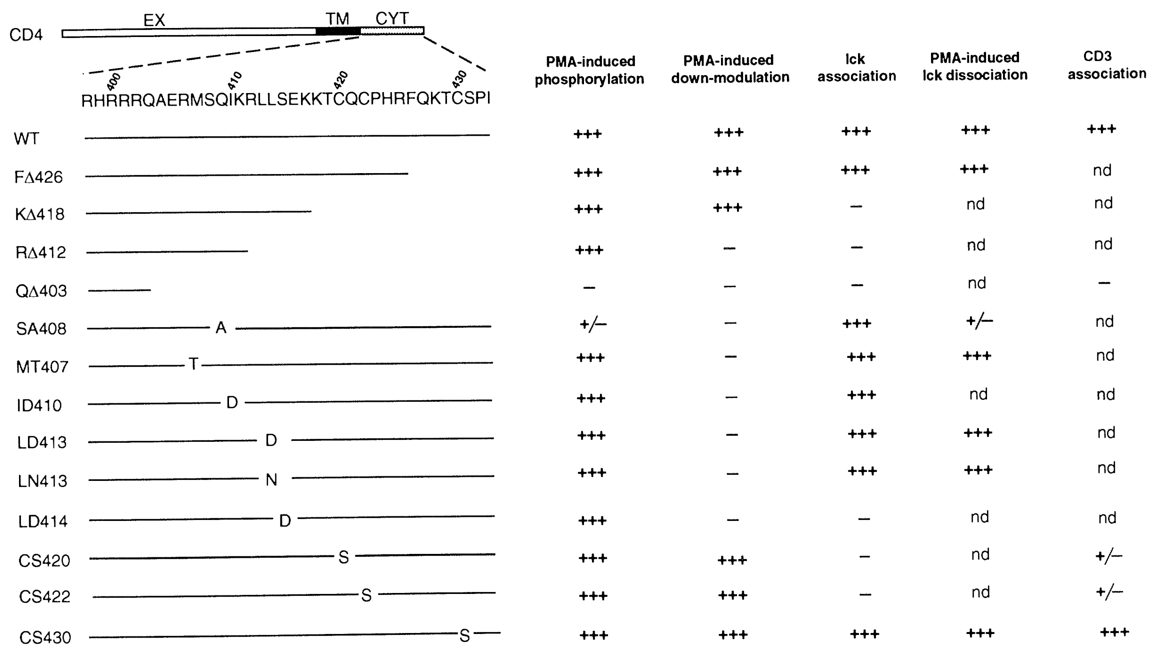


Figure 6. Characteristics of CD4 and its mutants. The amino acid sequence of the cytoplasmic domain of CD4 (residues 398 to 433) is shown. Substituted residues of individual mutants are indicated. All parameters were measured both in HeLa cells and murine thymoma BW5147 cells (Shin *et al.* 1990, 1991; Sleckman *et al.* 1992) except CD3-CD4 complex formation in activated murine T cell hybridoma BY155.16 (Collins *et al.* 1992).

Arg 412, or at Gln 403), point mutations of the potential seryl phosphorylation sites (Ser 408, Ser 415, Ser 431), and mutations in structurally important hydrophobic residues (Met 407, Ile 410, Leu 413, Leu 414) (figure 6).

Cytoplasmic features required for CD4 down-modulation obtained from these mutational analysis are as follows:

1. The membrane proximal region of the cytoplasmic domain from Arg 396 to Lys 417 is sufficient for phorbol ester-induced down-modulation of CD4; this segment is predicted to be an α-helix (Shin *et al.* 1991).
2. Ser 408 is the major PKC-mediated phosphorylation site, and this phosphorylation is required for CD4 down-modulation.
3. In addition to the Ser 408 phosphorylation, four hydrophobic residues (Met 407, Ile 410, Leu 413, Leu 414) are also required for CD4 down-modulation, although these mutations have no effect on Ser 408 phosphorylation. Interestingly, these hydrophobic residues are located on one side of the predicted α-helix, with a charged hydrophilic region on the other. Ser 408 lies near the intersection between these hydrophobic and hydrophilic regions.

Thus, our current model for CD4 down-modulation is that the hydrophobic face of the cytoplasmic domain is bound or occluded by a key molecule responsible for this process, perhaps $p56^{lck}$ in T cells. Phosphorylation of Ser 408 results in the dissociation of the CD4-$p56^{lck}$ complex, thereby exposing the hydrophobic face to the endocytic machinery.

As several groups have shown previously, two cysteine residues in the membrane distal region of the CD4 cytoplasmic domain are directly involved in association with $p56^{lck}$ (Shaw *et al.* 1989, 1990; Turner *et al.* 1990). In addition to this double cysteine motif, we have found that Leu 414 is also critical for CD4-$p56^{lck}$ complex formation. When Leu 414 is mutated to Asp 414, the CD4 mutant was unable to associate with $p56^{lck}$, while the same mutation of the Leu 413 did not show any affinity changes compared with wild-type CD4. Thus, the actual interaction between these two molecules may occur over a broader face than previously suggested. An alternative explanation would be that Leu 414 may regulate the relative position of the CD4 cysteine pair.

(*c*) *Role of* $p56^{lck}$ *in CD4 down-modulation*

Activation of PKC by phorbol ester treatment induces not only serine phosphorylation and down-modulation of CD4 but also disruption of the CD4-$p56^{lck}$ complex (Hurly *et al.* 1989). As mentioned above, dissociation of CD4 from $p56^{lck}$ may be critical for CD4 down-modulation. This relationship has been established by analysing CD4 mutants which were expressed on cell surface of the murine thymoma BW5147 (Sleckman *et al.* 1992).

1. Upon PMA treatment, wild-type CD4-$p56^{lck}$ complex was disrupted and CD4 was rapidly down-modulated.
2. The CD4.LD413 mutant, which was phosphorylated on Ser 408 but not down-modulated after PMA treatment (see above), forms a complex with $p56^{lck}$.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

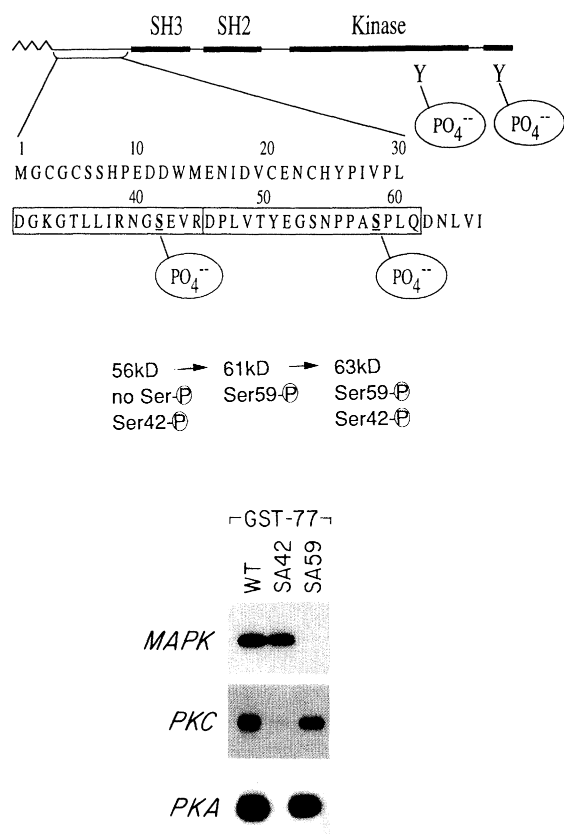


Figure 7. Serine phosphorylation in the N-terminal unique region of the p56^{lck}. (*a*) Schematic diagram of p56^{lck} and amino acid sequence of the unique N-terminal region. The src homology regions and the kinase subdomain are represented by filled boxes. The deduced phosphorylation sites at serines 42 and 59 are marked. Effects of serine phosphorylations on gel shift are also shown. (*b*) *In vitro* phosphorylation of Ser 42 and Ser 59 by purified kinases. GST fusion proteins containing the N-terminal 77 amino acids of wild-type and the serine-to-alanine mutations of Ser 42 and Ser 59 of p56^{lck} were used as substrates for purified MAP kinase, PKC, and PKA. After Winkler *et al.* (1993).

Incubation with PMA resulted in complete disruption of the CD4.LD413-p56^{lck} complex without measurable down-modulation. Thus, complex disruption precedes internalization.

3. The CD4.SA408 mutant, which lacks the major phosphorylation site and is not down-modulated by PMA treatment, also forms a complex with p56^{lck}. PMA treatment, however, induced neither disruption of the CD4.SA408-p56^{lck} complex nor down-modulation of the mutant CD4. Thus, Ser408 phosphorylation may induce disruption of the CD4-p56^{lck} complex.

4. p56^{lck} forms a complex with CD4 and CD4.CS430 but not with CD4.CS420 or CD4.CS422. When expressed in BW5147 cells, CD4 and CD4.CS430 are down-modulated at a slower rate in response to PMA treatment than CD4.CS420 and CD4.CS422. However, these four variants of CD4 molecules are down-modulated with the same rate in p56^{lck}-negative HeLa cells. Thus, the difference in kinetics may represent the time required for disruption of the CD4-p56^{lck} complex.

These data suggest that phosphorylation of Ser 408 on the CD4 cytoplasmic domain by activated PKC induces the dissociation of CD4 from p56^{lck}, and the subsequent endocytosis of CD4 mediated by phosphoserine and the exposed hydrophobic face.

(*d*) *Role of serine phosphorylations at the unique N-terminal region of the p56^{lck}*

Like other members of the *src* family of cytoplasmic tyrosine kinases, p56^{lck} has an N-terminal myristoylation site, two regulatory domains (the SH3 and SH2 domains), and a C-terminal catalytic domain (figure 7) (Hanks *et al.* 1988; Koch *et al.* 1991). With the exception of the N-terminal myristoylation site, the first 67 amino acids of p56^{lck} are unrelated in amino acid sequence to other members of the src family. Although the association of p56^{lck} with CD4 via the cysteine pair (Cys20 and Cys23) is thought to stimulate p56^{lck} kinase activity, little else is known about the regulatory function(s) of this region.

Treatment of T cells with PMA induces extensive serine phosphorylation in this unique N-terminal region and retardation of the p56^{lck} mobility on SDS-PAGE (Luo & Sefton 1990). Gel retardation has also been observed upon treatment of T cells with a number of different agents including IL2, CD4-TCR crosslinking, and calcium ionophore (Casnellie 1987; Horak *et al.* 1991). Thus, N-terminal phosphorylation could be involved in regulation of the p56^{lck}.

To identify the serine/threonine phosphorylation sites in p56^{lck}, all possible serine/threonine residues were mutated to alanines, and analysed in HeLa cells (Winkler *et al.* 1993). Only the alanine mutants at Ser 42 and Ser 59 produced changes in gel shift patterns of p56^{lck} after PMA treatment. *In vivo* ^{32}P-labelling, CNBr cleavage, and phosphopeptide mapping analysis showed that Ser 42 and Ser 59 are indeed the only major serine phosphorylation sites. Phosphorylation of Ser 59 results in a gel-shift from 56 kDa to 61 kDa. Simultaneous phosphorylation of Ser 42 and Ser 59 results in a further gel-shift to 63 kDa. *In vitro* kinase assays and immunodepletion studies showed that Ser 42 and Ser 59 may be phosphorylated by PKC and MAP kinase, respectively (figure 7).

In our preliminary experiments, the wild-type and serine phosphorylation site mutants of p56^{lck} have similar *in vitro* kinase activity, i.e. phosphorylations at Ser 42 and Ser 59 do not regulate the tyrosine kinase activity of p56^{lck} directly. However, when expressed in CD4+ HeLa cells, Ser 59 to Glu 59 mutant of p56^{lck} induced phosphorylation of a set of cellular proteins upon CD4 crosslinking. Our recent data also suggest that Ser 59 is a regulatory element for the binding of a specific phosphotyrosyl protein to the SH2 domain. Thus, N-terminal serine phosphorylation(s) may have a critical role in substrate recruitment of the p56^{lck}. Since the CD4 binding site (Cys 20 and Cys 23) is in close vicinity to the N-terminal serine phosphorylation sites (Ser 42 and Ser 59), dynamic changes in the CD4-p56^{lck} complex may also be involved in the regulation of p56^{lck}, perhaps by modulation of its non-catalytic domain(s).

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

5. CONCLUDING REMARKS

The multiple interactions between CD4 and MHC class II molecules, TCR and p56^{lck} exemplifies the pleiotropic function of this molecule. Studies described here have led to the following conclusions. First, cell adhesion mediated by CD4 and the β1 and β2 domains of MHC class II molecules appears to require active cellular processes. Second, in the absence of CD4, MHC class II-restricted TCR can only recognize a limited number of the naturally processed peptides derived from a single epitope. Third, phosphorylation of several sites on both CD4 and p56^{lck} regulate both their function and interaction. From its humble beginnings as a potential adhesion molecule, CD4 has emerged as a critical and integral part of the TCR/CD3 complex during T cell activation.

REFERENCES

Acres, R.B., Conlon, P.J., Mochizuk, D.Y. & Gallis, B. 1986 Rapid phosphorylation and modulation of the T4 antigen on cloned helper T cells induced by phorbol myristrate acetate and antigen. *J. biol. Chem.* **261**, 16210–16214.

Allen, P.M., Matsueda, G.R., Haber, E. & Unanue, E.R. 1985*a* Specificity of the T cell receptor: two different determinants are generated by the same peptide and the I-A^k molecule. *J. Immunol.* **135**, 368–373.

Allen, P.M., McKean, D.J., Beck, B.N., Sheffield, J. & Glimsher, L.H. 1985*b* Direct evidence that a class II molecule and a simple globular protein generate multiple determinants. *J. exp. Med.* **162**, 1264–1274.

Anderson, P., Blue, M.-L. & Schlossman, S.F. 1988 Comodulation of CD3 and CD4. Evidence for a specific association between CD4 and approximately 5% of the CD3:T cell receptor complexes on helper T lymphocytes. *J. Immunol.* **140**, 1732–1737.

Anderson, P., Blue, M.-L., Morimoto, C. & Schlossman, S.F. 1987 Cross-linking of T3 (CD3) with T4 (CD4) enhances the proliferation of resting T lymphocytes. *J. Immunol.* **139**, 678–682.

Atassi, H., Ulrich, R.G. & Atassi, M.Z. 1987 Immune recognition of human major histocompatibility antigens: localization of the continuous structure of the entire extracellular part of the chain. *Eur. J. Immunol.* **17**, 769–773.

Babbitt, B.P., Allen, P.M., Matsueda, G.R., Haber, E. & Unanue, E.R. 1985 Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature, Lond.* **317**, 359–361.

Bank, I. & Chess, L. 1985 Perturbation of the T4 molecule transmits a negative signal to T cells. *J. exp. Med.* **162**, 1294–1303.

Biddison, W., Rao, P., Talle, M.A., Goldstein, G. & Shaw, S.J. 1983 Possible involvement of the T4 molecule in T cell recognition of class II antigens. Evidence from studies of CTL target cell binding. *J. exp. Med.* **156**, 1065–1076.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L. & Wiley, D.C. 1987 Structure of the human class I histocompatibility antigen, HLA-A2. *Nature, Lond.* **329**, 506–512.

Brenner, M.B., Trowbridge, I.S. & Strominger, J.L. 1985 Cross-linking of human T cell receptor proteins: association between the T cell idiotype β subunit and the T3 glycoprotein heavy subunit. *Cell* **40**, 183–190.

Brown, J.H., Jardetzky, T., Saper, M.A., Samraoui, B., Bjorkman, P.J. & Wiley, D.C. 1988 A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. *Nature, Lond.* **332**, 845–850.

Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L. & Wiley, D.C. 1993 Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature, Lond.* **364**, 33–39.

Burgess, K.E., Odysseos, A.D., Zalvan, C., *et al.* 1991 Biochemical identification of a direct physical interaction between the CD4:p56lck and Ti(TCR)/CD3 complexes. *Eur. J. Immunol.* **21**, 1663–1668.

Buus, S., Sette, A. & Grey, H.M. 1987 The interaction between protein-derived immunogenic peptides and Ia. *Immunol. Rev.* **98**, 115–141.

Cammarota, G., Scheirle, A., Takacs, B., *et al.* 1992 Identification of a CD4 binding site on the β2 domain of HLA-DR molecules. *Nature, Lond.* **356**, 799–801.

Casnellie, J.E. 1987 Sites of in vivo phosphorylation of the T cell tyrosine protein kinase in LSTRA cells and their alteration by tumor promoting phorbol esters. *J. biol. Chem.* **262**, 9859–9864.

Chothia, C., Boswell, D.B. & Lesk, A.M. 1988 The outline structure of the T-cell αβ receptor. *EMBO J.* **7**, 3745–3755.

Chuck, R.S., Cantor, C.R. & Tse, D.T. 1990 CD4-T-cell antigen receptor complexes on human leukemia T cells. *Proc. natn. Acad. Sci. U.S.A.* **87**, 5021–5025.

Clayton, L.K., Sieh, M., Piuous, D.A. & Reinherz, E.L. 1989 Identification of human CD4 residues affecting class II MHC versus HIV-1 gp120 binding. *Nature, Lond.* **339**, 548–551.

Collins, T.L., Uniyal, S., Shin, J., Strominger, J.L., Mittler, R.S. & Burakoff, S.J. 1992 p56^{lck} association with CD4 is required for the interaction between CD4 and the TCR/CD3 complex and for optimal antigen stimulation. *J. Immunol.* **148**, 2159–2162.

Davis, M.M. & Bjorkman, P.J. 1988 T cell antigen receptor genes and T cell recognition. *Nature, Lond.* **334**, 395–402.

Dembic, Z., Haas, W., Zamoyska, R., Parnes, J.R., Steinmez, M. & von Boehmer, H. 1987 Transfection of the CD8 gene enhances T cell recognition. *Nature, Lond.* **326**, 510–511.

Doyle, C. & Strominger, J.L. 1987 Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature, Lond.* **330**, 256–259.

Eichmann, K., Jonsson, J.-I., Falk, I. & Emmrich, F. 1987 Effective activation of resting mouse T lymphocytes by cross-linking submitogenic concentrations of the cell antigen receptor with either Lyt-2 or L3T4. *Eur. J. Immunol.* **17**, 643–650.

Engleman, E.G., Benike, C.J., Grumet, C. & Evans, R.L. 1981 Activation of human T lymphocyte subsets: helper and suppressor cytotoxic T cells recognize and respond to distinct histocompatibility antigens. *J. Immunol.* **127**, 2124–2129.

Evavold, B.D. & Allen, P.M. 1991 Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science, Wash.* **252**, 1308–1310.

Gallagher, P.F., Fazekas de St. Groth, B. & Miller, J.F.A.P. 1989 CD4 and CD8 molecules can physically associate with the same T-cell receptor. *Proc. natn. Acad. Sci. U.S.A.* **86**, 10044–10048.

Gay, D., Buus, S., Pasternack, J., Kappler, J. & Marrack, P. 1988 The T cell accessory molecule CD4 recognizes a monomorphic determinant on isolated Ia. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5629–5633.

Golding, H., McCluskey, J., Munitz, T.I., Germain, R.N., Margulies, D.H. & Singer, A. 1985 T cell recognition of

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

a chimaeric class II/class I MHC molecule and the role of L3T4. *Nature, Lond.* **317**, 425–427.

Gorga, J.C., Knudsen, P.J., Foran, J.A., Strominger, J.L. & Burakoff, S.J. 1986 Immunochemically purified DR antigens in liposomes stimulate xenogeneic cytolytic T cells in secondary in vitro cultures. *Cell. Immunol.* **103**, 160–173.

Greenstein, J.L., Malissen, B. & Burakoff, S.J. 1985 Role of L3T4 in antigen-driven activation of a class I-specific T cell hybridoma. *J. exp. Med.* **162**, 369–374.

Greenstein, J.L., Kappler, J., Marrack, P. & Burakoff, S.J. 1984 The role of the L3T4 in recognition of Ia by a cytotoxic, H-2D[d]-specific T cell hybridoma. *J. exp. Med.* **159**, 1213.

Hanks, S.K., Quinn, A.M. & Hunter, T. 1988 The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science, Wash.* **241**, 4252.

Horak, A.D., Gress, R.E., Lucas, P.J., Horak, E.M., Waldmann, T.A. & Bolen, J.B. 1991 T-lymphocyte IL-2 dependent tyrosine protein kinase signal transduction involves the activation of p56lck. *Proc. natn. Acad. Sci. U.S.A.* **88**, 1996–2000.

Hurly, T.R., Luo, K. & Sefton, B.M. 1989 Activators of protein kinase C induce dissociation of CD4, but not CD8, from p56lck. *Science, Wash.* **245**, 407409.

Janeway, C.A. Jr 1991 The co-receptor function of CD4. *Semin. Immunol.* **3**, 153.

Janeway, C.A. Jr 1992 The T cell receptor as a multicomponant signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *A. Rev. Immunol.* **10**, 645–674.

Jorgensen, J.L., Esser, U., Fazekas de St. Groth, B., Reay, P.A. & Davis, M.M. 1992 Mapping T-cell receptor-peptide contacts by varient peptide immunization of single chain transgenics. *Nature, Lond.* **355**, 224–230.

Julius, M., Maroun, C.R. & Haughn, L. 1993 Distinct roles for CD4 and CD8 as co-receptors in antigen receptor signalling. *Immunol. Today* **14**, 177–183.

Kappes, D. & Strominger, J.L. 1988 Human class II major histocompatibility complex genes and proteins. *A. Rev. Biochem.* **57**, 991–1028.

Kinch, M.S., Strominger, J.L. & Doyle, C. 1993 Cell adhesion mediated by CD4 and MHC class II proteins requires active cellular processes. *J. Immunol.* (In the press.)

Koch, C.A., Anderson, D., Moran, M.F., Ellis, C. & Pawson, T. 1991 SH2 and SH3 domains; elements that control interactions of cytoplasmic signaling proteins. *Science, Wash.* **252**, 668–674.

Konig, R., Huang, L.Y. & Germain, R.N. 1992 MHC class II interaction with CD4 mediated by a region analogous to the MHC class I binding site for CD8. *Nature, Lond.* **356**, 796–798.

Krensky, A.M., Reiss, C.S., Mier, J.W., Strominger, J.L. & Burakoff, S.J. 1982 Long term human cytolytic T cell lines allospecific for HLA-DR and antigen are OKT4+. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2365–2369.

Kupfer, A., Singer, S.J., Janeway, C.A. Jr & Swain, S.L. 1987 Co-clustering of CD4 (L3T4) with the T cell receptor is induced by specific direct interaction of helper T cells and antigen presenting cells. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5888–5892.

Lamarre, D., Capon, D.J., Karp, D.R., Gregory, T., Long, E.O. & Sekaly, R.-P. 1989 Class II MHC molecules and the HIV gp120 envelope protein interact with functionally distinct regions of the CD4 molecule. *EMBO J.* **8**, 3271–3277.

Ledbetter, J.A., June, C.H., Rabinovitch, P.S., Grossman, A., Tsu, T.T. & Imboden, J.B. 1988 Signal transduction through CD4 receptors: stimulatory *vs.* inhibitory activity is regulated by CD4 proximity to the CD3/T cell receptor. *Eur. J. Immunol.* **18**, 525–532.

Lombardi, G., Barber, L., Aichinger, G., *et al.* 1991 Structural analysis of anti-DR1 allorecognition by using DR1/H-2E[k] hybrid molecules. Influence of the β2 domain correlates with CD4 dependence. *J. Immunol.* **147**, 2034–2040.

Luo, K. & Sefton, B.M. 1990 Analysis of the sites in p56lck whose phosphorylation is induced by tetradecanoyl phorbol acetate. *Oncogene* **5**, 803–808.

Maddon, P.J., McDougal, J.S., Clapham, P.R., *et al.* 1988 HIV infection does not require endocytosis of its receptor, CD4. *Cell* **54**, 865–874.

Marrack, P., Endres, R., Shimonkevitz, R., *et al.* 1983 The major histocompatibility complex restricted antigen on T cells. II. Role of the L3T4 product. *J. exp. Med.* **158**, 1077–1091.

Meuer, S.C., Schlossman, S.F. & Reinherz, E.L. 1982 Clonal analysis of human cytotoxic T lymphocytes: T4+ and T8+ effector cells recognize products of different major histocompatibility complex regions. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4395–4399.

Mittler, R.S., Goldman, S.J., Spitalny, G.L. & Burakoff, S. 1989 T-cell receptor-CD4 physical association in a murine T-cell hybridoma: induction by antigen receptor ligation. *Proc. natn. Acad. Sci. U.S.A.* **86**, 8531–8535.

Nel, A.E., Schabort, I., Rheeder, A., Bouic, P. & Wooten, M.W. 1987 Inhibition of antibodies to CD3 surface antigen and phytohemagglutinin-mediated T cellular responses by inhibiting Ca^{2+}/phospholipid-dependent protein kinase activity with the aid of 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride. *J. Immunol.* **139**, 2230–2236.

Norment, A.M., Salter, R.D., Parham, P., Engelhard, V.H. & Littman, D.R. 1988 Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature, Lond.* **336**, 79–81.

O'Rourke, A.M., Mescher, M.F. & Webb, S.R. 1990 Activation of polyphosphoinositide hydrolysis in T cells by H-2 alloantigen but not MLS determinants. *Science, Wash.* **249**, 171–174.

Parnes, J.R. 1989 Molecular biology and function of CD4 and CD8. *Adv. Immunol.* **44**, 265–311.

Piatier-Tonneau, D., Gastinel, L.-N., Amblard, F., Wojcik, M., Vaigot, P. & Auffray, C. 1991 Interaction of CD4 with HLA class II antigens and HIV gp120. *Immunogen* **34**, 121–128.

Potter, T.A., Rajan, T.V., Dick, R.F., II & Bluestone, J.A. 1989 Substitution at residue 227 of H-2 class I molecules abrogates recognition by CD8-dependent, but not CD8-independent, cytotoxic T lymphocytes. *Nature, Lond.* **337**, 73–75.

Ratcliffe, M.J.H., Coggeshall, K.M., Newell, M.K. & Julius, M.H. 1992 T cell receptor aggregation, but not dimerization, induces increased cytosolic calcium concentrations and reveals a lack of stable association between CD4 and the T cell receptor. *J. Immunol.* **148**, 1643–1651.

Rivas, A., Takada, S., Koide, J., Sonderstrup-McDevitt, G. & Engelman, E.G. 1988 CD4 molecules are associated with the antigen receptor complex on activated but not resting T cells. *J. Immunol.* **140**, 2912–2918.

Rogozinski, L., Bass, A., Glickman, E., *et al.* 1984 The T4 surface antigen is involved in the induction of helper function. *J. Immunol.* **132**, 735–739.

Rojo, J.M., Saizawa, K. & Janeway, C.A. Jr 1989 Physical association of CD4 and the T-cell receptor can be induced by anti-T-cell receptor antibodies. *Proc. natn. Acad. Sci. U.S.A.* **86**, 3311–3315.

Rudd, C.E., Anderson, P., Morimoto, C., Streuli, M. &

Downloaded from rstb.royalsocietypublishing.org

Schlossman, S.F. 1989 Molecular interactions, T-cell subsets and the role of the CD4/CD8: p56lck complex in human T-cell activation. *Immunol. Rev.* **111**, 225–266.

Ruegg, C.L., Rajasekar, S., Stein, B.S. & and Engleman, E.G. 1992 Degradation of CD4 following Phorbol induced internalization in human T lymphocytes. *J. biol. Chem.* **267**, 18837–18843.

Saizawa, K., Rojo, J. & Janeway, C.A. Jr 1987 Evidence for a physical association of CD4 and the CD3: α: β T-cell receptor. *Nature, Lond.* **328**, 260–263.

Salter, R.D., Norment, A.M., Chen, B.P., *et al.* 1989 Polymorphism in the a3 domain of HLA-A molecules affects binding to CD8. *Nature, Lond.*. **338**, 345–347.

Salter, R.D., Benjamin, R.J., Wesley, P.K., *et al.* 1990 A binding site for the T cell co-receptor CD8 on the α3 domain of HLA-A2. *Nature, Lond.* **345**, 41–46.

Sanchez-Madrid, F., Krensky, A.M., Ware, C.F., *et al.* 1982 Three distinct antigens associated with human T lymphocyte-mediated cytolysis: LFA-1, LFA-2 and LFA-3. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7489–7493.

Shaw, A.S., Amrein, K.E., Hammond, C., Stern, D.F., Sefton, B.M. & Rose, J.K. 1989 The cytoplasmic domain of CD4 interacts with the tyrosine protein kinase, p56lck, through its unique amino terminal domain. *Cell* **59**, 627–636.

Shaw, A.S., Chalupny, J., Hammond, C., *et al.* 1990 Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino terminal domain of the p56lck tyrosine protein kinase. *Molec. cell. Biol.* **10**, 1853–1862.

Shin, J., Doyle, C., Yang, Z., Kappes, D. & Strominger, J.L. 1990 Structural features of the cytoplasmic region of CD4 required for internalization. *EMBO J.* **9**, 425–434.

Shin, J., Dunbrack, R.L. Jr, Lee, S. & Strominger, J.L. 1991 Phosphorylation dependent down modulation of CD4 requires a specific structure within the cytoplasmic domain of CD4. *J. biol. Chem.* **266**, 16219–16225.

Sleckman, B.P., Shin, J., Igras, V.E., Collins, T.L., Strominger, J.L. & Burakoff, S.J. 1992 Disruption of the CD4 p56lck complex is required for rapid internalization of CD4. *Proc. natn. Acad. Sci. U.S.A.* **89**, 7566–7570.

Sussman, J.J., Mercep, M., Saito, T., Germain, R.N., Bonvini, E. & Ashwell, J.D. 1988 Dissociation of phosphoinositide hydrolysis and Ca^{2+} fluxes from the biological responses of a T-cell hybridoma. *Nature, Lond.* **334**, 625–628.

Swain, S. 1983 T cell subsets and the recognition of MHC class. *Immunol. Rev.* **74**, 129–142.

Swain, S.L., Dialynas, D.P., Fitch, F.W. & English, M. 1984 Monoclonal antibody to L3T4 blocks the function of T cells specific for major histocompatibility complex antigens. *J. Immunol.* **132**, 1118–1123.

Turner, J.M., Brodsky, M.H., Irving, B.A., Levin, S.D., Perlmutter, R.M. & Littman, D.R. 1990 Interaction of the unique N terminal region of tyrosine kinase $p56^{lck}$ with cytoplasmic domains of CD4 and CD8 is mediated by cystein motifs. *Cell* **60**, 755–765.

Ulrich, R.G., Atassi, H., Lutz, P., Cresswell, P. & Atassi, M.Z. 1987 Immune receptor of human major histocompatibility antigens: localization by a comprehensive synthetic strategy of the continuous antigen sites in the first domain of HLA-DR2 β chain. *Eur. J. Immunol.* **17**, 497–502.

Veillette, A., Bookman, M.A., Horak, E.M. & Bolen, J.B. 1988 The CD4 and CD8 T-cell surface antigens are associated with the internal membrane tyrosine protein kinase $p56^{lck}$. *Cell* **55**, 301–308.

Vignali, D.A.A., Moreno, J., Schiller, D. & Hammerling, G.J. 1992*a* Does CD4 help to maintain the fidelity of T cell receptor specificity? *Int. Immunol.* **4**, 621–626.

Vignali, D.A.A., Moreno, J., Schiller, D. & Hammerling, G.J. 1992*b* Species-specific binding of CD4 to the β2 domain of major histocompatibility complex class II molecules. *J. exp. Med.* **175**, 925–932.

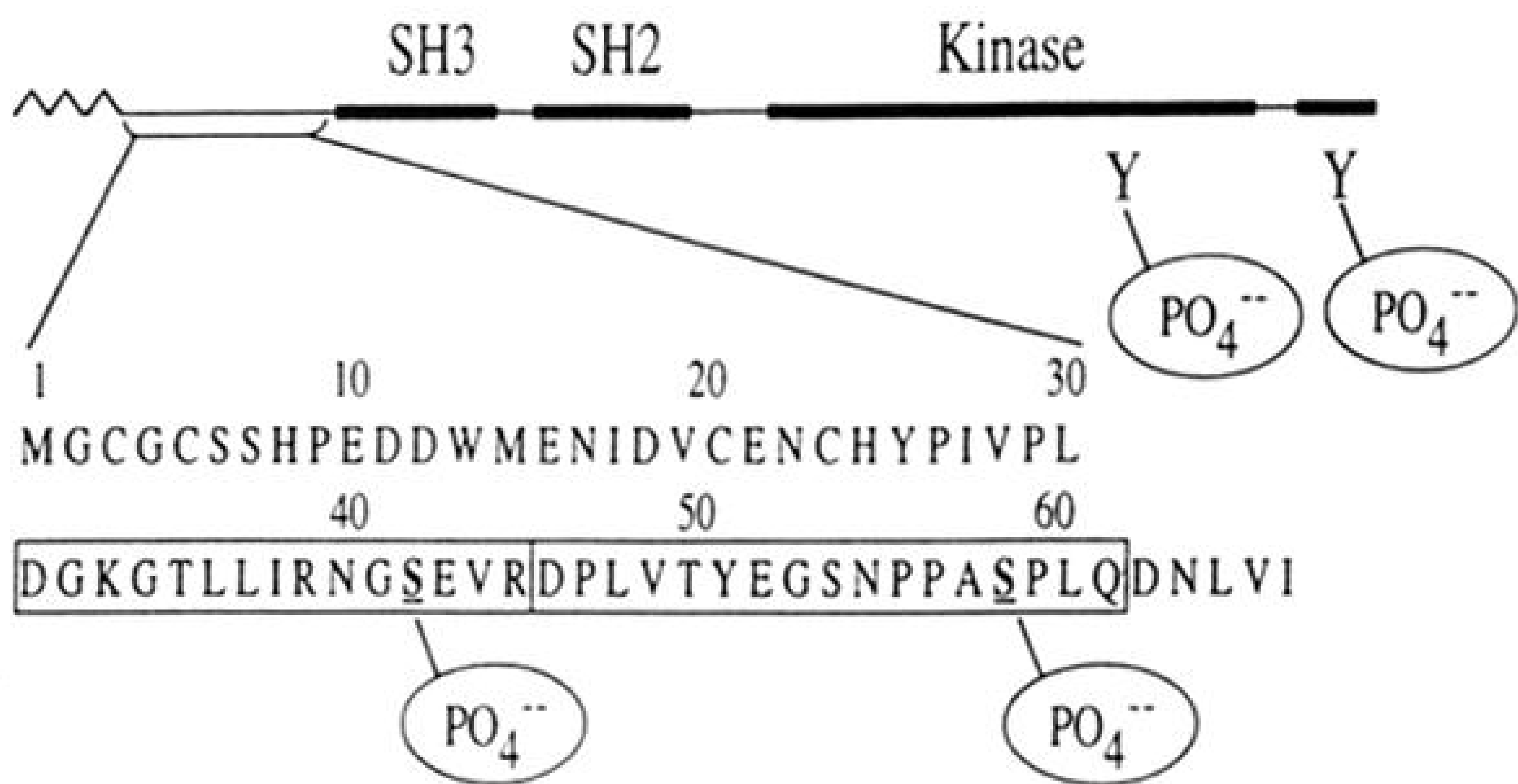
Wassmer, P., Chan, C., Logdberg, L. & Shevach, E.M. 1985 Role of the L3T4 antigen in T cell activation. II. Inhibition of T cell activation by monoclonal anti-L3T4 antibodies in the absence of accessory cells. *J. Immunol.* **135**, 2237–2242.

Weyland, C.M., Goronzy, J. & Fathman, C.G. 1987 Modulation of CD4 by antigenic activation. *J. Immunol.* **138**, 1351–1354.

Winkler, D.G., Park, I., Kim, T., *et al.* 1993 Phosphorylation of Ser42 mand Ser59 in th N-terminal region of the tyrosine kinase $p56^{lck}$. *Proc. natn. Acad. Sci. U.S.A.* **90**, 5176–5180.

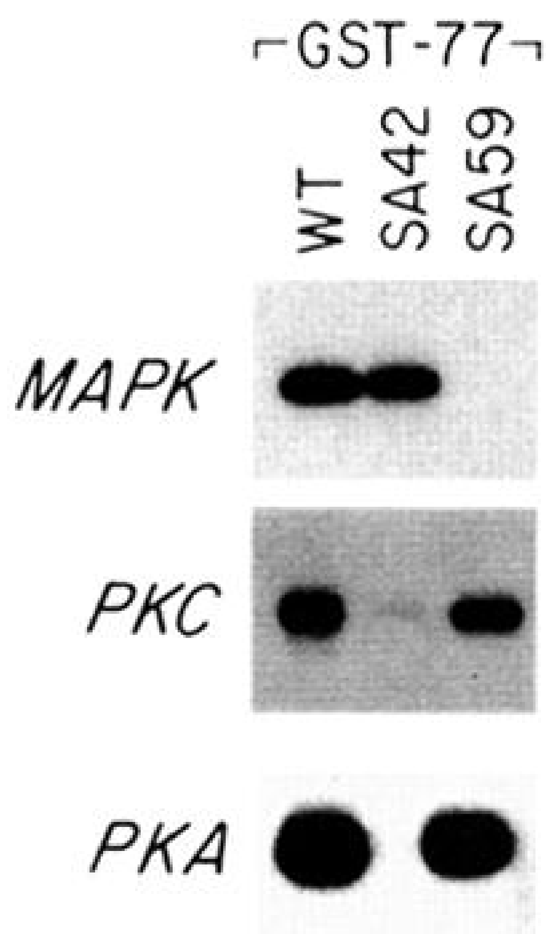
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



56kD → 61kD → 63kD
no Ser-Ⓟ Ser59-Ⓟ Ser59-Ⓟ
Ser42-Ⓟ Ser42-Ⓟ

Downloaded from rstb.royalsocietypublishing.org



PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

igure 7. Serine phosphorylation in the N-terminal unique gion of the $p56^{lck}$. (*a*) Schematic diagram of $p56^{lck}$ and nino acid sequence of the unique N-terminal region. The c homology regions and the kinase subdomain are reprented by filled boxes. The deduced phosphorylation sites at rines 42 and 59 are marked. Effects of serine phosphorylaons on gel shift are also shown. (*b*) *In vitro* phosphorylation Ser 42 and Ser 59 by purified kinases. GST fusion proteins ontaining the N-terminal 77 amino acids of wild-type and e serine-to-alanine mutations of Ser 42 and Ser 59 of $p56^{lck}$ ere used as substrates for purified MAP kinase, PKC, and KA. After Winkler *et al.* (1993).